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Functional bridging of protein disulfide bonds with maleimides

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I, Felix Franz Schumacher confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The application of chemical methods to biological systems has led to great advances in all life sciences and the discovery of novel approaches for therapy and diagnosis. Pivotal amongst these methods is the ability to chemically modify proteins to enhance their biophysical properties or add new functionality. Despite the success, the chemical toolbox of efficient and widely applicable protocols is relatively limited. In the work presented in this thesis the idea of protein modification via the targeting of solvent accessible disulfide bonds is explored. These are fairly common in secreted proteins and their reduction affords two cysteine residues, which are highly reactive nucleophiles. However, to preserve their native function – stabilisation and maintenance of the protein structure – ideally *bis*-reactive compounds are used that react simultaneously with both thiols and keep the covalent connection of the disulfide bond intact.

To this end a selection of maleimides substituted with good leaving groups in the 3 and 4 positions as well as their *N*-functionalised versions were synthesised and tested for their reactivity. The findings were transferred to the small peptide hormone somatostatin, which served as a model system to explore kinetics and feasibility of the proposed “functionalisation by bridging”. Changing the leaving groups from halides to thiols enabled the development of *in situ* protocols where the bridging reagent could be employed in tandem with the reducing agent, greatly decreasing the reaction time and unwanted side reactions such as dimerisation or unfolding.

The developed methods were then utilised to bridge the cystines of insulin as well as a selection of full length antibodies and antibody fragments. PEGylated, biotinylated, fluorescent or spin labelled analogues of these proteins were also synthesised. The biological activity, stability and functionality of the conjugates were assessed in biochemical and biophysical assays.

Overall the functionalisation of disulfide bonds with maleimides was found to be site-specific, fast, high yielding and the inserted bridge was stable under various conditions. The modification was well tolerated and all prepared analogues exhibited the desired functionality. The scope and potential of present and future applications of the method are discussed.

Publications and patent applications derived from this project:

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For Anneliese Schumacher

In loving memory of Jürgen Schumacher

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Abbreviations

µg	microgram
µl	microliter
µm	micrometre
µM	micromolar
Å	angstrom
aaRS	amino acid tRNA synthetase
Ac_2O	acetic anhydride
ACTH	adrenocorticotropic hormone
ADC	antibody-drug conjugate
ADCC	antibody-dependent cellular cytotoxicity
ADEPT	antibody-directed enzyme prodrug strategy
ATRP	atom transfer radical polymerisation
AU	arbitrary units
BiTE	bispecific T-cell engager molecule
CD	circular dichroism
CDC	complement-dependent cytotoxicity
CEA	carcinoembryonic antigen
CHCA	α-cyano-4-hydroxycinnamic acid
CI	chemical ionization
cm	centimetre
CW	continuous wave
d	day
Da	dalton
DCM	dichloromethane
DHA	dehydroalanine
DHB	2,5-dihydroxy benzoic acid
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsFv	disulfide-stabilised Fv
ds-scFv	disulfide-stabilised single-chain Fv
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EI	electron ionization
ELISA	enzyme-linked immunosorbent assay
EPR	electron paramagnetic resonance
equiv	equivalent(s)
ER	endoplasmatic reticulum

ES	electrospray
Et_2O	ether
Fab	antigen-binding fragment of an antibody
Fc	crystallisable fragment of an antibody
FDA	Food and Drug Administration
FRAP	Fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
Fv	variable fragment of an antibody
g	gram
GeV	gigavolt
GIRK	G protein-coupled inwardly-rectifying potassium channel
GPCR	G protein-coupled receptor
GSH	glutathione
GSSG	disulfide bridged dimer of glutathione
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
HRP/STREP	chemical conjugate of horseradish peroxidiase and streptavidin
Ig	immunoglobulin
IMAC	immobilised metal ion affinity chromatography
IR	infrared
ISMB	Institute of Structural and Molecular Biology
IUPAC	International Union of Pure and Applied Chemistry
kDa	kilodalton
kV	kilovolt
l	litre
LC	liquid chromatography
LCMS	liquid chromatography mass spectroscopy
M	molar mass
m/z	mass per charge
mA	milliampere
mAB	monoclonal antibody
MALDI-TOF MS	matrix-assisted laser desorption/ ionisation time-of-flight mass spectroscopy
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mmol	millimole

Mn	mean molecular mass
mPEG	methoxypolyethylene glycol
MS	mass spectroscopy
MSP1	merozite surface protein 1
MTSL	*S*-(2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methyl methanesulfonothioate
mU	milliunit
mV	millivolt
MW	molecular weight
MWCO	molecular weight cut off
MΩ	megaohm
n.d.	not determined
n.t.	not detectable
NCL	native chemical ligation
NEM	*N*-ethylmaleimide
NHS	*N*-hydroxysuccinimide
NHS-PEG	methoxypolyethylene glycol succinate *N*-hydroxysuccinimide
nm	nanometre
nM	nanomolar
NMR	nuclear magnetic resonance
OD_{XXX}	optical density at a wavelength of XXX nm
pA	picoampere
PBS	phosphate buffered saline
PDB	protein data bank
PDI	protein disulfide isomerase
PEG	polyethylene glycol
pF	picofarad
pH	pondus Hydrogenii (*lat*. “strength of hydrogen“)
ppm	parts per million
PTM	posttranslational modification
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
RTK	receptor tyrosine kinase
s	second
scFv	single-chain Fv
SDS	sodium dodecyl sulphate
SDSL	site-directed spin labelling
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography

SEC-MALS	size exclusion chromatography – multi angle light scattering
sinapinic acid (SA)	3,5-dimethoxy-4-hydroxycinnamic acid
siRNA	small interfering RNA
SPR	surface plasmon resonance
SST	somatostatin
sst_X	somatostatin receptor X
STREP	steptavidin
TCEP	*tris*(2-carboxyethyl)phosphine
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
T_m	melting temperature
TPO	2,2,5,5,-tetramethyl-1-pyrrolidinyloxy
UCL	University College London
UV	ultraviolet
V	volt
w/v	weight-in-volume

1 Introduction

The last decades have seen both a rapid increase in our understanding of biological systems and the development of new and powerful chemical methodologies. With chemists being able to synthesise almost any molecule[1] and biologists able to express tailor made proteins in large quantities[2] a new discipline – chemical biology – has been established at the interface between chemistry and biology, largely recognised since the 1980s.[3] In general this area of research is defined as the investigation of biological processes by chemical methods[4] and ranges from the synthesis of small molecule drugs to the immobilization of proteins on solid surfaces.[5]

One of the central aspects of chemical biology is the ability to chemically modify biomolecules as the applications of these enhanced natural molecules are numerous. They include: the mimicry and understanding of posttranslational modifications (PTMs),[6] the control of cellular response by cell surface modification,[7] the study of protein localisation and interaction-dynamics[8] and the development of biosensors and biocatalysts.[9] Probably the greatest challenge in the field is to overcome the common problems associated with the use of proteins and peptides as drugs[2] such as immunogenicity, proteolytic degradation as well as short half-life and to therefore realise the huge potential of biotherapeutics (or biologicals).[10]

Despite much recent advancement the chemical modification of proteins remains challenging. The often fragile nature and low concentrations of the biomolecules impose a number of restrictions on the chemistry used, such as reactivity and solubility in aqueous media, at ambient or lower temperatures and near neutral pH, as well as tolerance to salt, surfactants and metals.[11] Efficient methods also have to offer rapid and preferably complete conversion of the precious starting material. Finally, as chemical selectivity is crucial to the naturally occurring modifications of proteins (i.e. PTMs),[6] so it is for its artificial analogues. Reactions with high selectivity and site-specificity which would yield well defined products[12] and thus avoid the loss of functionality[13] are therefore highly desirable. Although there are many reagents that fulfil the majority of these requirements, site-specificity is rarely obtained in the presence of the rich functionality of the side chains of the 20 naturally occurring amino acids, which are

also usually present in multiple copies. Thus a key focus of the ongoing research is the development of both highly selective and site-specific methods for protein-conjugation.[1, 14]

1.1 Selective chemical modification of proteins

Selectivity in the chemical modification of proteins can be obtained via two different approaches. Either the reactivity of a chemical reagent is adjusted to be selective for a single natural amino acid or a non-natural group is incorporated into the target protein, which offers a unique reactivity that is orthogonal to the surrounding groups.[7] As this second method has been used to generate very promising results it should be explained in more detail: The introduction of a non-natural group – more than 40 have been successfully used so far,[15] often as amino acid analogues with a specially designed side chain – can be achieved via a range of different methodologies:

Total synthesis: Peptides and small proteins of up to 40 residues can be prepared completely via solid phase synthesis with the non-natural amino acid inserted during this process at the desired position.[16-18] Recent developments in this field have even allowed for the preparation of complicated systems such as insulin.[19]

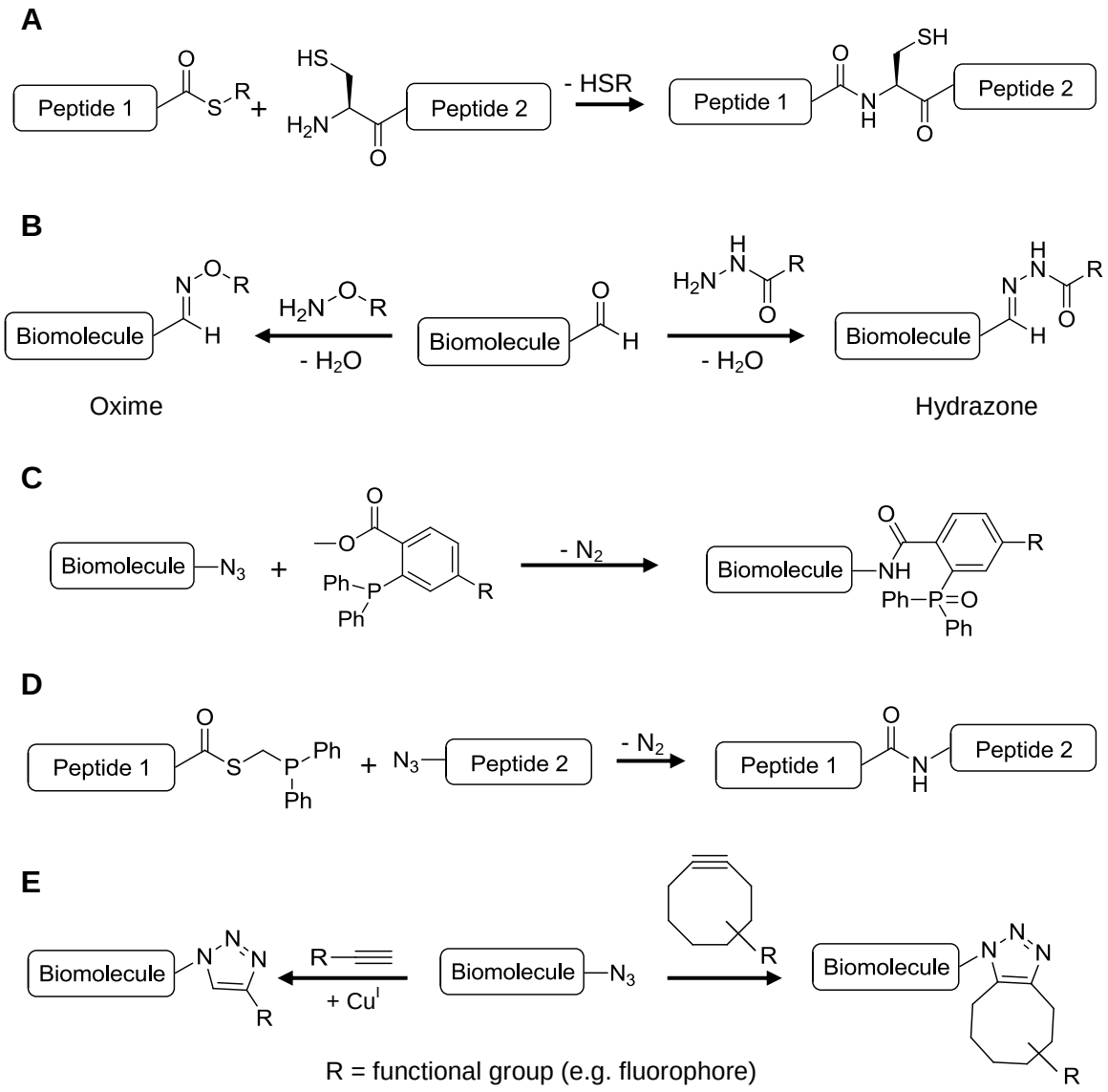
Native chemical ligation: Native chemical ligation (NCL) is the reaction of a thioester with an N-terminal thiol, often the amino acid cysteine (Scheme 1.1 A).[4] It can be used to link peptides derived from total synthesis or recombinant expression, which contain non-natural groups, to assemble full length proteins.[20]

Metabolic modification: During ribosomal protein synthesis the genetic code is translated into a polypeptide sequence via the correct pairing of the tRNA to its specific codon on the DNA in the ribosome. Before this event the tRNA has to be charged with the correct amino acid, a process which is facilitated by specialised enzymes, the amino-acyl tRNA synthetases (aaRSs). These carrier proteins, although specific for their natural amino acid, exhibit a certain degree of tolerance towards non-natural substrates.[8] By feeding an organism with an excess of such a substrate, e.g. selenalysine,[21] during expression it can become incorporated into the translated proteins and can be targeted after purification.

Genetic encoding: Instead of relying on the promiscuity of the aaRSs, an artificial pair of an aaRS and a tRNA are introduced into the organism in order to

insert the non-natural amino acid of interest into the target protein. The corresponding codon is often a rarely used stop codon (the so called amber stop codon) and is placed at the desired position in the DNA sequence of the protein, which has also to be introduced genetically into the expression strain.[22]

Commonly used non-natural groups that offer water compatible chemistry[23] and a good degree of bio-orthogonality are aldehydes, ketones[8] and the more popular azides[24] or alkynes.[25] Aldehydes and ketones react with aminooxy or hydrazine compounds to give stable oximes or hydrazones (Scheme 1.1 B). Various chemical methods have been established for azides. These undergo bio-orthogonal reactions, including the Staudinger ligation of azides and triarylphosphines (C), the traceless Staudinger ligation (D) and the [3+2] cycloadditions of azides and alkynes (E) which are, depending on the alkyne, copper-catalysed or copper free. Many of these reactions have good efficiencies and are therefore referred to as “click reactions”. This term describes reactions that are modular, stereospecific, wide in scope, high yielding, come with inoffensive by-products, have simple reaction conditions and generate products that are easy to purify.[26]



Scheme 1.1: Examples of the chemical modification of peptides and proteins after incorporation of non-natural chemical groups. (A) Native chemical ligation. (B) Reaction of aldehydes (and ketones). (C) Staudinger ligation. (D) Traceless Staudinger ligation. (E) [3+2] copper-catalysed and strain-induced cycloaddition.

The described methodology has been broadly accepted in the scientific community, has generated a wealth of literature and applications and many groups continue to develop it further. Still it is not without disadvantages. Most obvious is the substantial amount of work that has to be invested into the preparation of the protein or organism before the actual modification can be carried out.[27] The introduced modifications can also lead to disappointing yields of expression.[28] In addition, the current chemical methods themselves are not efficient enough.[29] Drawbacks include: slow reaction kinetics,[23, 30] making the use of high reagent

concentrations necessary,[31] toxicity and induced protein-cleavage of the copper-catalysts[32] as well as often non-quantitative conversions,[33, 34] making the removal of unreacted material difficult. In addition, aldehydes and ketones can react undesirably with biological nucleophiles such as amines, thiols or alcohols.[8] Finally it is generally accepted that changing the chemical character of an amino acid side chain too drastically should be avoided in order to minimise the negative impact on the structure and activity of the protein of interest.[3]

Due to this, continuous interest exists in the development of methods of "traditional" chemical modification of proteins,[1] which focuses on targeting the 20 available natural amino acids. Most of the hydrophilic residues have been explored in this context and reagents for the modification of histidine, tyrosine, tryptophan, arginine, lysine, cysteine, serine and threonine as well as aspartic acid and glutamate and even methionine have been developed (Fig. 1.1).[3, 35]

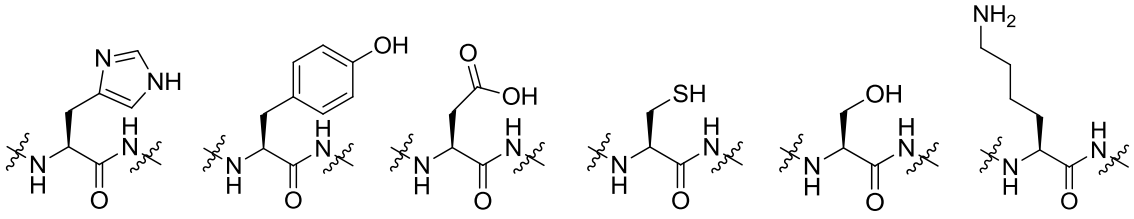


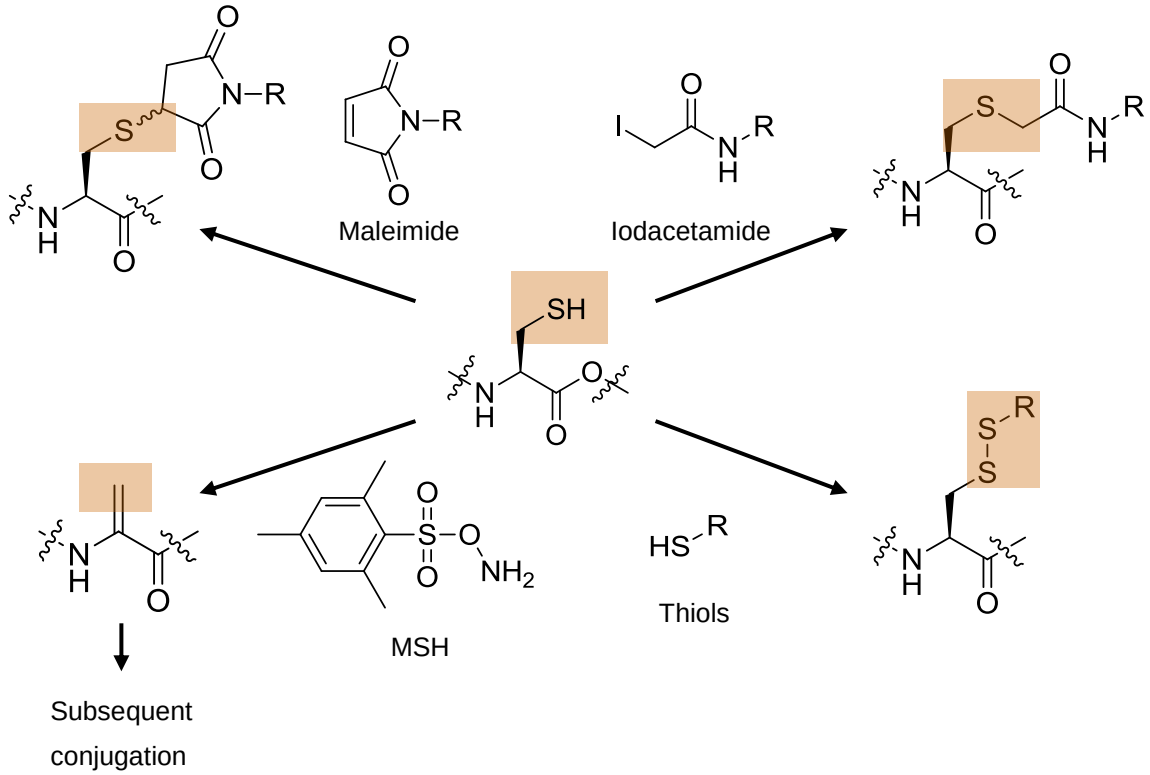
Fig. 1.1: Proteinogenic amino acids targeted for chemical modification. Left to right: histidine, tyrosine, aspartic acid, cysteine, serine and lysine.

Classically attention has been focused on the modification of cysteine and lysine[8] because these two residues offer the best reactivity due to their nucleophilic side chain groups. Although the majority of chemically modified biologicals that have obtained FDA approval to date have been prepared via the targeting of lysines, this technique is now often considered to be non-ideal. The main problem is the high abundance of this amino acid (up to 10%).[3, 35] Virtually all proteins contain several solvent exposed lysines in addition to the amine of the N-terminus, leading to a heterogeneous mix of products with a potentially wide range of loading levels.[36] The result is often a narrow therapeutic window of these conjugates[27] as too many modifications per protein raise problems with solubility[37] and the loss in surface charges induces faster clearance rates from the patients' blood.[38] In addition, lysines in or near the active or binding site of

the protein might be targeted, which leads to loss or abrogation of activity.[39, 40] Furthermore, the heterogeneity complicates the product analysis,[41] and the most widely used reagents for lysine-modification, *N*-hydroxysuccinimide (NHS) esters, have limited storage stability due to moisture sensitivity.[42] Control over the reaction can only be achieved to a certain extent if carried out under strict pH control[43] or extremely slowly[44] to allow only the most reactive lysine present to react. Against this background the naturally occurring amino acid that offers the greatest potential for site-specific modification is cysteine.

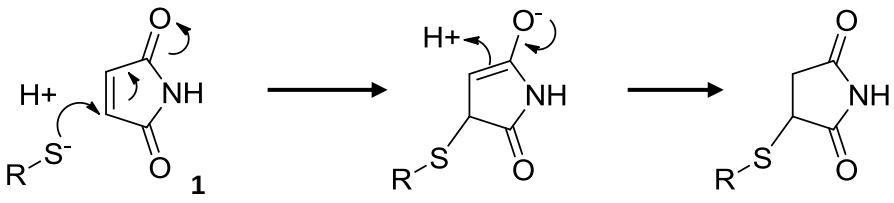
1.2 Modification of cysteine residues

Cysteine is, after tryptophan, the most highly conserved amino acid.[45] Free cysteines are extremely rare in proteins[46] and make up only 0.2% of all naturally occurring amino acids.[47] This, together with the fact that the thiol side chain contains the most reactive nucleophile of all proteinogenic groups at physiological conditions,[48] make it a useful target for the selective and site-specific modification of proteins. Cysteine-targeting chemistry is well established and widely employed to create bioconjugates,[49-52] probe enzyme activity,[53] label proteins,[54] probe the cellular oxidation status[55] or obtain structural information.[56-58] Employed cysteine-reactive groups include α,β-unsaturated compounds, haloalkanes, activated disulfides[59] and reagents that convert the thiol side chain into dehydroalanine (DHA) (Scheme 1.2).[60]



Scheme 1.2: Reagents for the selective modification of cysteine. R = functional group, MSH = O-mesitylenesulfonylhydroxylamine.

The choice of reagent depends on the application and necessities of reactivity. The most widely used alkylation compounds are maleimides **1**.[48] These compounds act as Michael-acceptors upon reaction with cysteine forming a covalent bond (Scheme 1.3).



Scheme 1.3: Reaction of maleimide (**1**) with a thiol to form a thiosuccinimide. The thiol acts as the donor and the maleimide as the acceptor in this Michael-Addition.

Maleimides offer good thiol-specificity[61] and a very limited cross-reactivity with lysine is only observed at high pH.[62] The reaction is usually fast[63] and robust over a broad range of pH.[64] In addition, the imide is synthetically readily acces-

sible to attach functional molecules[65] and some maleimides are membrane permeable.[66, 67]

Alternative alkylation reagents are halo-alkanes with iodacetamide being the most widely used since iodine offers the best reactivity.[68] These compounds react via a S_N2 reaction with free thiols forming an irreversible covalent bond. Halo-alkanes are typically not as reactive as maleimides[69, 70] and therefore often need to be used in large excess, exhibit some unwanted cross-reactivity with amines[71] and can be light sensitive.

DHA is a naturally occurring PTM arising via β-elimination from serine, cysteine, threonine and seleno-amino acids.[16, 72] It is found in natural products such as lantibiotics and thiopeptide antibiotics but also in proteins such as human serum albumin.[21, 73] Its artificial introduction into proteins and peptides has been extensively studied.[74] The conversion of cysteine to DHA gives rise to a chemical handle that can react with selected nucleophiles; a relatively mild protocol for its synthesis has been published recently.[60] However free DHA is not stable, especially in aqueous conditions, which limits its utility[75] and reactions with this PTM yield diastereomeric products due to the newly installed chiral centre.[76]

The most bio-similar method for the modification of a cysteine residue is the formation of a disulfide bond – a naturally occurring structural motif in proteins (see 1.4). The bond formation can happen via oxygen-induced oxidation catalysed by traces of metal ions,[77] but as this reaction is very slow the reagent is usually activated e.g. with a methanethiosulfonate group.[78] Due to its negligible influence on the protein structure this method is often used for spin labelling[79] and additionally, because of its reversible nature by thiol mediated cleavage, for the conjugation of groups that should be detachable under certain conditions.[80, 81] The same property makes the modification of cysteines via a disulfide bond unsuitable for some *in vivo* applications; although the human blood contains only small amounts (typically around 18 µM) of free small thiol-bearing compounds such as cysteine or glycine,[82] the concentration of free cysteines on plasma proteins can reach up to 500 µM.[83] Furthermore the intracellular environment has even stronger reducing properties with concentrations of the cysteine-containing tripeptide glutathione (GSH) up to 10 mM.[84] It is thus necessary to stabilise the protein-cargo disulfide bond, which is possible by the addition of steric hindrance around the conjugation site in the form of methyl groups.[85, 86]

Often overlooked by many users of cysteine-targeting chemistry is the fact that the reactivity of the amino acid side chain can vary significantly.[3] The reactive species is not the thiol but rather the thiolate anion which exhibits an increased reaction speed up to 5 x 10^9 times that of the thiol.[87] Thus both the pK_a of the thiol and the pH of the environment have an impact on the modification reaction.[88] Typical pK_as of protein thiol groups are in the range between 8 and 9[70, 89] but can be influenced by the ionization state of neighbouring groups, hydrogen-bonding and electrostatic effects.[90] Especially positively charged groups in proximity to the thiol can decrease the pK_a.[91] For example in the protease papain the active site cysteine has a pK_a of 4.2.[92] In addition reactivity can be governed simply by the accessibility of the amino acid side chain[66, 93] and it is thus not unusual that two or more cysteine residues within the same protein differ significantly in their reactive behaviour.[3, 94] To control a reaction the pH can be adjusted, for example for the modification of cysteine with *N*-ethylmaleimide (NEM) an increase of the pH from 3.0 to 4.95 accelerated the reaction by a factor of 30.[95] Limitations to the utilisation of cysteine reactivity are the stability of the target protein and its tolerance to the conjugation chemistry used. Depending on the application, maleimide is often considered the best choice.

1.3 Maleimide protein labels

As stated, the maleimide group is amongst the most widely used for cysteine modification and every new generation of protein labels has been attached to this compound to facilitate cysteine-coupling.

1.3.1 Maleimide

Maleimide **1** (IUPAC name: pyrrole-2,5-dione) is a small organic compound with a molecular weight of 97.07 g/ mol that comprises several features which are responsible for its unique activity:

- it is a 5-membered ring
- the ring contains an imide
- two carbonyls are in the 2 and 5 positions
- the 3 and 4 carbons are connected by a double bond
- the double bond and the carbonyls are in conjugation

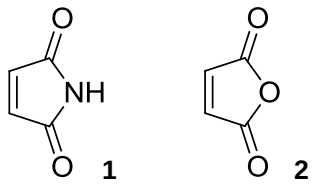
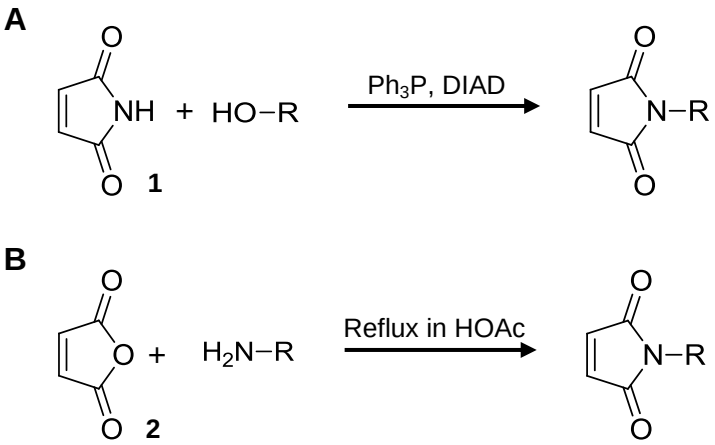


Fig. 1.2: Maleimide (**1**) and maleic anhydride (**2**).

The reactive groups of maleimide (**1**) are the imide and the double bond.[96] The α,β-position of the double bond relative to the two carbonyls make the compound a very good electrophile[97] as this relationship creates very electron-poor carbon atoms in the 3- and 4-positions. Maleimide reacts at physiological conditions[98] but also over a broad range of pH[64] and at high concentrations of chaotrophic substances.[99] Despite this high activity, thiol-nucleophiles are clearly favoured over amines and alcohols as reaction partners of maleimide, due to their "softness".[100]

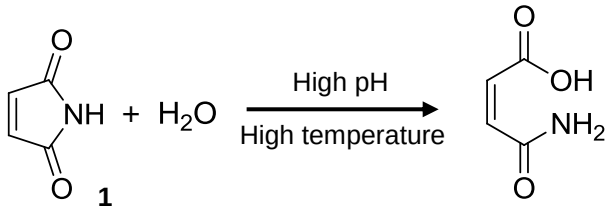
Various methods for the synthesis of *N*-functionalised maleimides have been described. *N*-alkylation can be carried out by the use of silver or mercury salts with simple alkyl bromides.[101] An alternative route is via the Mitsunobu reaction which can be utilised due to the relatively low pK_a of the imide (around 10),[102, 103] where a maleimide is added in place of the acid used in the standard procedure (Scheme 1.4).[104, 105] A more general method is the reaction of maleic anhydride **2** with an amine carrying the desired functional group under acidic conditions.[106]



Scheme 1.4: Synthesis of *N*-functionalised maleimides. (A) Mitsunobu reaction with maleimide (**1**). DIAD = diisopropyl azodicarboxylate. (B) Reaction of maleic anhydride (**2**) with an amine under acidic conditions.

The presence of the conjugated system of the carbonyls with the double bond adds another useful feature to the compound. Maleimides exhibit a relatively strong absorbance (ε_{300} = 620 M^{-1} cm^{-1}) for a molecule of its size and were used to quantify protein free thiols[107] before the advent of Ellman's reagent.[108] The technique was carried out by monitoring the loss of absorbance with a photometer upon conversion of maleimide to succinimide and the accompanied loss of the double bond. The compound is, to some degree, water soluble and can be easily solubilised in aqueous buffers by addition of small amounts (typically around 5%) of organic solvents such as DMF.[109]

Despite these advantages two well-known problems are associated with maleimides. A minor one is the polymerisation of the compound initiated by the double bond, leaving the imide ring intact.[110] This process can be radical- or base catalysed.[111] The second is the hydrolysis of maleimides which results in a heterogeneous mixture of products[112] (Scheme 1.5).



Scheme 1.5: Hydrolysis of maleimide (**1**).

This process is temperature dependent and is induced by high pH to which a linear relationship exists.[113] The half-life of maleimides at 30 °C and pH 7.0 is around 42 h but decreases to less than a day if the pH is elevated to 8.5.[114, 115]

A third problem has come to attention of academic as well as industrial researchers recently during work on maleimide-based conjugation of highly potent cytotoxic drugs to antibodies; under certain conditions the thiosuccinimide adducts are unstable and can slowly release their cargo either via hydrolysis and loss of the maleamic acid, β-elimination after oxidation of the thioether to the sulfoxide,[116] or retro-Michael addition driven by the transfer of the regenerated maleimide unit to another thiol.[117, 118] All these processes are dependent on the solvent accessibility of the thiosuccinimide[119] and the pH of the solution.

Despite these issues maleimides have been used in the synthesis of a host of complex molecules[120-122] and have even been found to be a part of natural products.[123-125] The Michael-acceptor has been employed to target almost any type of biological molecule such as lipids,[126] sugars[50, 51] and siRNA[127] as well as drugs[128, 129] and organometallic complexes.[130] The connection of two maleimide units via a linker affords compounds that can be used for the cross-linking of proteins.[131, 132] With respect to the functionalisation of proteins the most common groups found attached to maleimide are fluorescent molecules,[133, 134] biotin,[52] spin labels[135] and polyethylene glycol (PEG) chains, which will be discussed in turn.[136]

1.3.2 Fluorescein

Fluorescence is the physical phenomenon of light emission that occurs within nanoseconds after the absorption of light that was typically of higher energy (shorter wavelength). During this process an electron is excited upon absorption into a higher energy state. This is followed by its return to the ground state accompanied by the release of light. The advantage of fluorescence is the difference in absorbed and emitted light which makes it possible to excite a sample and obtain the read-out with the same source of energy (light), at the same time and without loss of signal. This system has been realised in a large number of applications; one of the most common experiments is the localisation of biological molecules in cells after labelling with a fluorescent probe or even by genetically encoded fusion to a fluorescent protein. Much more sophisticated experimental settings include fluorescence recovery after photobleaching (FRAP) or Förster resonance energy transfer (FRET) to measure cell membrane dynamics and protein-protein interactions respectively.

Many organic compounds have intrinsic fluorescence-properties whose basis is often a system of aromatic rings and/ or conjugated electron-systems. Because of the importance of this technique more than 3,000 fluorescence probes have been developed.[137] One of the most commonly used is fluorescein (Fig. 1.3) due to its high quantum yield of 0.9.[138]

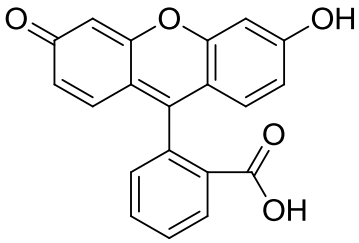


Fig. 1.3: Fluorescein.

The dye is typically exited at 488 nm and has an emission maximum of 518 nm giving a bright green fluorescence. Fluorescein has a good safety profile and has been in clinical use for the imaging of angiography for three decades.[139]
Most techniques for the fluorescence-labelling of proteins are focused on the modification of cysteine[140] since, for many experimental setups, the quantitative and spacial control of the labelling reaction is essential.

1.3.3 Biotin

Biotin is a small organic molecule which consists of a tetrahydroimidazole ring fused to a tetrahydrothiophene ring that carries a valeric acid substituent (Fig. 1.4).

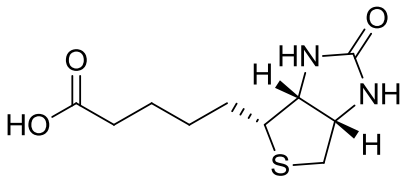


Fig. 1.4: Biotin.

It functions as a coenzyme in different metabolic pathways and is essential for higher organisms (vitamin B_7). Biotin shows the strongest known non-covalent interaction in nature (K_d = 1.0 x10^{-15} M) with the small protein avidin (*Gallus gallus domesticus*) and the closely related streptavidin (*Streptomyces avidinii)*.[141] Due to the reversible nature of the binding event the (strept)avidin-biotin pair can be used after conjugation to a target protein for affinity based separations.[142] The system is very popular and a range of structurally related compounds are commercially available for these and other applications such as diagnostic assays or metabolic quantification.[52] A limitation of the use of the (strept)avidin-biotin technology are the harsh conditions that are needed to sub-

sequently disrupt the interaction and a cleavable linker between the protein and biotin to circumvent this problem would be desirable.[143]

1.3.4 Spin labels

Spin labels are small organic probes that consist of a stabilised radical (often a nitroxide) and a chemical moiety for conjugation with a biomolecule. These compounds are often used for electron paramagnetic resonance (EPR). This is a branch of spectroscopy where electrons with unpaired spins are placed in a magnetic field where they absorb electromagnetic radiation. Thereby they are transferred from a low to a high energy level and emit electromagnetic waves when they return to the ground state. This effect is directly influenced by their immediate environment. EPR is similar to nuclear magnetic resonance (NMR) but instead of proton transitions, electron transitions are observed and the radio frequency, which is lower than in NMR,[144] is usually fixed, while the magnetic field is tuned to obtain resonance.[145] As in NMR the read-out of EPR spectroscopy contains information on the environment of the probe, its dynamics and, if present, the distance from a second spin label. For example, site-directed spin labelling (SDSL) has been used to obtain structural information on proteins where crystallisation trials were not successful.[79, 135]

The position of the spin label on the protein is even more important for EPR spectroscopy than in fluorescence applications. Spin labels with short linkers are desired to reduce segmental mobility as valuable information is comprised in the orientation between the protein and the label.[54] The majority of the conjugation chemistry in this field is cysteine-targeted and common groups such as maleimide (e.g. maleimide-2,2,6,6-tetramethylpiperidine-1-oxyl (maleimide-TEMPO)) or activated disulfide bonds (e.g. *S*-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanethiosulfonate (MTSL)) are used (Fig. 1.5).

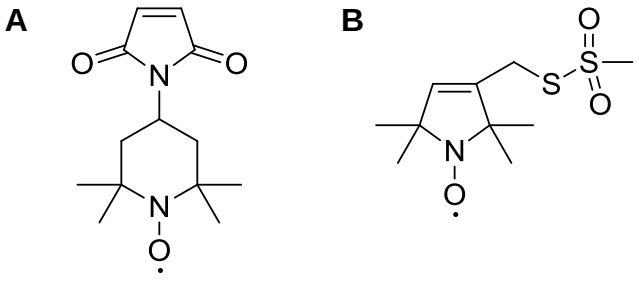


Fig. 1.5: Popular spin labels. (A) Maleimide-TEMPO. (B) MTSL-spin label.

The labels are usually added in large excess and maleimide-conjugated compounds exhibit the best reaction profile.[78] A disadvantage is the need for iterative purification steps of the spin labelled material: Proteins with free cysteines often have to be treated first with a reducing agent, due to the unwanted formation of disulfide bonds. These then have to be removed as nitroxides are sensitive to the presence of thiols.[146, 147] Finally the labelling reaction can be carried out and the excess of label has to be removed by a second purification step as the remaining free spin label would interfere with the measurements.

1.3.5 Polyethylene glycol

PEG is a polymer that is composed of iterative units of ethylene glycol (Fig. 1.6).

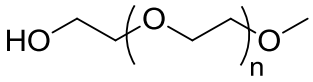


Fig. 1.6: Methoxy-PEG (mPEG) chain.

It has been in use for a long time in everyday products such as toothpaste, shampoo, deodorants and food and drink.[148] PEGylation was discovered as a useful modification for proteins during the first trials with peptide- and protein-derived drugs. These had shown that biomolecules suffer in general from fast proteolytic degradation, short circulating half-lifes facilitated by rapid renal and hepatic clearance and immunogenicity in the human body.[149, 150] When a PEG chain was attached to one of these early biologicals there were a number of improvements; the solubility, absorption, physical and thermal stability as well as the overall half-life increased, the plasma concentrations became more stable, enzymatic degradation as well as renal clearance decreased significantly and the immunogenicity and antigenicity were greatly reduced.[151] This effect is generated by the interaction of the polymer with water. Each monomer subunit binds two or three molecules of H_2O which increases the hydrodynamic size of the PEG chain up to five times larger than a globular protein of the same molecular weight.[43] The theory is that any protein conjugated to a large enough PEG polymer is surrounded by a flexible “conformational cloud” that shields it from the immune system and protects it from degradation and renal filtration.[152] In contrast, a recent structural study found that PEG chains seem to adopt a

domain-like conformation, forming a “dumbbell” with the protein.[153] Nevertheless the polymer is, after initial optimisation of the industrial processes, simple to synthesise, convenient to functionalise[154] and soluble in some organic solvents. PEG chains with variable length, branched PEGs and PEGs with cleavable linkers are available.[155, 156] The FDA has approved the polymer for general use and in 2009 a total of six PEGylated proteins and two PEGylated recombinant antibody fragments had their admission to the market[157] with at least 30 more in clinical trials. PEG was successfully attached to lipids,[158] siRNA,[159] aptamers,[160] nanoparticles[161] and drugs,[162] and has been exploited as a tag for mass spectroscopy (MS).[163]

Besides minor issues with polymer separation and biodegradability,[152] a major problem has prohibited PEGylation from becoming a general modification for biotherapeutics; the activity of most biomolecules depends on physical interaction with its target or substrate and this is easily impaired by the presence of the polymer. Furthermore, the main method for PEG-conjugation utilises random attachment to the lysines and the N-terminus of the protein of interest (6 of the approved PEGylated drugs are prepared in this way)[164] which yields a heterogeneous mixture of isomers.[43] Although it is well known that in some cases the increased half-life offsets the reduced activity,[2, 150] numerous reports exist where the activity was lost completely upon conjugation.[149, 165, 166] This is especially critical in the case of antibodies where the antigen binding site nearly always contains lysine residues.[13]

To obtain more control over the modification reaction and thus reduce the amount of randomised conjugation, site-specific chemical modification can be used.[157] The usual thiol-targeting groups (maleimide, iodacetamide, orthopyridyl disulfides) attached to the polymer are available.[154] Although the length and position of the PEG chain is still an issue, many groups have shown successful protein-PEGylation via this route without significant losses of activity and in some cases even the generation of fully active proteins.[80, 167]

1.4 Protein disulfide bonds

It is obvious that a highly site-specific conjugation method is desirable for many types of protein modifications. If the labour-intensive preceding incorporation of a non-natural amino acid should be avoided or is technically not achievable, the

best choice is the use of cysteine modification chemistry. Unfortunately, despite several advantages, such as the possibility of the pH dependent control of reactivity or the well-established and variable chemistry, cysteine is not the ideal target for the chemical modification of proteins. Despite its low level abundance being the key to selectivity, the amino acid is too rare to be of general use and, if present, is often part of the active site, plays an essential role in the redox-chemistry of the protein[168] or is buried in its hydrophobic core. Although cysteines can be easily engineered genetically into proteins by standard methods[11, 169] and can be well tolerated, especially when inserted in place of a serine,[89] an additional amino acid of this type can interfere with the correct folding,[135, 170] induce disulfide scrambling,[171, 172] unwanted dimerisation[47, 93] or destabilise the protein.[173] For these reasons the production yields of proteins with engineered cysteines can drop significantly.[174-176] Furthermore a substantial amount of these additional amino acids form disulfide bonds with low molecular weight thiols such as GSH during expression, which have to be carefully removed before the subsequent conjugation reaction.[177]

Free cysteine has an abundance of around 0.2% but the total frequency of this residue is 1.7%.[4] The remaining 1.5% are tied up in disulfide bonds – pairs of covalently connected cysteines. Due to the thiol reactivity in combination with the increased abundance, these disulfide bonds (or cystines) present themselves as an attractive target for the chemical modification of proteins.

Cysteine-containing polypeptides typically comprise an even number of this residue to form disulfide bonds.[47] The covalent thiol-linkages can be divided into two sub-groups. The first are structural cystines, which are one of the most common and important structure-stabilisation motifs.[178] Each such disulfide bond adds 2.4–4.5 kcal/ mol to the stability of the protein fold.[179] The amount of stabilisation can vary and mainly depends on the distance in sequence between the two involved cysteines,[180] whereby less than 3 amino acids in between are usually disfavoured due to conformational constraints.[181] The stability gain is generated by the confinements on the distance and angle between the C^{β} and S^{γ} atoms, a destabilisation of the unfolded state by reduction of its conformational entropy and the stabilisation of favourable local interactions.[182] The second type, in contrast, are the redox-sensitive disulfide bonds, which usually do

not provide stability and sometimes even destabilise proteins. They are involved in regulatory processes and sensing of the oxidative status of the cytoplasm.[183] Their function is not yet fully understood but as more and more data on the cellular response to oxidative stress is accumulated, the importance of this type of cystines in the regulation of numerous metabolic pathways becomes apparent.[184]

Intracellular proteins and enzymes normally do not contain disulfide bridges[172] as the cytoplasm is a reducing environment. If they do, such as the bacterial expression factor OxyR or the human chaperone Hsp33, the cystines are often of the redox-sensitive type.[185-187] Secreted and cell-surface proteins on the other hand typically contain disulfide bonds.[188, 189] Small proteins usually have more than one to compensate for the lack of a distinct hydrophobic core[190] and peptides with under 30 amino acids rely on cyclisation via disulfide bonding to maintain a biological active structure.[191] Overall approximately 20% of the proteins expressed in the human body contain cystines[192] – many contain more than one[193] – and the human proteome seems to be enriched with this structural motif in comparison to the protein-set of other organisms.[194]

Disulfides are formed during the maturation of newly synthesised proteins in the endoplasmatic reticulum (ER) or, in case of prokaryotes, in the periplasm[195] and are considered to be a PTM.[196] The required electron-acceptor is provided by a complex machinery of chaperones and protein disulfide isomerases (PDIs),[197] which usually contain disulfide bonds and/ or iron-sulphur clusters[198] and are independent of the presence of small thiol reducing equivalents such as the GSH/ GSSG redox system.[188] The formation of the correct disulfide bonds is often a crucial step in the folding pathway of proteins. Once formed these can provide a folding nucleus for the next steps of structural rearrangement as well as add considerable stability to the folding intermediate.[199] The correct pairing of the free cysteines is ensured by the structural information contained in the polypeptide chain: random cystine formation is followed by disulfide reshuffling; as soon as a first correctly paired disulfide bond is formed it is permissive for further structural rearrangements and becomes buried.[200] Upon this event the two cysteines involved are no longer available for random thiol-exchange reactions and the process is repeated for all other remaining disulfides.[182] Due to this folding process around 50% of all protein disulfide bonds are buried in hy-

drophobic regions[46] and become accessible only after partial or complete unfolding. These cystines often play an important role in the maintenance of the structure while more solvent exposed disulfide bridges add extra stability to the protein.[190] Disulfide bonds between different domains are rarely found.[45] A well-known exception are antibodies where the intermolecular cystines play a crucial role in the formation of the protein complex and its ability to activate the complement system.[201]

1.5 Functionalisation of disulfide bonds

Although the presence of disulfide bonds is limited mainly to secreted proteins, many of which are of therapeutic interest fall into this category.[202] Any method designed for the functionalisation of cystines has to consider three key points: how the disulfide bond is opened, how the reduction of the thiol-thiol bridge is tolerated by the target protein and what type of chemistry is used to modify the free thiols.

1.5.1 Reduction of disulfide bonds

Disulfide bridges, although covalent bonds, are considered to be weak links[172] and in some cases efforts have been made to the develop more stable disulfide bond mimetics.[203, 204] Cystines can be opened in a reducing environment, by heat or alkaline pH.[205] To exercise as much control as possible usually reducing agents are used to cleave disulfide bonds. Here the rate of reaction depends on the compound used, the temperature, the pH, the electrostatic environment and the torsional strain present in the target protein.[206] Most commonly small thiols, which reduce cystines in S_N2 type reactions,[207] such as 2-mercaptoethanol or dithiothreitol (DTT) (Fig. 1.7 A and B) are employed in up to 1000x excess.[208] DTT is often favoured as, in contrast to other thiols, it does not form stable mixed disulfides with free protein cysteines but exhibits a rapid self-cyclisation to form a 6-membered ring.[209] As an alternative to thiols, phosphines are well known to cleave disulfide bridges but many of them are malodourous and/ or not water soluble.[210] In contrast *tris*(2-carboxyethyl)phosphine (TCEP) is both soluble and odourless (Fig. 1.7 C).[211]

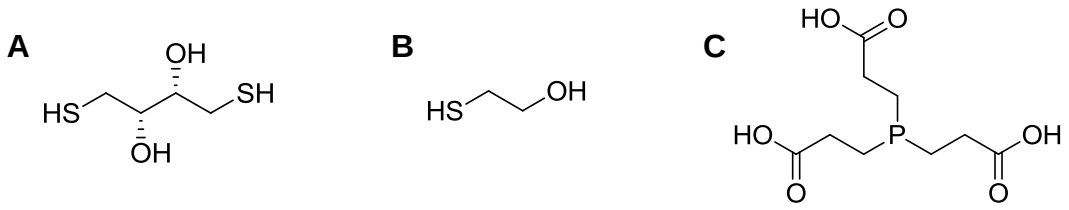


Fig. 1.7: Popular reducing agents. (A) DTT. (B) 2-mercaptoethanol. (C) TCEP.

TCEP reduces disulfide bonds in near stochiometric amounts, is active over a broad range of pH, reacts rapidly (reduction occurs within minutes) and is compatible with many alkylating reagents.[212] The phosphine is more expensive than thiol-based reducing agents and some cases have been reported where it was unable to reduce partially buried disulfide bonds[213] as the spatial arrangement of up to 4 molecules of H_2O around the TCEP-cystine intermediate are necessary to enable the reaction.[214]

An interesting alternative is the use of selenols. These reagents are not able to open disulfide bridges on their own but accelerate the thiol-mediated cleavage up to 90x thereby decreasing the amount of excess needed.[215, 216] This effect is based on the increased nucleophilicity of selenium compared to sulfur. Both elements are similar in their quality as leaving groups, their electronegativity, ionic radius and oxidation states but selenium is softer and selenols have a lower pK_a[217] which makes it easier for these groups to interact with a disulfide bond because of the presence of more selenolate at a given pH.

Thiols and phosphines are considered mild reducing agents.[170] Other reagents such as sodium borohydride or alkali metals are also able to open disulfide bridges but are often avoided due to side reactions or even deleterious effects on proteins.[218]

1.5.2 Effects of disulfide bond reduction

The disulfide bonds of a protein are generally considered to be vital for its stability and activity[185] and accordingly the cleavage of these covalent links destabilises many proteins.[219] Even more problematic is the fact that unpaired free thiol groups, e.g. from a reduced cystine, can act as nucleophiles attacking the remaining intact disulfide bridges forming mixed thiol-thiol bonds with them. This process is called disulfide bond scrambling.[47, 172] Together with the reduction of

one or more cystines, this often results in protein unfolding, potentially followed by aggregation.[220]

On the other hand many proteins exist that contain accessible disulfide bonds that upon reduction do not lose their structure, or in some cases their activity.[45] Sometimes the protein is even able to fold correctly without the formation of its cystines.[221] Often a native-like structure is adopted upon cleavage of a single disulfide bridge[182] and the overall flexibility of this intermediate determines whether unfolding occurs and if the activity is lost or retained. An interesting example is the trypsin inhibitor protein family, which contains two solvent exposed disulfide bonds. The family member found in the soybean retains its structure upon reduction but loses all catalytic activity.[178] In contrast a closely related trypsin inhibitor from the flower plant erythrina is folded and fully active after breakage of both cystines.[222] Despite the retention of structural integrity or even activity, a loss of stability against heat or chaotropic agents usually accompanies the reduction of a cystine.[223]

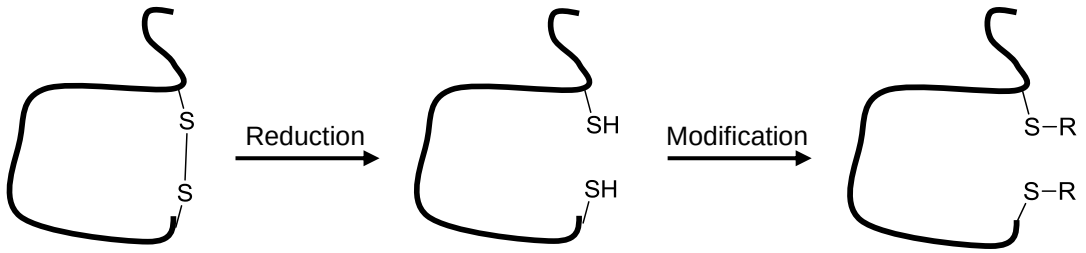
It should be mentioned that the insertion of artificial disulfides in the context of protein engineering to create analogues with increased stability has been attempted by academic and industry-based groups with varying success.[224] In most of the failed attempts the additional cystine was not able to adopt the strict geometrical requirements that are necessary for this PTM to achieve an overall gain in stability[225] or had introduced strain on the native structure thereby offsetting the benefits of additional stability.[226]

In conclusion, every protein has to be treated as an individual case. It seems evident that often the reduction of one or more solvent exposed disulfide bonds is relatively well tolerated, while the cleavage of a buried cystine, for example after partial unfolding, frequently leads to denaturation and aggregation. Only in a few cases single disulfide bridges could be selectively reduced and structurally vital cystines kept intact at the same time in the same protein.[170, 216, 218]

1.5.3 Chemical methods for the modification of protein disulfide bonds

For the mentioned reasons disulfide bonds have been rarely considered as useful targets for bioconjugation. In the simplest case the protein of interest tolerates the loss of the cystine and retains its structure and function upon reduction.

This would afford two free cysteines, which can then be targeted to attach two functional groups with conventional chemistry (Scheme 1.5).



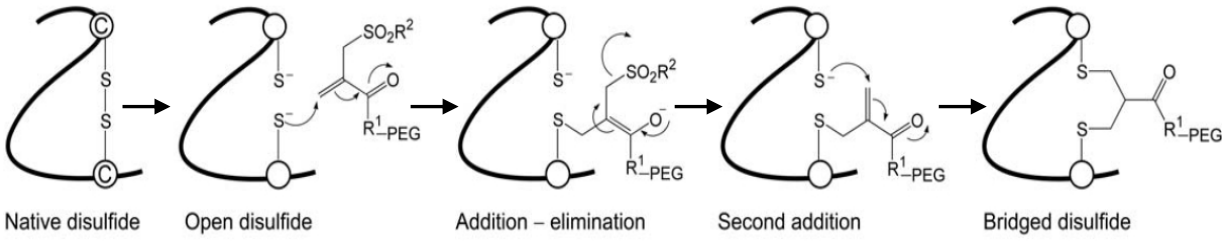
Scheme 1.5: Schematic of the reduction-modification strategy to target protein disulfide bonds for bioconjugation. R = thiol-reactive compound with functional group attached.

An example of this procedure is the attachment of two PEG chains to the peptide hormone calcitonin[227] and it is also used extensively for the preparation of antibody-drug conjugates (ADCs, see 1.6.3). Hereby maleimide-conjugated cytotoxic compounds are linked to the target antibody after complete or limited reduction of their interchain disulfide bonds.[228-230]

As the reduction of a disulfide bond can have a negative impact on the structural integrity of the target protein, it is desirable to re-connect the free thiols during the reaction to regenerate the original covalent link. This could be accomplished by the insertion of an artificial bridge into the disulfide bond which would join the two thiols and also directly carry either the functional group of interest (e.g. a fluorophore or polymer) or a chemical handle to introduce those in a following reaction step. Following this logic, a small number of attempts to modify reduction-generated or a pair of engineered cysteines by bridging have been published. These have usually focused on the introduction of functionality rather than the regeneration or creation of a native disulfide bond, and mainly well-established thiol-targeting groups have been used. Packard and colleagues inserted a fluorophore, which was attached to two free thiol groups into the reduced disulfide bonds of an antibody by formation of two cystines.[231] Spin labels modified with two thiol-reactive groups were inserted between two cysteines via a similar mechanism into other model compounds.[232, 233] Griffin and co-workers used the chelatisation of arsenic groups by thiols to fluorescently label proteins with 4 recombinant cysteines inside living cells.[234] The ability of bisarylmethylenebromides to bridge two cysteines enabled Muppidid *et al.* to maintain

the structure of the helix of a cell penetrating peptide[235] while Caron and colleagues connected two maleimide moieties to a fluorophore to label proteins which had been expressed with a cysteine-containing peptide tag. Finally, tris-(bromomethylene) benzene has long been known to be able to connect three free cysteine residues and has been used to create cyclic peptides.[236] Although the yields are poor and the benzene ring is unsuitable to mimic a disulfide bridge, the method has been commercialised to prepare rigid peptide-templates for the affinity-maturation of antibodies.[237, 238]

The first, and so far only, successful attempt to modify proteins via disulfide bonds whilst retaining their near-native structure has been reported by Shaunak and co-workers in 2006.[239] In this work, an enone-sulfonyl reagent was synthesised that reacts via an addition-elimination step followed by a second addition reaction with both thiols of a cleaved disulfide bridge, thereby re-connecting the cysteines with a three carbon bridge (Scheme 1.6).

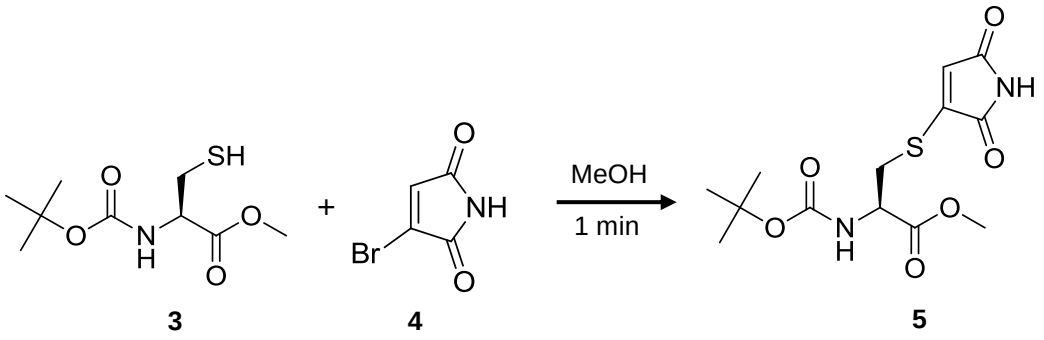


Scheme 1.6: Mechanism of the insertion of a three-carbon bridge into a disulfide bond. Modified from Shaunak *et al.* 2006, Scheme S1.[239]

This re-bridging of the peptidyl-thiol groups prevented the denaturation of the protein (here interferon α-2b) and thus represents a general mechanism for the functionalisation of cystine. It has been shown in subsequent work that this technique allows the insertion the PEGylated compound into a series of proteins of therapeutic interest without too much impact on their activity,[170, 240] which in contrast had been observed when the enzymes were PEGylated by amine-coupling.[169] However, with this method a single S-S bond with an average length of around 2.02 Å[45] is replaced by two S-C and two C-C bonds and the developers suggest the application of bioinformatics tools to determine the suitability of a protein before application of their method.[190, 241] Furthermore, the reaction produces diastereomers by the introduction of a new chiral centre,[242]

seems to be slow (up to 16 h), which would be problematic in the re-bridging of unstable proteins and the preparation of the reagents require a number of work intensive steps with an overall medium yield.

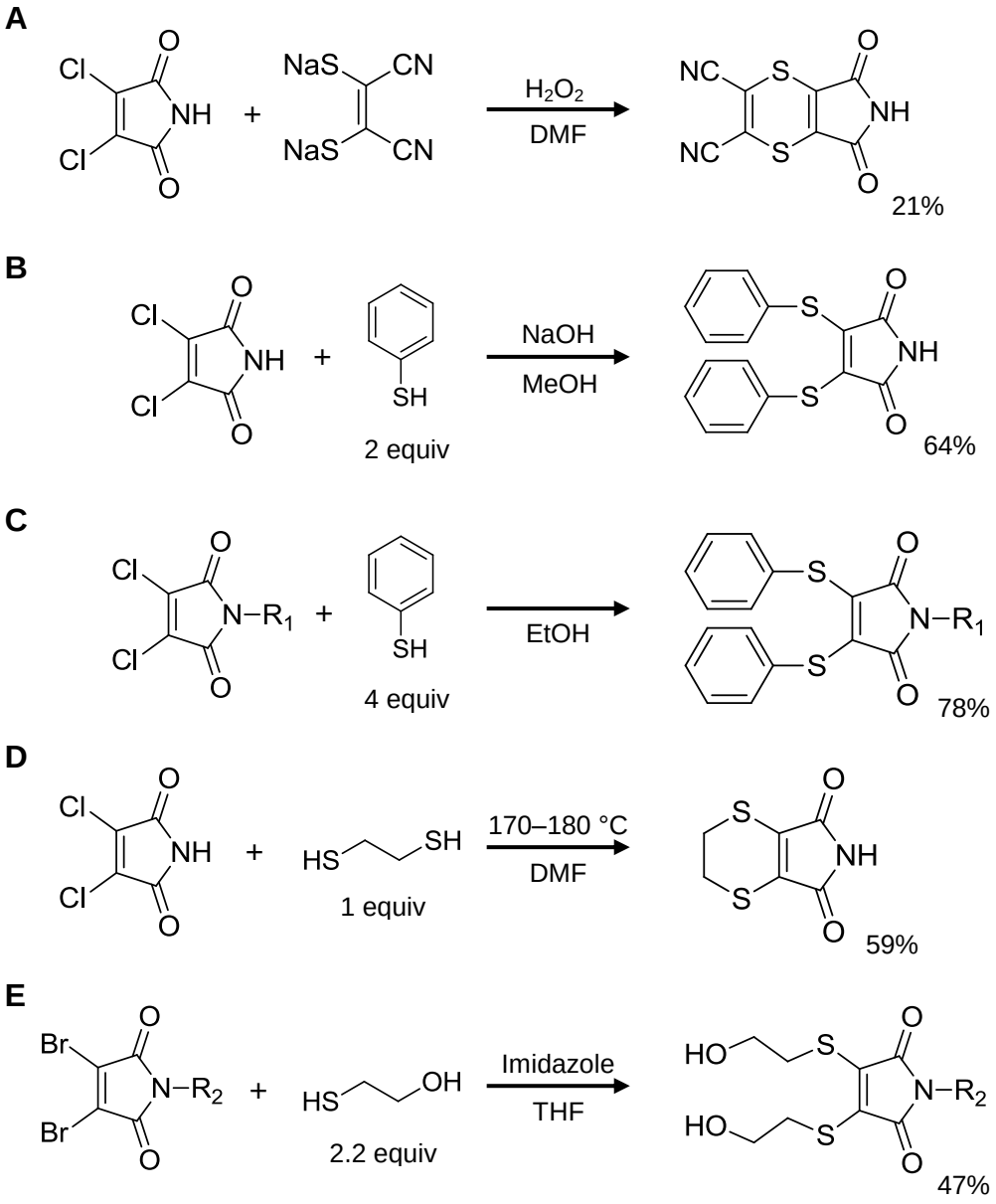
In 2009, Tedaldi and colleagues reported a chemical methodology using maleimide reagents that might overcome some of the issues and serve as a more efficient route to the functionalisation of disulfide bonds.[243] In this work the 3-position of maleimide was substituted with bromine to furnish monobromomaleimide **4**. The bromine atom was displaced upon reaction with a protected cysteine mimic **3** by the thiol yielding a thiomaleimide **5** instead of a thiosuccinimide (Scheme 1.7).



Scheme 1.7: Reaction of monobromomaleimide (**4**) with protected cysteine (**3**) yields a thiomaleimide (**5**).

The reaction was quantitative, highly selective for cysteine over simple amines and monobromomaleimide proved to exhibit a higher reactivity than simple maleimide in a competition experiment. Furthermore, the addition of cysteine was almost completely reversible upon treatment of **5** with TCEP, due to the conservation of the double bond.

Together with earlier reports on reactions of dichloro- and dibromomaleimides with low amounts of small thiols[244-248] or dithiols,[249, 250] (Scheme 1.8) this suggests that dihalomaleimides might be a useful class of compounds to target disulfide bonds, combining the advantages of conventional maleimide chemistry with this new approach.



Scheme 1.8: Selection of the published reactions of dihalomaleimides with mono- and dithiols. (A) Fickentscher 1969.[249] (B) Lynch and Crovetti 1972.[246] (C) Augustin and Müller 1985.[244] R_1 = various amino acids. (D) Mørkved 2007.[250] (E) Muus *et al.* 2010.[245] R_2 = various aryl and arylalkyl groups.

In order to establish a new method for the modification of disulfide bonds, which should be broadly applicable, it is crucial to test it on suitable model systems. These would ideally have a clinical background, in order to obtain detailed information on the impact on stability and biological activity.

1.6 Peptides and proteins with various types of cysteine networks

Several factors will increase the difficulty of targeting a disulfide bond for protein modification. These include the presence of additional disulfide bridges or free cysteines, the level of hydrophobic shielding against the solvent and the importance of the individual cystine in stabilising the protein fold or connecting different domains of a complex. A chosen set of model proteins should thus reflect these possible obstacles.

1.6.1 Somatostatin

Many secreted peptide hormones comprise one or more disulfide bonds.[251] Somatostatins are a family of cyclic peptides that are produced by cells of the endocrine, gastrointestinal, immune and neuronal system as well as various tumours.[252] The molecule known as somatostatin (SST) is a 14 amino acid peptide (1,638 Da) (Fig. 1.8) derived by proteolytic cleavage from a 116 residue precursor. The hormone inhibits the release of growth hormone, insulin, glucagon and gastrin as well as acting as a neurotransmitter and neuromodulator in the brain.[253]

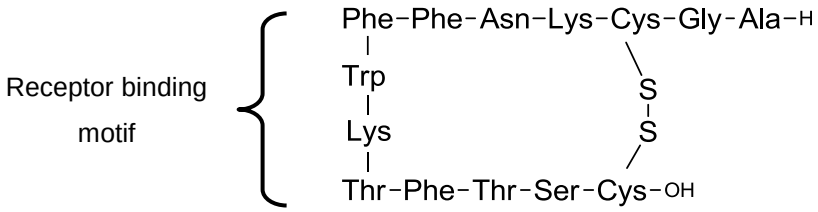


Fig. 1.8: Sequence and structural arrangement of somatostatin.

The peptide is highly flexible and exists in an equilibrium of rapidly interchanging conformations,[254] which has hindered any efforts to obtain a NMR or crystal structure. It exerts its function through binding to five somatostatin receptors (sst_{1-5}), all of which are G protein-coupled receptors (GPCRs) and which have been cloned and expressed.[255] SST receptors are distributed throughout the body but only sst_2 and sst_5 have been explored with reference to their physiological role.[256] The binding motif in SST is known and comprises the residues Phe7, Trp8, Lys9 and Thr10 which are arranged in a β-loop structure.[257]

Due to its inhibitory activities on various pro-proliferative systems and the overexpression of its receptors in a range of tumours,[258] a lot of interest in SST ex-

ists as a potential candidate for oncology. Unfortunately the half-life of SST in the human body is between 4 and 8 minutes and the synthesis of stable and selective analogues has been a challenge.[259] So far no non-peptide agonists have been developed.[260] Stabilising strategies have been adopted instead such as a prodrug approach, the synthesis of peptidomimetics, the covalent attachment of polymers and lipids or the chemical modification of the original peptide.[261] These led to the discovery of the small cyclic peptidomimetic octreotide (Sandostatin) which is an agonist of the receptors $sst_{2,3}$ and sst_5.[262] Octreotide is in clinical use to treat acromegaly, neuroendocrine tumours and hyperinsulinemia[257, 263] and has been further modified by PEGylation[264, 265] as well as by conjugation to radio-labels[266, 267] or fluorescent dyes[268] for diagnosis.

SST contains a single disulfide bond – Cys3 to Cys14 – which is known to enhance its metabolic stability[251] but is difficult to maintain during some of the applied modification processes.[256] The cystine is critical to keep the loop structure of the binding siteintact[269] and all functional analogues either contain the disulfide bond (as in Octreotide) or exchange it for a more stable bridge.[270] It has been previously modified by disulfide bond bridging[240] and would be a simple target to generate a new generation of stabilised SSTs. Clinical demand for these does exist as the full length peptide hormone is, beyond its current use, also a possible drug-precursor for indications such as epilepsy, pain and diabetes.[271]

1.6.2 Insulin

Insulin is a vital endrocrine peptide hormone of 5.8 kDa molecular weight. It is produced and stored by pancreatic β-cells and comprises two peptide chains – an A chain with 21 amino acids and a B chain of 30 amino acids – that are structured by three disulfide bonds. Insulin is synthesised from a 110 residue prepropeptide (12.0 kDa) and maturated by disulfide bond formation and cleavage of the signal peptide as well as the C chain in the ER. Its overall globular structure is mainly helical (45%) with two short α-helices in the A chain (GlyA1 – CysA8 and SerA12 – CysA20) and one in the B chain (GlyB8 – CysB19) (Fig. 1.9). Despite its small size the hormone has a well-defined hydrophobic core.[272]

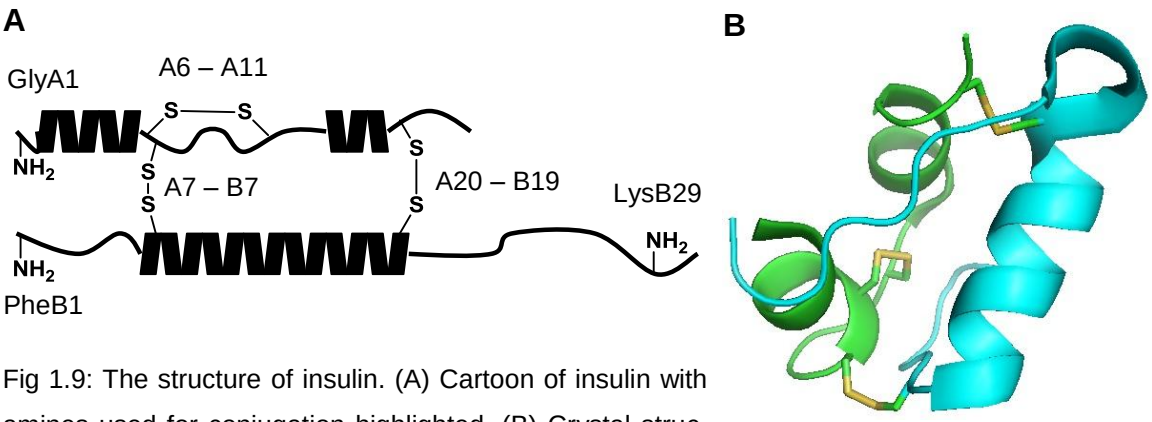


Fig 1.9: The structure of insulin. (A) Cartoon of insulin with amines used for conjugation highlighted. (B) Crystal structure of insulin. Green = A chain, blue = B chain, yellow = disulfide bonds.

Insulin is released from the pancreas upon sensing of increased blood glucose levels and subsequently binds its receptor, which is present mainly on skeletal muscle cells and adipose tissue with high affinity (K_d = 1.4 x10^{-9} M).[273] Evidence exists that suggests the receptor binds a first insulin molecule with high affinity and possibly a second, due to negative cooperativity, with low affinity.[274] A batch of aromatic and hydrophobic amino acids on the surface of the hormone are involved in the receptor-interaction (GlyA1, GlnA5, TyrA19, AsnA21, ValB12, TyrB16, GlyB23, PheB24, PheB25, TyrB26).[275] Insulin quickly forms dimers and trimers of dimers (hexamers) in the presence of zinc[276] (complexed by HisB10) but is only active in the monomeric form as the dimerisation site partially overlaps with the receptor-binding site.[277] Upon binding to its receptor, which is accompanied by substantial conformational changes in the C-terminus of the B chain,[278] the receptor tyrosine kinase (RTK) is activated. The following signal cascade inhibits gluconeogenesis, glycogenolysis, lipolysis as well as protein breakdown and activates glucose uptake from the blood, glycogen and protein synthesis as well as lipogenisis.[279]

Physiological concentrations of insulin are in the medium picomolar to low nanomolar range.[280] Loss of the producing cells, pathological low concentrations and cellular immunity towards insulin result in Diabetes type I and II while an increase in hormone blood levels is associated with obesity, hypertension, cancer and heart failure.[281] Because of growing numbers of diabetes patients worldwide, insulin plays an increasing role as a therapeutic in replacement therapy since its discovery in 1922. The peptide is available relatively inexpensively

from recombinant production as well as from conversion of porcine insulin and increasingly high yielding methods for the total synthesis of the hormone have been discovered in the last decade.[19, 282] Unfortunately, native insulin has a poor pharmacodynamic and pharmacokinetic profile with low physical and chemical stability[283] and short plasma half-life.[284] Additionally, the protein can form insoluble fibrils upon partial unfolding, which are highly immunogenic and inactive.[285] The insulin formulations that are used for the treatment of diabetes today are mainly based on the manipulation of its oligomerisation state.[286] Multimerisation is either induced to create slow-releasing and thus long-acting insulin-types or inhibited to obtain fast-acting hormone preparations. This can be accomplished by mixing insulin with zinc to enhance hexamer-formation or by the introduction of small changes to the peptide sequence (e.g. exchange of LysB29 for Asp) or its surface (e.g. by alkylation) to activate or prevent self-association. Even the most advanced regimes of replacement therapy are insufficient to simulate hormone levels that match the physiological profile and, since all insulin analogues have to be administered subcutaneously, patient compliance is poor.[276]

The chemical modification of insulin might yield the possibility to create stable, long acting and even inhaled or orally available analogues.[287] A significant amount of research in this field has been done, mainly focusing on conjugation of the hormone to macromolecules such as sugars,[288, 289] serum proteins,[290] nanoparticles[291] and, in the majority of cases, PEG chains of various sizes. In most approaches the amino groups of insulin – the two chain N-termini GlyA1 and PheB1 and a single lysine side chain LysB29 – were targeted. Initial attempts of random modification showed that conjugation at the GlyA1 position completely abrogated binding activity and yielded, in general, inseparable heterogeneous mixtures.[284, 292, 293] To avoid these problems a protection strategy was adopted where the critical amino-groups (often GlyA1 and LysB29) were protected selectively by careful pH control, the peptide PEGylated and the protection groups later removed.[285, 294-296] The same strategy was utilised to PEGylate GlyA1 selectively but an additional self-hydrolysing linker was included to slowly release unmodified insulin.[297] In general all these methods are low-yielding, labour intensive and result in heterogeneous mixtures of products. Although some residual activity is often reported, it lies well below that of the un-

modified hormone. In addition, as the interaction of the hormone with its receptor is governed by ionic interactions[298] the modification via amines seems problematic in general. Interestingly conflicting reports exist on the influence of the size of the polymer chain on the retention of activity.[294, 299] Recently Vinther and co-workers successfully introduced an additional free cysteine residue into insulin (at position A21), which was subsequently used to site-specifically PEGylate the hormone, but the group also encountered the usual problems of engineered free thiol groups and the overall synthetic yield was extremely low.[300]

The most important question with regards to the modification of insulin via its cystines is whether the overall structure will tolerate the temporary loss of the interchain disulfide bonds. The two peptide chains of the hormone are held together by two salt bridges, five intermolecular H-bonds, distinctive interchain hydrophobic contacts and the disulfide bridges CysA7 – CysB7 and CysA20 – CysB19.[301] A third, intramolecular disulfide bond, CysA6 – CysA11, is also present. All three cystines are necessary for full biological activity[302] and the hormone starts to unfold upon reduction promoted by disulfide scrambling through free cysteines of the B chain.[303] The thiol groups of insulin are highly reactive and disulfide exchange is observed even under optimised storage solutions.[304] After complete cleavage the A chain is soluble but tends to cyclise while the B chain quickly aggregates.[305] Surprisingly insulin with non-native mixed disulfides retains partial activity as long as the A20 – B19 bond is left untouched.[306] This observation, together with mutational studies,[307, 308] revealed a “hierarchy” amongst the insulin disulfide bonds; if the intramolecular disulfide bridge A6 – A11 is lost then the A1 – A8 α-helix unfolds but the overall structure and a partial activity is maintained. The mutation of the A7 – B7 cystine leads to the unfolding of both A chain α-helices upon which the activity is essentially lost, but the protein is still able to fold otherwise. Without the A20 – B19 disulfide bond insulin missfolds and aggregates.

Taking the available crystal structures of the peptide hormone into account, this behaviour seems like a reversion of the paradigm that the solvent exposed disulfides of a protein have a small impact on its structure after reduction; the non-essential A6 – A11 bond is buried completely in the hydrophobic core of insulin while the A7 – B7 cystine is solvent exposed and the A20 cysteine is

accessible due to a small distortion while the B19 cysteine is buried.[301] Modifying insulin via its disulfide bonds can be expected to be challenging but also rewarding due to the therapeutic importance of the hormone and the limited success of previous attempts at conjugation.

1.6.3 Antibodies

Antibodies are macromolecular protein complexes of 150 kDa and above. They are produced in the course of genetically driven affinity-maturation against a unique target structure (the antigen), often found on the surface of bacteria or viruses, by plasma cells in the blood upon activation of the adaptive immune system. In this context they have three major functions: activation of the complement system to induce complement-dependent cytotoxicity (CDC), cross-linking followed by neutralisation of the target by antibody-dependent cellular cytotoxicity (ADCC) and passive immunisation.

Antibodies consist of four peptide chains, two identical heavy and two identical light chains, which are connected by several disulfide bonds (see below). Each heavy chain is folded in four immunoglobulin (Ig) domains and each light chain in two Ig domains. The overall shape of the complex lies between a “Y” and a “T” as the angle between the “arms” can vary widely (Fig. 1.10).[309, 310]

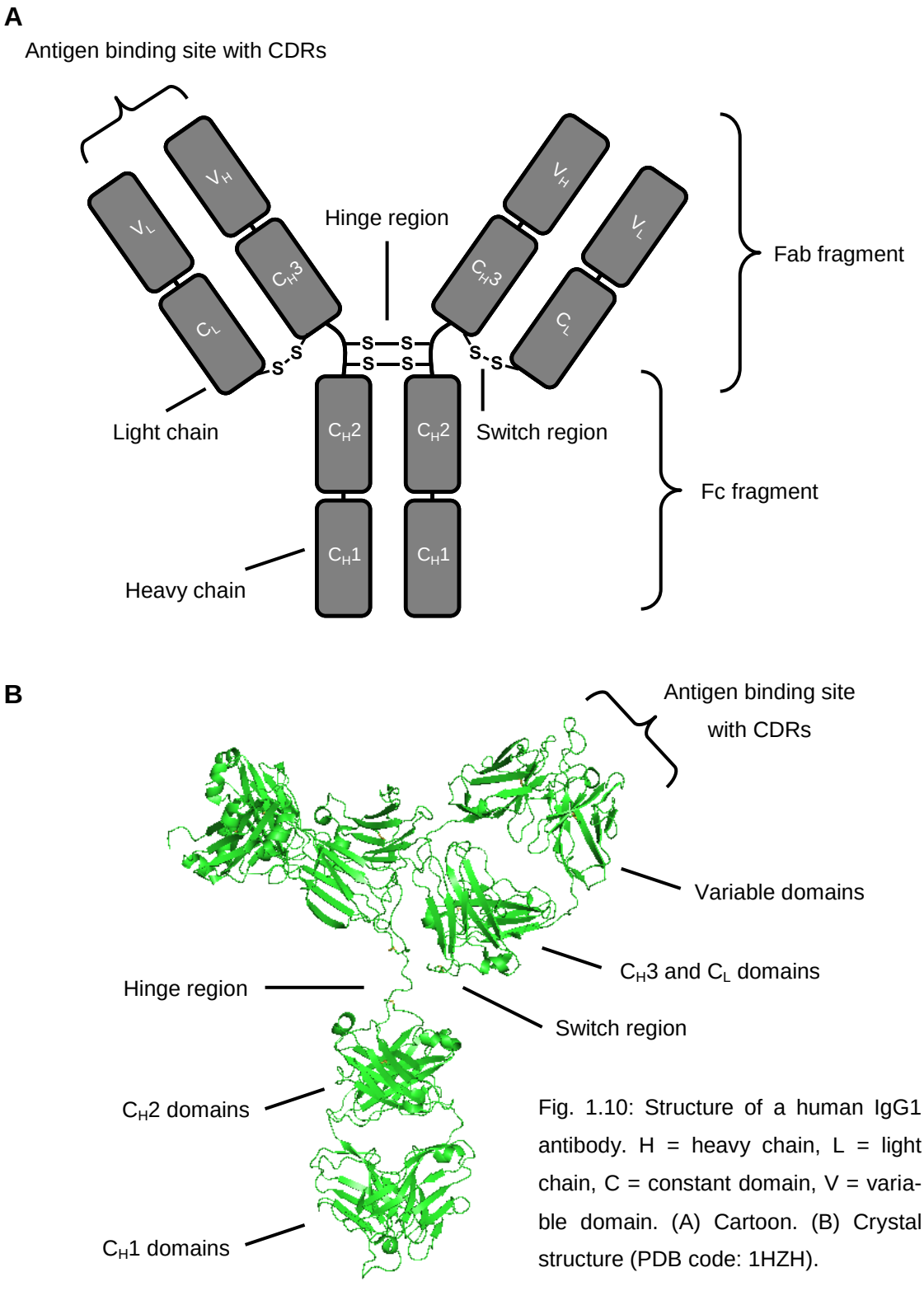


Fig. 1.10: Structure of a human IgG1 antibody. H = heavy chain, L = light chain, C = constant domain, V = variable domain. (A) Cartoon. (B) Crystal structure (PDB code: 1HZH).

Two binding sites for the antigen are located at the ends of the “arms”, which are thus called Fab (fragment, antigen binding). The recognition element resides in the complementarity determining regions (CDRs) – three hypervariable peptide loops on the top of each of the variable domains, which give antibody

binding affinities usually down to nano- or picomolar range. The effector functions of an antibody – binding and activation of the C1q component of the complement system as well as of the Fc receptor on immuno-competent cells – are located on the base, termed Fc (fragment, crystallisable). Due to variations in the heavy chains, antibodies are divided into five different isotypes (IgA, D, E, G and M) and are usually glycosylated on the C_H2 domain. In addition, four subtypes of IgG exist (IgG_1 to IgG_4), which differ in the architecture of the hinge region and number of cystines present within it.[311]

Overall, the antibody disulfide bonds can be divided into three types: i) Each Ig domain comprises a single disulfide bond which adds additional stability to its structure[198] and is buried in the hydrophobic core of the mainly β-barrel structure and thus is not solvent accessible.[312] ii) The two heavy chains are connected by between one and up to 15 disulfide bridges[169] in the hinge region where the two short arms of the Y structure meet and which are sequentially accessible by reducing agents.[313] iii) The single cystines that connect the heavy and the light chain in the switch region, which are often less solvent accessible than the hinge region disulfide bonds.[311]

The sequence of amino acids comprising the cysteines that form the hinge and switch region disulfide bonds is highly conserved within species and isotype and is usually very rich in proline residues.[314] Although these introduce some rigidity into this otherwise flexible part of the antibody, only a small number of crystal structures exist with atomic resolution of the hinge region.[315, 316]

Because of their useful ability to bind a single target with high selectivity and specificity, methods for the maturation and production of antibodies were quickly developed. The proteins were initially prepared via the hybridoma technology, where a B cell, which produced the desired protein, was fused with a cancer cell to prepare an immortalised cell line.[317] Monoclonal antibodies (mABs) with high specificity, affinity and solubility are now rapidly selected from millions of clones in phage-, ribosomal- or bacterial surface displays[318] and isolated in gram per litre quantities from mammalian cell cultures.[319]

With this material a broad range of applications in research and *in vitro* diagnostic methods has been established in the last 3 decades. Antibodies are the essential part of the ELISA and Western Blot techniques, are used on Biacore

chips and array plates and are the basis of immunohistochemistry, immunofluorescence, immunoprecipitation and many flow cytometry protocols to name just a few. Due to their biological activity antibodies are also used in therapies where the killing of a specific cell type or population is a possible treatment option, such as in cancer or autoimmune diseases.[320] Although this idea has generated a multi-billion dollar market and one third of all biotechnology products under development today are antibodies,[321] only around 30 of these molecules have obtained FDA approval to date and most of them have to be used in combination with other forms of treatments.[10] Initial problems with antibody-based therapies were the high immunogenicity of murine immunoglobulines used and low target affinities.[322] These have been solved with the advent of chimeric, humanised or fully human antibody technologies and the development of the above mentioned ultra-high throughput maturation assays. Today the main reason for the failure of many mABs in clinical trials is poor efficacy based on insufficient cell-killing activity.[323, 324] Various solutions to this problem are under development but most of them are based on what has become the standard for the majority of research and diagnostic applications – the covalent linkage of the antibody to an effector- or reporter-molecule:[325, 326]

Antibody fragments and multi-valent constructs: The modular structure of antibodies allows the allocation of functions to domains. Thus as the binding activity is confined to the CDR regions, small fragments of a full antibody can be produced which retain full target specificity and affinity. Initially these were generated by proteolytic digest of the complete proteins with papain and pepsin, enzymes which cleave above the hinge region (generating Fabs) or just below the hinge region (generating $F(ab')_2$) respectively.[327, 328] Modern biotechnology has made the creation of a vast number of formats of fragments possible and a comprehensive description is beyond the scope of this introduction and has been reviewed elsewhere.[329, 330] The most robust and popular fragment is the variable fragment (Fv), comprising only the variable domains of the heavy and light chain (V_H and V_L) (Fig. 1.11).

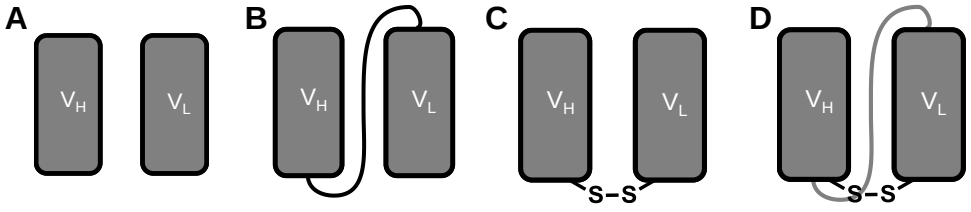


Fig. 1.11: Cartoon of Fv based antibody fragments. (A) Fv. (B) scFv. (C) dsFv. (D) ds-scFv.

Although the domains are held together by a number of non-covalent interactions, which can be enhanced by certain mutations,[331] the Fv is usually too unstable for *in vivo* applications.[332, 333] Therefore the Ig domains are usually cross-linked either chemically,[334] by a peptide linker[335] (resulting in a single-chain Fv, scFv), or by a disulfide bond[336] (resulting in a disulfide-stabilised Fv, dsFv). To combine the high expression yield of the scFv and the stability against unfolding and aggregation of the dsFv, a number of groups have prepared fragments with both stabilising features – ds-scFvs.[337-339]

The advantages of antibody fragments are their high production yields in microbial systems, reduced immunogenicity and good tissue penetration,[340, 341] which is of special importance for the treatment of solid tumours due to their high internal pressure.[342] Although desirable for many imaging applications, the fast renal clearance rate of the small fragments[343] together with the loss of its natural effector functions (as the Fc is usually not present) are the main disadvantages. The short circulation time of antibody fragments can easily be overcome by PEGylation,[169] however an attractive alternative is the linkage of two or more fragments. This does not only increase the size of the construct but also affords superior binding activities due to an increase in avidity. Multimerisation can be accomplished by chemical coupling,[344] the introduction of disulfide bonds,[345] the attachment of naturally dimerising structures such as helix bundles[346] or the special design of the peptide linker in an scFv, which induces the formation of non-covalent dimers.[347] The last concept, known as diabodies, is especially attractive due to its simple construction and reasonable production yields.[348]

To equip antibody fragments with effector-functions they can be coupled to chemical compounds in analogy to full antibodies (see ADCs below). Alternatively, the multi-valent formats offer the possibility to combine two fragments

with different target specificity – so called bispecifics[349] (although similar constructs have been designed with full antibodies).[350, 351] Besides the obvious advantages of targeting two antigens simultaneously, even more interest has been generated by the connection of a cancer-epitope targeting scFv with a T-cell targeting scFv (such as anti-CD3).[352] These bispecific T-cell engager molecules (BiTEs) can stimulate resting T-cells to an immunreaction against cancerous tissue[353] with cell-killing activities in the sub-picomolar range.[354] The main hurdles to overcome for this technology to reach the market are low production yields and the generation of ill-defined by-products.[355]

Antibody-directed enzyme prodrug strategy (ADEPT): In this approach the antibody is coupled, either through genetic fusion, chemically or by non-covalent interactions (such as a biotin/ streptavidin pair) as a targeting-device to an enzyme. After administration and clearance of the unbound antibody-enzyme constructs a potent pro-drug is administered which is cleaved into its active form by the enzyme.[356] The localisation by the antibody thus targets the drug indirectly to the tumour site allowing the use of highly potent compounds. Besides poor efficacy[357] another key problem is the complexity of the multi-component system: coupling of antibody and enzyme is often not efficient, mammal proteins can be present in other tissue while non-mammalian material is immunogenic[322] and the clearance of the large constructs is difficult to control.

Immunoconjugates and ADCs: The idea of an immunoconjugate is relatively simple: An antibody is coupled to a cytotoxic compound – typically a radionuclide, a toxin or a small molecule drug. Therefore, unlike conventional therapy, this compound is not free upon administration to circulate the patient's blood system and thereby to reach and damage healthy tissue,[358] but is directed by the antibody only to the diseased cells (Fig. 1.12). The conjugate then enters by endocytosis after antibody-binding to the antigen, which is localised on the cell-surface. The cytotoxic compound is released and induces cell death.[359]

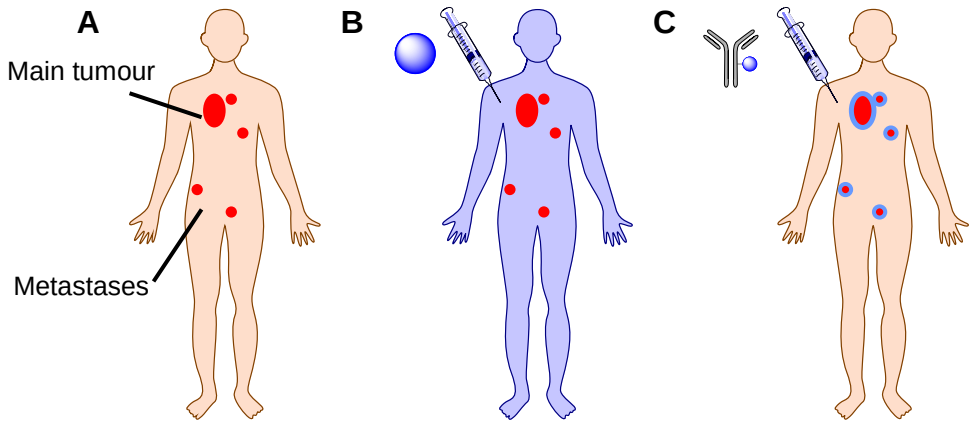


Fig. 1.12: Principle of antibody-based targeted therapy (here for the treatment of cancer). (A) Distribution of tumour and metastases (red) throughout the patient body. (B) Systemic distribution of a cytotoxic compound (blue) during conventional chemotherapy. (C) Targeted delivery of the cytotoxic compound by conjugation to an antibody.

Despite a long development time marked with many failures,[318] ADCs are now the most successful approach to enhance the efficacy of antibody therapeutics with two radio- and two small molecule drug-conjugates approved by the FDA (although one ADC has been voluntarily withdrawn from the market).[10, 360] As radioactive compounds are difficult to handle and immunotoxins are often highly immunogenic,[361] most research interest exists in antibody-drug conjugates. Similar to the ADEPT-constructs, every component of an ADC has to be considered carefully; the drug must be very potent as the delivery efficiency is very low[362] and ideally its mechanism has been validated. Besides the usual requirements to the antibody its antigen must be highly and specifically expressed on the target cells and able to internalise.[363] The linker has to be stable in blood and release the cargo inside the cell although non-cleavable linkers, where the drug is separated from the antibody by proteolytic digest of the protein, are also used successfully.[85, 364] The method of drug conjugation to the antibody has long been underestimated in its importance to the production and properties of ADCs. It is now agreed that heterogeneity is an issue at all steps of the process of development[41] and one of the reasons for unwanted off-site toxicity – the main problem of immunoconjugates.[38]

The majority of the described methods require the covalent connection of domains, proteins and chemical compounds as do many of the other emerging

technologies to improve biologicals. Although genetic fusion is sometimes possible, chemical conjugation methods are usually considered more efficient and easier to control. Here different routes have been explored in antibody fragments in comparison to full length antibodies.

Initially conjugates of antibody fragments were prepared by lysine-targeting chemistry. Problematic here is, that with shrinking size of the antibody construct the share of the (lysine rich) antigen binding site on the total protein surface is increasing, which leads more frequently to the formation of inactive products.[365] For this reason site-specific conjugation becomes even more important when working with antibody fragments. As the cystine in the Fab fragment is not important for its binding activity,[366] some attempts have been made to use the cysteines of the reduced disulfide bond for conjugation but the stability of these constructs was greatly decreased[367] and cleavage and fast blood clearance rates were observed.[368, 369] The production of scFv and other small fragments in bacterial expression systems makes them ideally suited for the introduction of additional functionality. Thus the engineering of free cysteines as handles for site-specific chemical modification has become standard and is used for various application such as multimerisation,[370] biotinylation[371] or radio labelling,[372] but faces the usual drawbacks of low expression yields or unwanted dimerisation.[312] As an alternative, but with its own disadvantages (see 1.1), non-natural amino acids can be introduced and have been used, for example, for immobilisation[373] or the preparation of bispecifics.[374]

The options for the modification of full antibodies are more limited (Fig. 1.13).

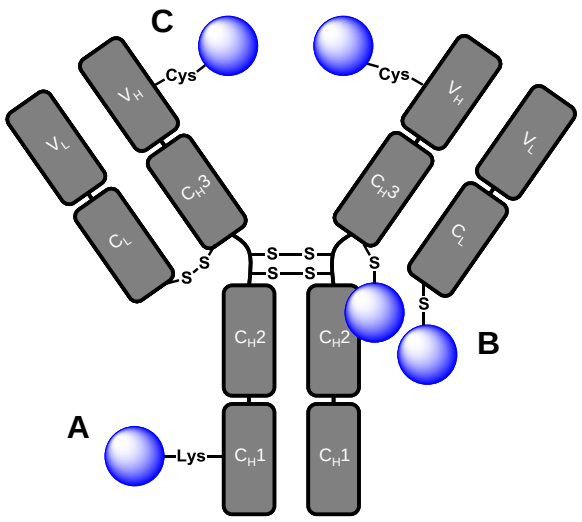


Fig. 1.13: Methods for the chemical modification of full antibodies. (A) Targeting of surface lysines. (B) Reduction and modification of intrachain disulfide bonds. (C) Introduction of free cysteine residues.

Still the most popular method is the conjugation via amino-groups.[375] This material is usually active enough for most immuno-based research or *in vitro* diagnostic methods but the production of immunoconjugates is more difficult. Although carefully developed protocols yield products with an average of 3-4 drugs per antibody, these still exist in heterogeneous mixtures that range from molecules without any cytotoxic compound to those attached to 8 or more drugs.[38] While the naked antibodies are inactive and block potential binding sites, the overlabelled material is prone to aggregation, is quickly cleared and toxic.[37]

To achieve increased homogeneity the disulfide bonds of antibodies can be targeted. The covalent connections between the peptide chains are not essential for the stability of the complex, which is held together by a large number of hydrophic as well as Van der Waals interactions and salt bridges.[376] Therefore the dissociation half-life of a fully reduced antibody is weeks to several months.[377] Initially all 4 cystines of an IgG1 were targeted[228] but these constructs exhibited high toxicities and, despite good binding activity, existed as fragments after the conjugation reaction[378] with an increased uptake in the kidneys of the light chains in particular.[379] Partial reduction with only a few equivalents of DTT or TCEP can be employed to overcome this problem. Such treatment opens only a limited number of the antibody disulfide bonds and yields, relatively consistently, an average of 3.5 drugs per antibody.[229, 380] This technology has been marketed

very successfully and is used to produce the only FDA approved clinically used ADC at the moment (Brentuximab Vedotin by Seattle Genetics).[360] Despite these achievements this conjugation protocol is not ideal and production yields can be as low as 10%. In addition, although selectivity for the hinge region has been claimed, contradictory observations have been published[230, 313, 379] suggesting that a certain heterogeneity exists with respect to which disulfide bonds have been reduced and that the number of attached drugs varies. Furthermore, reduction and mutation studies have shown that intact hinge region disulfide bonds are vital for the activation of CDC,[381, 382] it's modulation,[201, 383] as well as initiation of ADCC and the activation of phagocytosis.[384] Finally, intact disulfide bonds stabilise antibodies under non-native conditions such as heat or chaotropic substances[223] which could be important, for example, during storage.

To achieve real homogeneity it would be desirable to introduce a specific chemical handle into full antibodies for chemical modification. The engineering of free cysteine residues has been difficult due to its potential interaction with the many native disulfide bonds and need for solvent accessibility.[385] Although earlier reports exist,[326, 386] the first successful attempt to make an ADC via this route has been published by Junutula and colleagues in 2008.[177] Despite carrying only two drugs per antibody (one free cysteine per heavy chain) the potency of these "Thiomabs" is similar to lysine-conjugates[387] and ADCs derived from the reduction of interchain disulfide bonds.[388] They show better efficacy and have a better safety profile as well as an increased stability and plasma half-life. Drawbacks are complicated production protocols due to capping of the additional thiol groups, low expression yields and problems with the stability of maleimide adducts (see 1.3.1).[119]

In summary, antibodies and their fragments seem to be the perfect representation of the long sought after "magic bullet" due to their high binding specificities and selectivity. The "targeting half" of this device can now be made relatively easily thanks to advances in biotechnology but, until now, chemistry has failed to attach the "killing half" efficiently. Amongst other approaches the development of new methods for the chemical modification of antibodies is of high interest and targeting their cystines by selective bridging seems feasible and ideally suited to solve a number of the present problems.

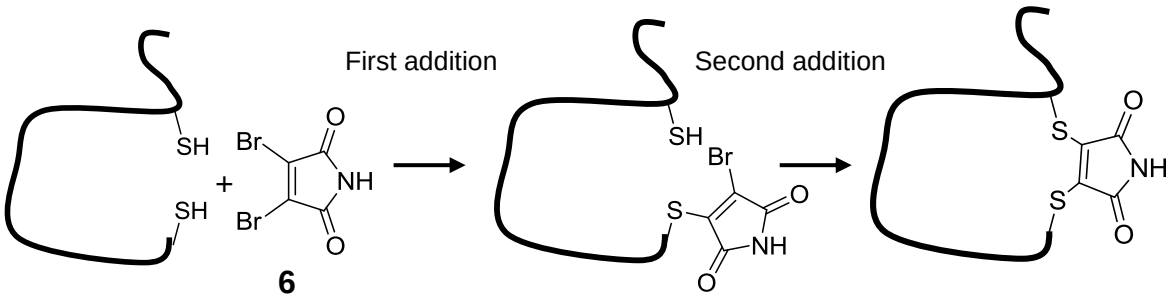
1.7 Project aims

Disulfide bonds are an important feature of numerous secreted proteins, many which are of therapeutic interest, and a good knowledge base also exists to introduce them artificially. Targeting cystines offers two important advantages – site-specificity and high selectivity. It avoids many of the problems associated with the introduction of additional cysteines or non-natural amino acids since it is present as a naturally occurring structural motif in many proteins and the reactive groups (the two thiols) are masked in its oxidised state. Due to their function (see 1.4), an ideal modification process has to regenerate the covalent linkage between the two thiol groups and Brocchini and colleagues have shown strikingly that this can be achieved by intelligent design of the bridging compound (see 1.5.3).[170]

As the published method suffers a number of disadvantages, the aim of the project at hand is the development of a new method for the efficient "functionalisation by re-bridging" of peptide and protein disulfide bonds. The starting point is the selection of an alternative chemical scaffold with better adapted properties. The bridging compound is ideally small in order not to elongate the native cystine too much and thus has two thiol-reactive centres close to each other. High electrophilicity will guarantee good reactivity, which should not be decreased after the first addition reaction. A symmetric compound avoids the introduction of regioisomers, and the reaction would ideally not introduce chiral centres to avoid the formation of diastereomeric products. Any rigidity within the structure should mimic the geometric constraints of native disulfide bonds. An attachment point for functional or linking groups should be synthetically easily accessible. The ideal compound is also reasonably water soluble and an inexpensive precursor should be commercially available.

Despite this long list of requirements an ideal starting compound was identified at the start of this project. Inspired by basic maleimide chemistry the Baker group had synthesised monobromomaleimides, which showed high reactivity and specificity towards thiols (see 1.5.3).[243] While the properties of the maleimide moiety fulfil many of the listed points (see 1.3.1) addition of a second halide turns mono-substituted maleimides into symmetric compounds with two thiol-reactive centres (see Scheme 1.8). Amongst these, dibromomaleimide is commercially available. In theory it could be used to connect the cysteines of a

reduced disulfide bond with a two-carbon bridge after displacement of the bromines by the thiol groups (Scheme 1.9).



Scheme 1.9: Theoretical bridging of a reduced disulfide bond with dibromomaleimide (**6**).

The retention of the double bond should minimise the length of the introduced spacer. The crystal structure of a simple dithiomaleimide compound shows a distance of 3.47 Å between the thiols[248] instead of the usual 2.04 Å of a disulfide bond[241] and such small changes in the length of cystines can be well tolerated even in small peptide hormones.[389]

With this background the working hypothesis of this project is: Dibromomaleimide can be used to efficiently bridge protein disulfide bonds. Initial tests of this idea will be carried out in a small molecule setting by analogy to the mentioned work by Tedaldi *et al.*, to understand reactivity and reaction conditions.[243] If successful the findings will be transferred to a simple peptide system. Somatostatin (see 1.6.1) seems ideal here, both as a representation for the many cystine-containing small peptide hormones as well as for a direct comparison with the work carried out by the Brocchini group. In case dibromomaleimides can compete with the established methodology following three aspects will be further examined: i) The chemistry by preparation of *N*-functionalised halomaleimides and exchange of the bromines for other suitable leaving groups. ii) The biology in form of activity testing of the synthesised somatostatin analogues and iii) interfacing issues such as the kinetics of the reaction or the stability of the products. After this first round of optimisation, translation of the findings will be attempted, potentially now based on other types of 3,4-substituted maleimides, to more complex systems. Insulin (see 1.6.2) will be used as a target with multiple disulfide bonds, which have different solvent accessibilities. Cystine contain-

ing antibody fragments and full length antibodies (see 1.6.3) are macromolecular targets with an immediate clinical background and their modification should enable the expansion of the comparison of the developed chemistry with other bioconjugation techniques.

Although it is desirable that most of the prepared protein analogues will have direct applicability for research, diagnosis or even therapeutic methods, the aim of the work at hand is not to follow up on these but to understand and partially optimise the aspects of the envisaged disulfide bridging chemistry. Hence the focus lies on the development of efficient and realistic conjugation protocols that yield products with desirable properties such as good stability, solubility and full activity. It is hoped that this will create a solid and attractive technology platform for a multitude of applications which will help to realise the full potential of biotherapeutics and to fulfil the promise that these might be a core principle of the next generation of therapeutics.

2 Results and Discussion

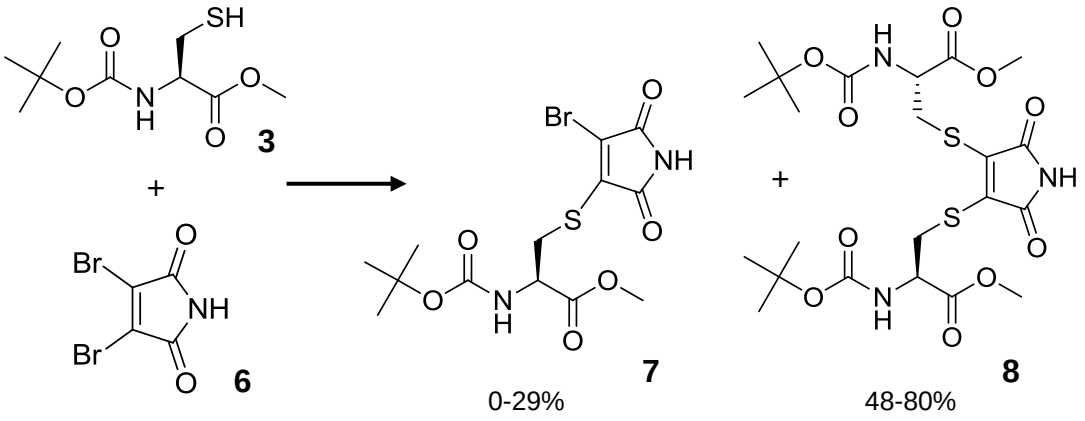
2.1 Reactions of dihalomaleimides with small thiols

2.1.1 Synthesis

Dibromomaleimide is commercially available. Diiodomaleimide was synthesised as described in the literature.[390] Dichloromaleimide was kindly provided by Dr James Baker (UCL Chemistry), who followed a published procedure for this synthesis.[391]

2.1.2 Dibromomaleimide bridges two cysteines

As a test of the working hypothesis that a small molecule model system was set up. Dibromomaleimide **6** was reacted with a protected L-cysteine analogue (*N*-Boc-L-Cys-OMe) **3** in organic solvent (MeOH) (Scheme 2.1) to mimic the reaction of the maleimide with peptidyl thiol groups. A few variations of the reaction were performed (Table 2.1).



Scheme 2.1: Reaction of dibromomaleimide (**6**) with protected L-cysteine (**3**) and proposed products. Both the mono- (**7**) and the di-addition (**8**) product were observed.

Table 2.1: Optimisation of the reaction of dibromomaleimide with protected L-cysteine. Buffer = 150 mM NaCl, 100 mM sodium phosphate, pH 8.0, 7.5% DMF. *Product contains up to 5% impurities. N.d. = not determined.

Reaction	Yield mono-addition product **7** [%]	Yield di-addition product **8** [%]	Solvent	Equiv maleimide	Equiv L-cysteine	Base
1	n.d.	n.d.	MeOH	1.0	2.2	none
2	29.1*	47.9	MeOH	1.0	4.0	$NaHCO_3$
3	7.1	69.6	buffer	1.1	1.0	buffer
4	n.d.	45.0	buffer	10.0	1.0	buffer
5	0.0	80.0	buffer	1.0	2.2	buffer

Upon addition of the cysteine analogue **3** to dibromomaleimide **6** the reaction turned yellow immediately indicating the formation of the mono- **7** and di-addition **8** products. All the starting material had been consumed after approximately one minute, as detected by thin layer chromatography (TLC) analysis. When the reaction was initially set up without a base (entry 1), reversion of the addition of cysteine to bromomaleimide occurred and starting materials were detected by TLC analysis after 24 h at 4 °C in the dried product mixture. The competition between the thiol group and the hydrobromic acid, which is formed during the reaction, for the electrophilic centres of the maleimide is potentially the reason for this observation. Reversion was no longer found by TCL analysis when a base was added in excess before mixing both reactants and the di-addition product was isolated pure from reaction 2.

The model system was then transferred into an aqueous solution to test its efficiency in an environment that would be suitable for proteins. A buffer system was chosen which contained a concentration of NaCl commonly used in biochemical methods as well as a strong phosphate buffer system at a basic pH (8.0) to increase the population of the thiolate anion. DMF (up to 7.5%) had to be added to solubilise the organic compounds.

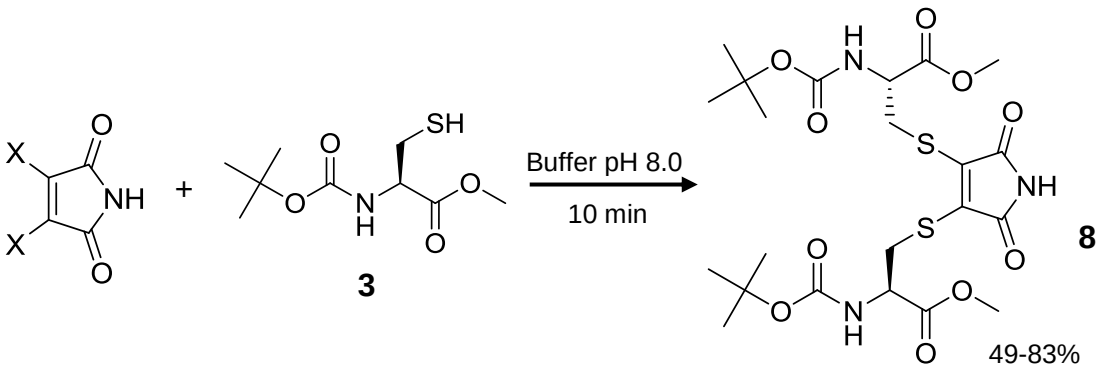
The reaction proceeded very rapidly and the products crashed out of solution as a bright yellow precipitate. The results of reaction 3 indicated that, as long as free cysteine is available, the addition of the thiol to the maleimide yields mainly the dicysteinemaleimide. The second addition seemed to be very fast as even reaction 4, in which a large excess of the maleimide was added, still contained considerable amounts of this compound.

To synthesise larger quantities of the dithiomaleimide **8** a bigger batch of the basic reaction was prepared (entry 5) and, with slightly more than a 2x excess of cysteine, complete conversion of dibromo- to the dicysteinemaleimide was achieved.

Overall cysteine reacts readily and very rapidly with dibromomaleimide. The reaction from the mono-addition product to the two-carbon bridged dicysteine-maleimide is unhindered and takes place at the presence of the slightest excess of free thiol groups. Inclusion of a base seems to be important as reactions performed in buffered solutions were in general higher yielding, especially compared to the available literature reports (see Scheme 1.8).[244-246]

2.1.3 Influence of the halide on the reaction with cysteine

To investigate the influence of the type of halogen on the reactivity towards cysteine a series of reactions with dichloro-, dibromo- and diiodomaleimide was carried out (Scheme 2.2). The three halomaleimides were reacted for 10 min with 0.9 equiv or 2.2 equiv of protected L-cysteine **3** in aqueous buffer (150 mM NaCl, 100 mM sodium phosphate, 7.5% DMF, pH 8.0). In the case where 0.9 equiv were employed, in one set of reactions the cysteine was added into the stirring halomaleimide solution and in another the addition of reagents was the other way around. The results are summarised in Table 2.2.



Scheme 2.2: Reactions of halomaleimides (X = Cl, Br or I) with protected L-cysteine (**3**) to yield dicysteinemaleimide (**8**).

Table 2.2: Reaction of various dihalomaleimides with protected L-cysteine. The yield of maleimide is relative to its starting concentration. All other yields are relative to the used amount of cysteine. *The mono-addition product and cystine were obtained as an inseparable mixture. Relative yields were estimated from the corresponding ^{1}H NMR spectra.

Halogen (X)	Equiv cysteine	Added compound	Mono-addition product **7** [%]*	Cystine **3** [%]*	Di-addition product **8** [%]	Cysteine left [%]	Maleimide left [%]	Succinimide by-product [%]
chlorine	0.9	maleimide	4	1	75	0	27	2
bromine	0.9	maleimide	8	3	68	0	24	2
iodine	0.9	maleimide	23	4	49	0	22	2
chlorine	0.9	cysteine	8	1	83	0	6	4
bromine	0.9	cysteine	20	1	68	0	21	1
iodine	0.9	cysteine	14	5	60	0	42	2
chlorine	2.2	maleimide	5	1	70	7	0	5
bromine	2.2	maleimide	6	2	65	2	0	3
iodine	2.2	maleimide	11	5	56	6	0	8

The first set of reactions showed that all three halomaleimides react rapidly with cysteine as the di-addition product was formed in higher quantities than the mono-addition product despite the sub-stochiometric amounts of the thiol added. Dichloromaleimide seems to favour the second attack of a thiol the most. The formation of a succinimde by-product suggests that the electrophilic positions in the maleimide exhibit reactivity towards nucleophiles even after replacement of the halide. It should be noted that a substantial amount of the unreacted maleimide was not recovered and was potentially lost in washes during the purification procedure as the halomaleimides are partially water soluble.

In the second set of reactions an increase in the amount of the mono-addition product would have been expected, due to the changed order of addition. This was observed only in the case of dichloro- and dibromomaleimide but mainly due to a more thorough consumption of the maleimide. This result suggests again that the second addition of cysteine must be very fast and might even be quicker than the first addition.

While in the first two sets of reactions the amino acid had been consumed completely, in the last three reactions the maleimides were fully reacted. The presence of a higher concentration of thiol led to the formation of increased amounts

of the succinimide product. In summary, all three halomaleimides favour the second addition of thiol with a more pronounced effect observed with dichloro- than dibromo- than diidomaleimide.

2.1.4 Reversibility of the bridging reaction

To investigate the potential reversibility of the reaction between cysteine and maleimide the synthesised and purified dicysteinemaleimide were subjected to the reducing agents DTT and TCEP as well as to the tripeptide GSH, which is present in high amounts intracellularly,[84] and the small dithiol 1,2-ethanedithiol. The results are summarised in Table 2.3.

Table 2.3: Treatment of dicysteinemaleimide **8** with reducing agents and thiols. †Obtained as impure mixture. ‡Compound observed in the dried aqueous phase by LCMS.

Reducing com-pound	Equiv reducing com-pound	Reac-tion time	Dicysteine-maleimide **8** left [%]	Cysteine isolated [%]	Other products observed
TCEP	2	60 min	14	25	cystine, dicysteinesuccinimde
TCEP	10	3 d	0	86	dicysteinesuccinimide, mono-cysteine-succinimide†
DTT	10	30 min	0	96	monocysteinemaleimide, cyclic DTT-maleimide adduct‡, cy-clisised DTT‡
GSH	2	24 h	20	16	cystine, GSH-monocysteinemaleimide‡, GSH-cysteine‡
1,2-ethanedi-thiol	10	60 min	0	57	pyrrole-5,7-dione, none-6,8-dione, dicysteineethanedithiol‡

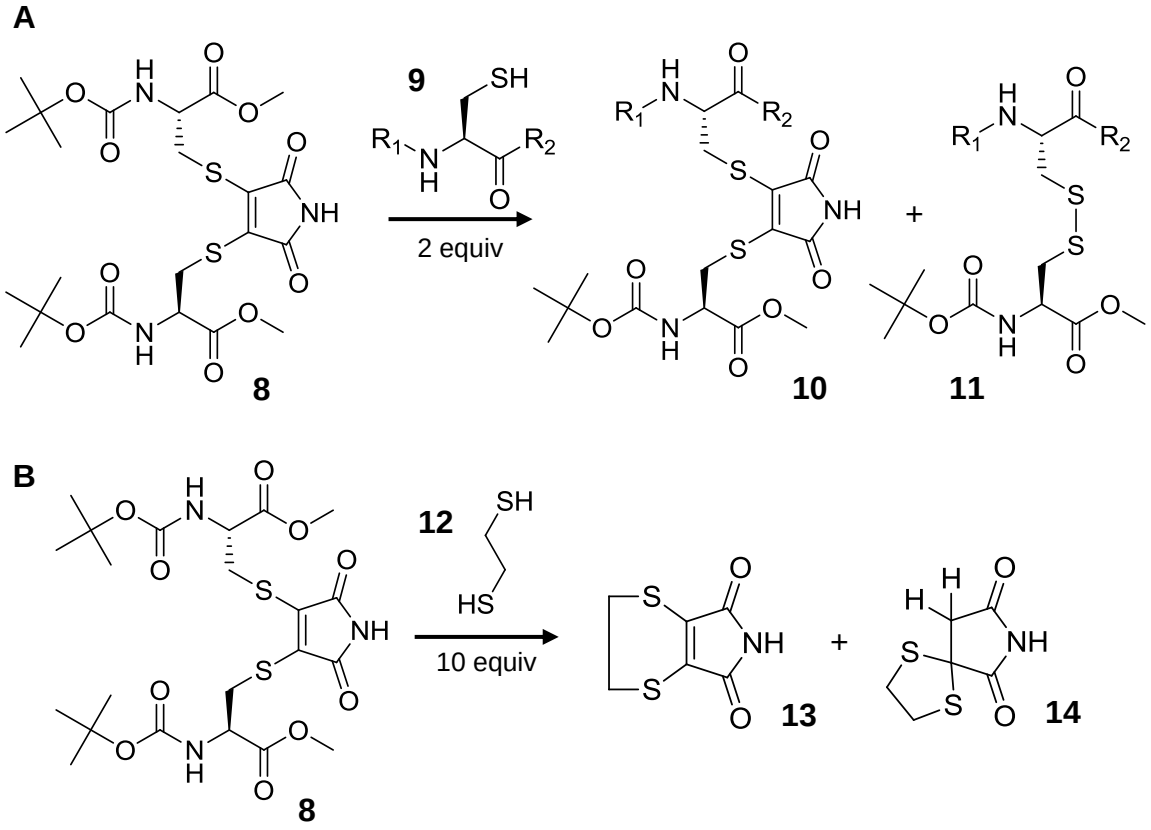
In an initial test dicysteinemaleimide **8** was exposed to 2 equiv of TCEP over 3 days at ambient temperature in MeOH. No additional product spots besides the starting material were found by TLC analysis and 98% of the dicysteinemaleimide was recovered. This result underlines the dependency of the reactivity of the phosphine on the presence of water.[214]

When the same reaction was prepared in the buffer system described in 2.1.2 but with 40% of acetonitrile (MeCN) to solubilise the dicysteinemaleimide **8**, free cysteine appeared in the solution within 2 min and a strong spot corresponding to the amino acid analogue was observed by TLC after 60 min. While 2 equiv of TCEP liberated only around one quarter of the cysteine present in the deployed dicysteinemaleimide, increased reducing agent and time yielded 86% of the free

thiol and the previously bright yellow solution turned colourless. A certain quantity of impure di- and monocysteinesuccinimide were present as determined by NMR and MS spectroscopy. It has been shown before that phosphines can reduce maleimides to give succinimide under the right conditions.[392, 393] Overall the reaction showed that the phosphine can reverse the addition of cysteine to dibromomaleimide and that the presence of a buffered aqueous solution (or a base) is necessary to enable the cleavage.

When 10 equiv of DTT (Fig. 1.7 A) were added to dithiomaleimide **8**, the yellow colour disappeared within minutes. The purification of the organic phase showed that most of the cysteine had been released (no cystine was found), a certain amount of monocysteinemaleimide **7** was left and the remaining DTT had cyclised.

As GSH **9** is not soluble in non-polar organic solvents but a certain amount was necessary to solubilise dicysteinemaleimide **8** in the buffer, the dithiolmaleimide was exposed only to 2 equiv of the tripeptide **9** (Scheme 2.3 A).



Scheme 2.3: Disulfide exchange of dicysteinemaleimide (**8**) and reducing agents. (A) Reaction with GSH (**9**, R_1 γ-glutamate, R_2 glycine) yields GSH-monocysteinemaleimide (**10**) and the mixed disulfide GSH-cysteine (**11**). (B) Reaction with 1,2-ethanedithiol (**12**) yields the cyclic pyrrol-dione (**13**) and spiro-nonane-dione(**14**) products.

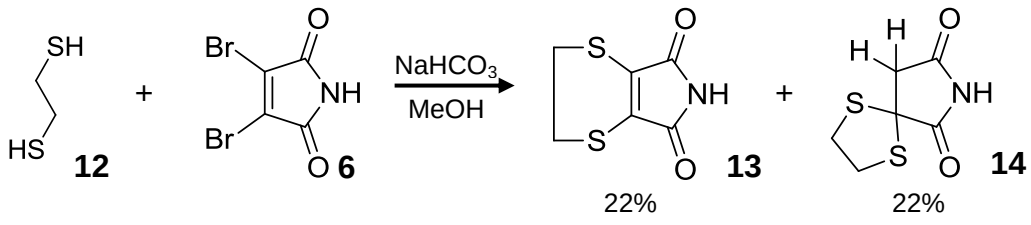
Upon the addition of GSH **9** to **8**, free cysteine was quickly observed by TLC analysis. After work-up and flash chromatography 24% of the cysteine (4% as cystine) and 20% of the starting material were isolated from the organic phase. The aqueous phase comprised the remaining products – the mixed disulfide species **10** and **11**. Di-GSH-maleimide was not observed but control reactions with dibromomaleimide **6** and the tripeptide **9** had revealed that the di-addition product is barely detectable by LCMS (data not shown) and thus the compound could have been present in the sample.

As the cyclisation reaction observed with DTT (Fig. 1.7 A) might indicate a useful mechanism for the cleavage of dithiomaleimide bridges, the shorter dithiol 1,2-ethanedithiol **12** was reacted with dicysteinemaleimide (Scheme 2.3 B). After 1 h at ambient temperature more than 50% of the cysteine was recovered during purification and most of the starting material had reacted to give the expected cyclic adducts **13** and **14** (see 2.1.5).

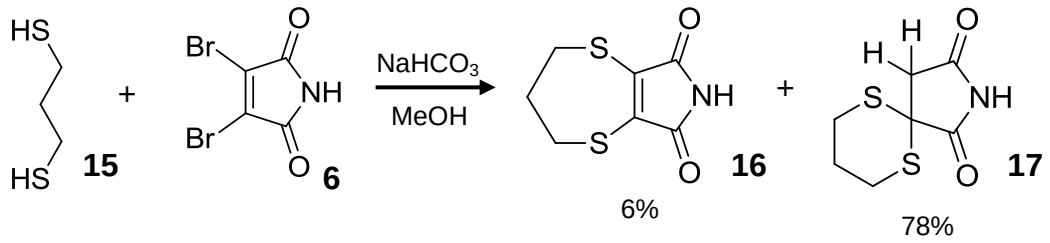
Overall, these reactions suggested that the developed method for disulfide bridging is reversible. DTT acts as the strongest reducing agent followed by 1,2-ethanedithiol and TCEP. The exchange reaction observed with GSH implied that maleimide-conjugated peptides could potentially be used as drug delivery systems which release their “cargo” upon entering the cell, where the concentration of the tripeptide is very high.

2.1.5 Small dithiols form cyclic structures with dibromomaleimide

The shortest connection between two cysteines in naturally occurring disulfides is one amino acid.[45] Directly neighboured residues of that type rarely form disulfide bridges as the distance between the two C_α-atoms is too short for PDIs to facilitate a cystine. To further investigate the behaviour of small dithiols, dibromomaleimide **6** was mixed with 1,2-ethanedithiol **12** or 1,3-propanedithiol **15** (Scheme 2.4 and 2.5).



Scheme 2.4: Reaction of dibromomaleimide (**6**) with 1,2-ethanedithiol (**12**). Both the pyrrol-dione (**13**) and the spiro-nonane-dione rings (**14**) were formed.



Scheme 2.5: Reaction of dibromomaleimide (**6**) with 1,3-propanedithiol (**15**). Dithiepino-pyrrol-dione (**16**) and the spiro-decane-dione (**17**) were isolated as products.

The expected pyrrol-dione product **13** was isolated from the reaction with **12** in 22% yields, showing that small dithiols can displace both bromines. Intriguingly the same amount of the spirocyclic compound **14** was also found and must be formed by a reductive side reaction. It could possibly derive from thiol attack on the bromide of an intermediate species where the halogen of the starting material in the 4-position of the maleimide ring (in compound **14**) is still present. Similar observations were made with 1,3-propanedithiol but here the formation of the spiro-compound was clearly favoured with a yield of 78% over the pyrrol-dione (6%). Formation of an energetically favourable 6-membered ring in comparison to a 7-membered ring might play a role here.

Interestingly, the addition of 1,2-ethanedithiol to dichloromaleimide has been described before, but in that instance the compounds were heated to 170–180 °C for 1.5 h with a yield of the pyrrole-dione of approximately 60%.[250] This might suggest that the formation of the expected compounds **13** and **16** is kinetically favoured over the other products. Overall these results demonstrate that dibromomaleimide can form disulfide bridges with thiol groups that are as few as two carbon atoms apart from each other.

2.2 Modification of the somatostatin disulfide bond

2.2.1 Synthesis

N-methyldibromomaleimide **18** is commercially available. Monobromomaleimide **4** was synthesised following a literature procedure.[120] *N*-methylmonobromomaleimide **19** was kindly provided by Mr Ramiz Nathani (UCL Chemistry).[243]

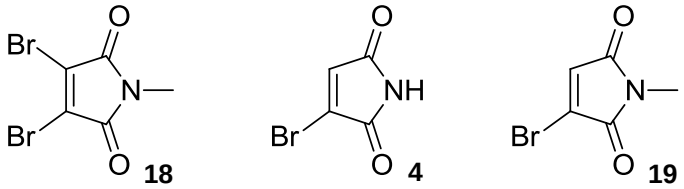
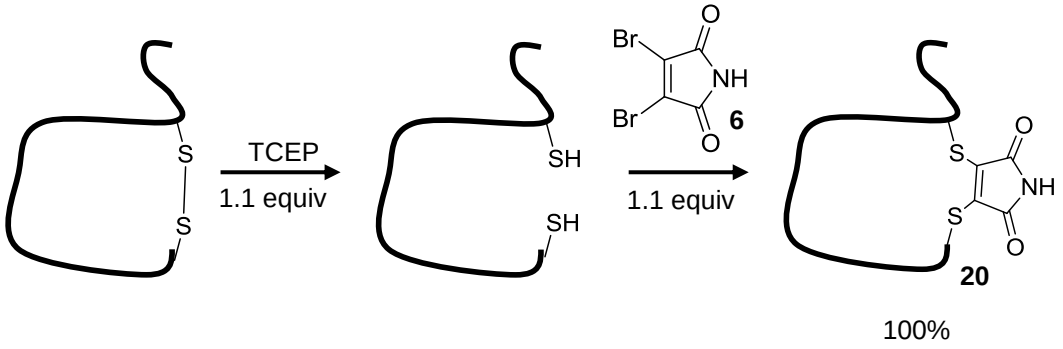


Fig. 2.1: Halomaleimide derivatives. *N*-methyldibromomaleimide (**18**), monobromomaleimide (**4**) and *N*-methylmonobromomaleimide (**19**).

2.2.2 Re-bridging of reduced somatostatin

After the connection of two cysteine residues via dibromomaleimide was demonstrated successfully the next step was to apply this technique to a simple peptide model system. Initial tests on somatostatin were performed as described by Balan and co-workers,[240] but the protocol was soon adapted to the superior reactivity of bromomaleimides.

Commercially available somatostatin was reduced with 1.1 equiv of TCEP for 60 minutes at ambient temperature. Without further purification the cleaved disulfide bond was reacted with 1.1 equiv of dibromomaleimide (Scheme. 2.6). The reaction was monitored by LCMS.



Scheme 2.6: Reaction of dibromomaleimide (**6**) with reduced SST yields the two-carbon bridged peptide (**20**). Buffer = 50 mM sodium phosphate, pH 6.2, 40% MeCN, 2.5% DMF.

The change from the original peptide (1,638 Da) to the reduced state (1,640 Da) and the maleimide bridged molecule **20** (1,734 Da) was clearly detectable. High resolution MALDI-TOF MS spectra confirmed the identity of the peptides (Fig. 2.2).

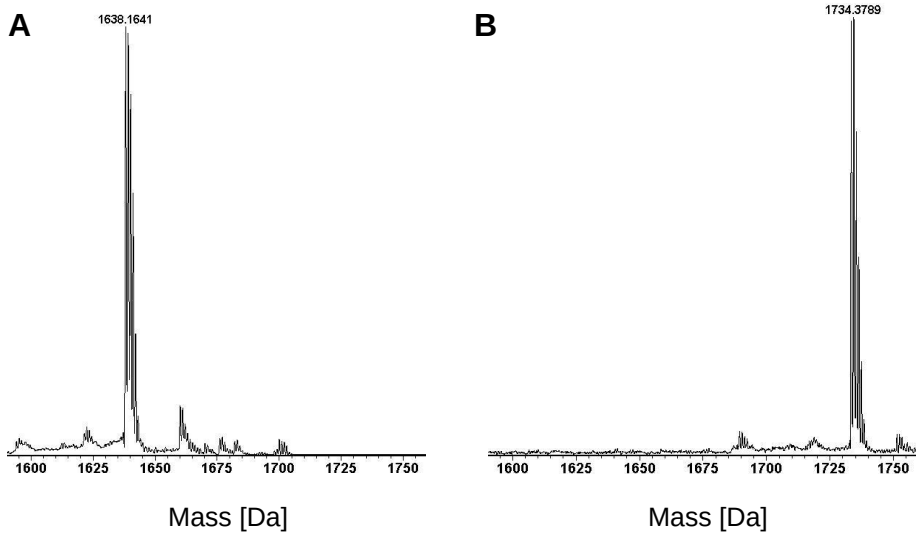


Fig. 2.2: Bridging of somatostatin with dibromomaleimide as observed by MALDI-TOF MS. (A) Somatostatin. (B) Maleimide bridged somatostatin (requires 1,734 Da).

The formation of the product was very fast and the bridged SST accounted for ca. 80% of the present peptide species after 1 min and more than 95% after 5 min. With ongoing product formation the solution turned pale yellow which yielded the possibility of monitoring the insertion of the maleimide bridge into proteins spectrophotometrically. The retention times on the LC column of the various forms of the peptide did not change substantially.

A control reaction with unreduced SST mixed with an excess of dibromomaleimide showed that the small compound does not react with the peptide if there is no free thiol available. When this mixture was incubated at ambient temperature for 28 days a species accounting for approx. 15% of the SST with a mass of 1,896 Da was detected. This mass can result from the addition of dibromomaleimide to the hormone without loss of a bromine atom and might indicate a slow side reaction with one of the amines (SST comprises two lysine residues) present. The bridged somatostatin was stable for at least 8 weeks at 4 °C.

2.2.3 Influence of the halide on the bridging reaction

Since the variation of the halide on the maleimide ring had made a small but observable difference in the reaction with protected L-cysteine in the small molecule model system, dichloro-, dibromo-, and diiodomaleimide were added to reduced SST under comparable conditions. A smaller time scale was chosen to

monitor the fast reaction (Fig. 2.3, in this figure, as in all following figures showing data derived by mass spectroscopy, the signal in % refers to the relative population of the indicated peptide species amongst all other detectable peptide species such as unreacted SST, reduced SST, etc. See 4.2 for details on the quantification of MS data. If no error bars are shown the data represents a single repetition of the experiment).

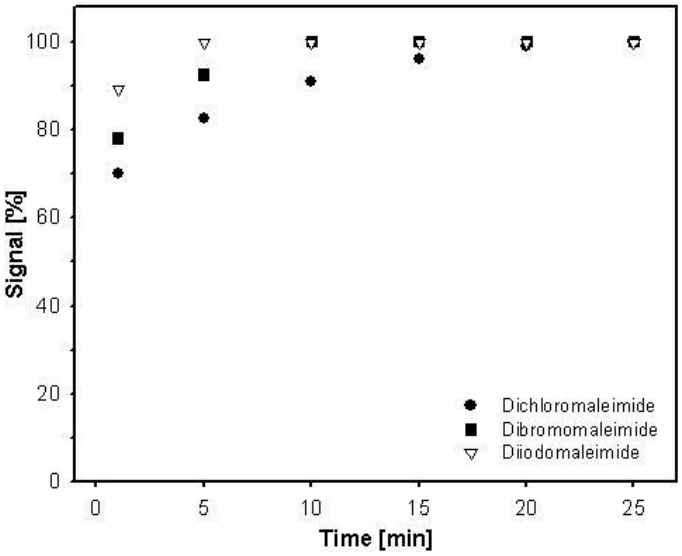


Fig 2.3: Reaction of reduced somatostatin with various dihalomaleimides as observed by LCMS. The signal % corresponds to the relative population of the maleimide bridged somatostatin **20**.

As observed in the model reaction, all three maleimide analogues were able to bridge the two free cysteines of reduced somatostatin efficiently. Within the limit of the assay, diiodomaleimide seemed to be the most reactive reagent with completion reached after 5 min, followed by dibromo- (full conversion in the 10 min sample) and dichloromaleimide (20 min). A similar behaviour of bromine and chlorine in di-halo compounds has been observed.[394] The high activity of the iodine-substituted maleimide might be assigned to the qualities of the halogen as a better leaving group[395] or its ability to deactivate the maleimide ring less. The order of reactivity found here is different from what had been observed with the small molecule system but with the latter, the experimental set-up had not allowed any conclusions on the reaction kinetics to be drawn.

2.2.4 Stopped-flow analysis of halomaleimide mediated bridging of somatostatin

To quantify the phenomenon discovered in the bridging of somatostatin, kinetic data was obtained for this reaction. Absorbance scans had revealed that the maleimide bridged somatostatin has a strong absorbance band at 395 nm (data

not shown) enabling the possibility to monitor the reaction in great detail via UV/Vis spectroscopy. Initial tests showed that the reaction proceeded too fast for slow mixing methods. Thus, with the kind support of Dr Tina Daviter (Birkbeck Department of Biological Sciences), stopped-flow techniques were used to measure the reaction rates. SST was reduced as described and mixed with halomaleimides at 20 °C. The increase in absorbance at 395 nm was recorded for 12 min. The final concentration of peptide was 34.7 µM and 173.5 µM (5x excess) of the bridging reagent. A two-exponential fit gave the best results when the apparent rate constants were calculated (Table 2.4).

Table 2.4: Apparent rate order constants calculated from stopped-flow kinetics for the halomaleimide mediated bridging reaction of reduced somatostatin. Errors are derived from triplicate experiments.

Halogen	$k_{app}1$ [1/s]	Error $k_{app}1$	$k_{app}2$ [1/s]	Error $k_{app}2$	Total amplitude
chlorine	0.080	± 0.001	0.013	± 0.001	0.107
bromine	0.148	± 0.003	0.018	± 0.001	0.112
iodine	0.204	± 0.002	0.019	± 0.002	0.100

The results confirmed the observations from the previous experiment. Diiodomaleimide reacts slightly faster than dibromomaleimide which again is approximately twice as fast as dichloromaleimide. The good quality of a two-exponential fit suggested that the reaction takes place in a two-step process or that two slightly different reactions generating the same product happen at the same time. Although the first possibility is certainly true as Michael-Additions feature an addition-step followed by an elimination-step, only the final product is observable via absorbance (due to the presence of the double bond). Monocysteinebromomaleimide **7** can not be detected under these conditions at the chosen wavelength. On the other hand Rabenstein and Weaver[251] have measured the pK_as of the two cysteines of somatostatin and found them to be quite different (8.15 for Cys3 and 9.92 for Cys14). This suggests that the observed two-step process can be a result of the unequal reactivity of the different products arising from the first addition to either the higher (lower pK_a) or lower (higher pK_a) reactive thiol. Further experiments would be necessary to determine the reaction order. Unfortunately the poor solubility of reduced somatostatin at

higher pH[240] imposes restrictions on a more in-depth investigation of the pH effect in this system.

With respect to dibromomaleimide the overall bridging reaction of somatostatin compares well with the activity reported for NEM[95] (with a single cysteine) and is several orders of magnitude faster than α-halogen alkylating compounds[70] and the reaction rates of biocompatible click reactions.[23]

2.2.5 Reversibility of the bridging of somatostatin

To examine the potential of reversibility as observed in the small molecule system, bridged SST was treated with common reducing agents (DTT, 2-mercaptoethanol and TCEP) as well as GSH and 1,2-ethanedithiol at ambient temperature (Fig. 2.4). The generation of unmodified peptide was monitored by LCMS. As most biochemical protocols suggest the use of an excess of reducing agent over protein, 100 equiv of these compounds were added.

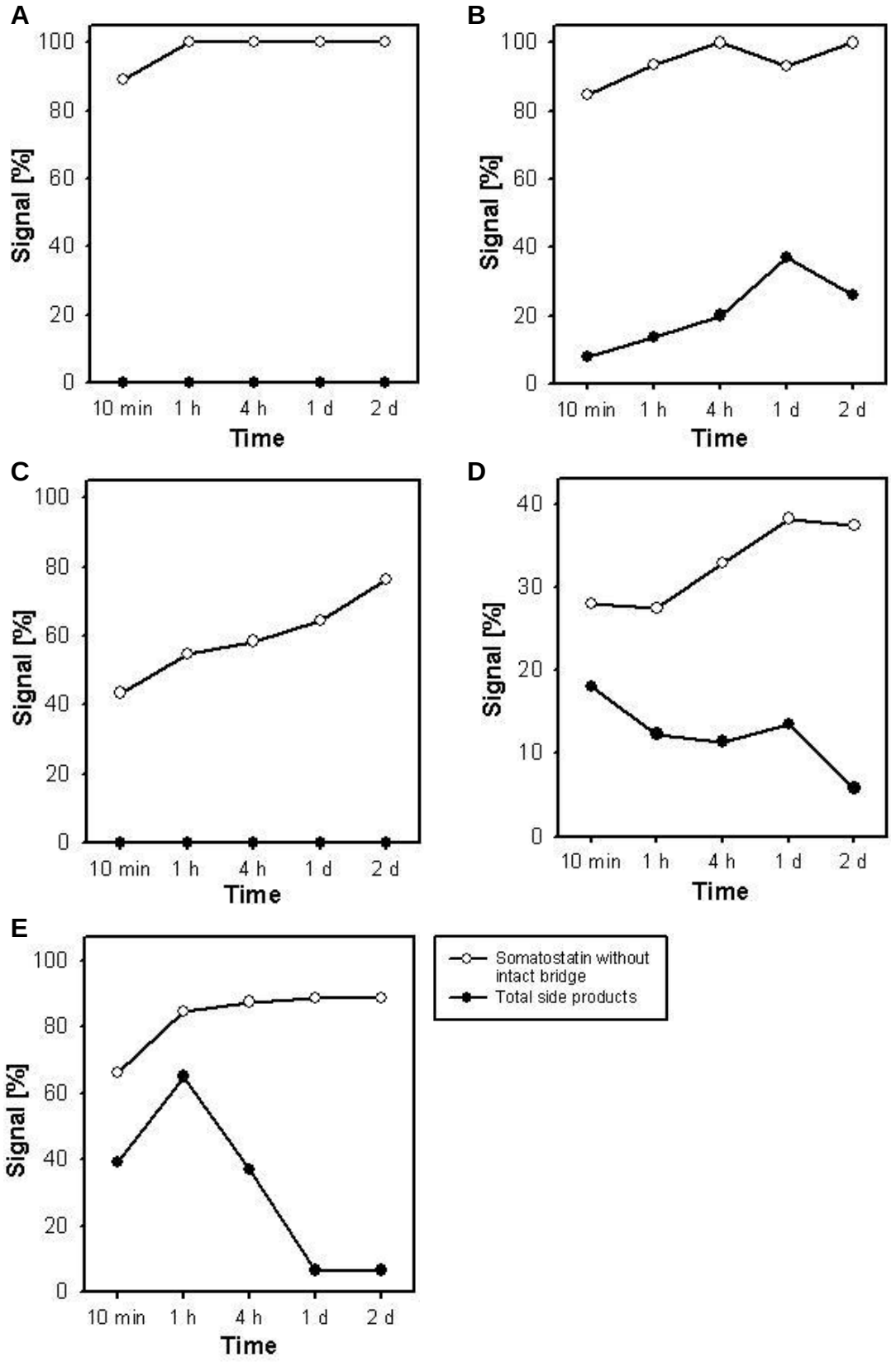


Fig. 2.4: Cleavage of maleimide bridged somatostatin **20** with reducing agents and thiols monitored by LCMS. Side products are mixed disulfides of SST with the reducing agents or addition products of reducing agents to the bridged SST. (A) DTT. (B) 2-Mercaptoethanol. (C) GSH. (D) TCEP. (E) 1,2-Ethanedithiol.

Both DTT and 2-mercaptoethanol cleaved the maleimide bridge completely within 1 and 4 hours respectively. As expected, the dithiol was not involved in the formation of side products while signals for the mixed disulfides of 2-mercaptoethanol and somatostatin were observed. In contrast TCEP was not effective in the reversal of the bridging reaction and various addition products (see 2.5.2) were formed. GSH cleaved the maleimide bridge slowly but constantly over 24 h. The assumed mixed disulfide products were not visible by LCMS but could be detected by MALDI-TOF MS to make up for the remaining SST (data not shown). 1,2-Ethanedithiol quickly added to the bridged peptide and then cleaved the compound slowly, presumably via the formation of cyclic structures on the maleimide ring (see 2.1.5). In summary, this experiment confirmed that the unmodified somatostatin can be regenerated by addition of an excess of reducing agent.

To test the stability of the maleimide bridge against smaller amounts of the best performing reducing agents, SST **20** was incubated with 10–50 equiv of DTT and 2-mercaptoethanol (Fig. 2.5).

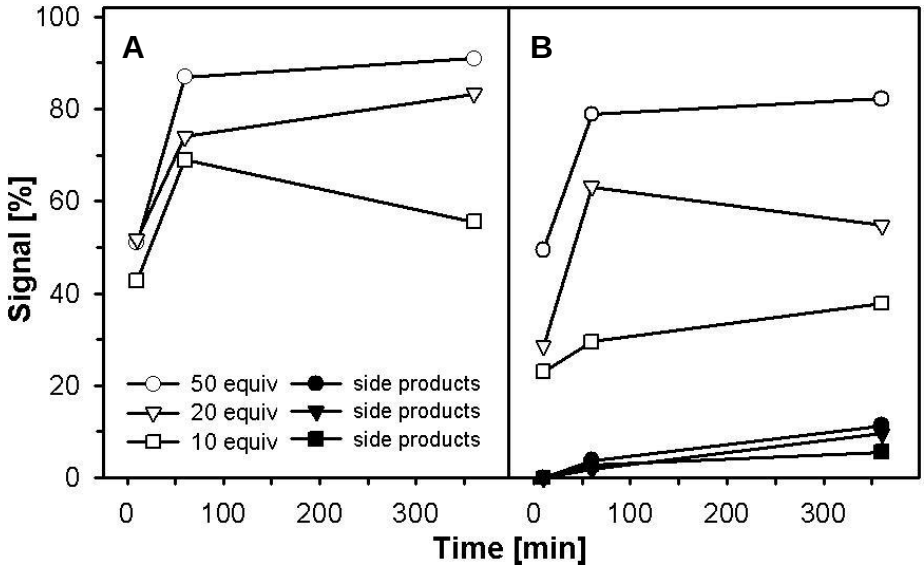


Fig. 2.5: LCMS monitored cleavage of maleimide bridged somatostatin with smaller amounts of reducing agents. Indicated signals are the unmodified peptide (empty symbols) and side products (filled symbols). (A) DTT (no side products were observed). (B) 2-Mercaptoethanol.

In all cases, the reduced amounts of reagents were not able to reverse the bridging reaction completely. These results show that a large excess of free thiols is necessary to cleave a maleimide bridged disulfide bond and indicates

that proteins modified in such a way might be stable in the human blood where plasma levels of free thiols (cysteine, homocysteine or GSH) are in the low µM range.[82, 83, 251]

2.2.6 Catalysed cleavage of the maleimide bridge of somatostatin

Due to the well-known positive effect of selenols on the thiol-mediated cleavage of disulfide bonds[215] and previously observed reversion taking place when diiodomaleimide was used to generate the SST analogue **20** (data not shown), 5 equiv of both a selenol (commercial available benzeneselenol) or iodide (in the form of sodium iodide) were used together with 20 equiv of 2-mercaptoethanol to cleave maleimide bridged SST (Fig. 2.6).

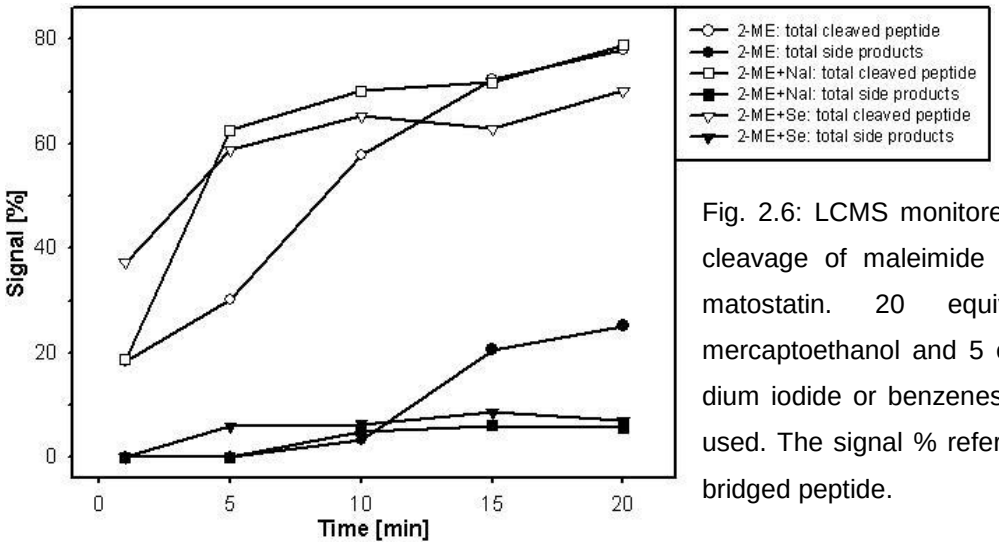


Fig. 2.6: LCMS monitored catalysed cleavage of maleimide bridged somatostatin. 20 equiv of 2-mercaptoethanol and 5 equiv of sodium iodide or benzeneselenol were used. The signal % refers to the unbridged peptide.

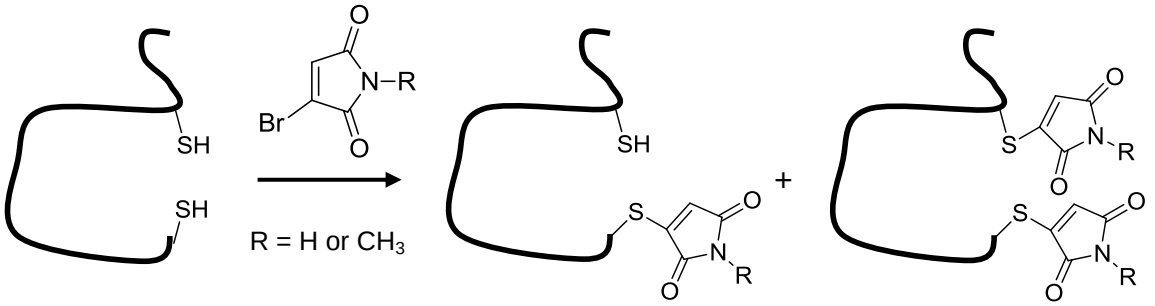
None of the substances increased the amount of cleavage with only 20 equiv of the reducing agent significantly. Still, as usually observed with catalysed reactions, the rate of the formation of the product is enhanced and a near maximal amount of cleavage was reached within five minutes. A similar effect was found when the amount of 2-mercaptoethanol was reduced to five or even 2 equiv (data not shown). No cleavage was observed when no thiol was present.

In summary, the modification of a disulfide bond with halomaleimides is reversible by an excess of reducing agent yielding the potential of stability in an *in vivo* scenario as well as the regeneration of a target protein *in vitro*. Additional research might yield substances that decrease the amount of necessary reducing

agents to a point were a maleimide bridge can be removed without harming other disulfide bonds present.

2.2.7 Reactions of various halomaleimides with somatostatin

To gain more insight into the reactivity of monobromomaleimides, monobromomaleimide **4** and *N*-methylmonobromomaleimide **19** were reacted with the reduced form of SST (Scheme 2.7). To confirm the addition of one or two molecules in the case of the monosubstituted compounds the products were mixed with “plain” maleimide after a first LCMS run, to test if further conversion of the single-addition products was observable.



Scheme 2.7: Proposed reaction of reduced somatostatin with monobromomaleimides.

As the imide of the maleimide ring is the designated anchor point to add functional molecules to a disulfide bridge, *N*-methyldibromomaleimide **18** was also included into the investigation. The simple modification of the basic framework had no negative influence on the bridging reaction (Fig. 2.7).

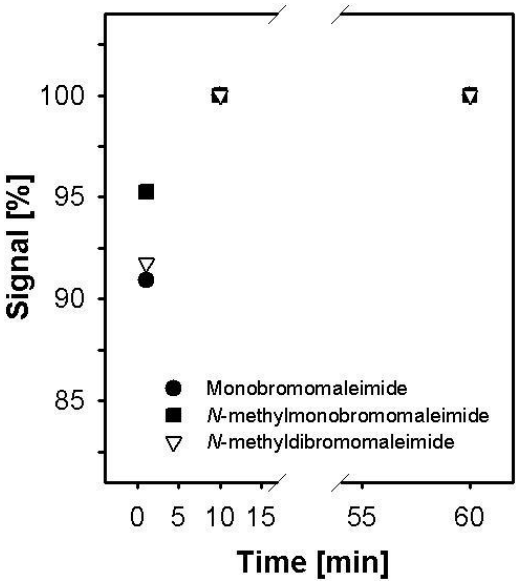
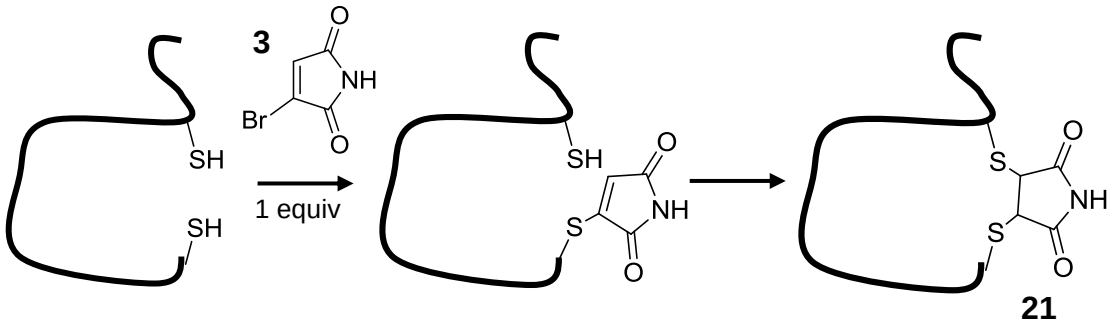


Fig. 2.7: LCMS monitored reaction of reduced somatostatin and various bromomaleimides. The signal % corresponds to the relative population of the maleimide bridged somatostatin **20**. Alternatively results where 2 equiv of monobromomaleimides were added are shown and here the referred peptide species is the double-addition product.

When 0.5 equiv of monobromomaleimide **4** was added to the reduced peptide only the mono-addition product and unmodified somatostatin were detected. Using 1 equiv of maleimide **4** resulted in free peptide (27%), the mono-addition (61%) as well as small amount (12%) of the double-addition product. This last compound was the only one found upon addition of 2 equiv of maleimide **4.** The reaction took place at the same speed as the bridging of somatostatin by dihalomaleimides. The behaviour of the *N*-methylated version of monobromomaleimide was essentially the same.

In order to investigate if the second free thiol of the mono-addition product can attack the maleimide double bond, thereby forming somatostatin bridged by a succinimide **21**, the reduced peptide was reacted very slowly with 1 equiv of monobromomaleimide (Scheme 2.8) and incubated for 60 min.



Scheme 2.8: Reaction of 1 equiv of monobromomaleimide with reduced SST leading to the formation of succinimide bridged peptide (**21**).

Indeed treatment of the reaction mixture after 60 min with maleimide revealed the presence of mainly (93%) a peptide species with an increased mass (+96 Da) but without any free thiols. This indicated that a maleimide attached to one of the cysteines can still act as a Michael-acceptor and that the succinimide bridge had been formed. Dimerisation of the hormone was not observed.

Overall, these reactions suggest that the reactivity of halomaleimides towards peptidyl thiols is robust. The application of monobromomaleimides to form succinimide bridges might be useful to generate reagents that could be more resistant towards cleavage of the artificial disulfide bridge due to the loss of the double bond.

2.3 Functionalisation of the somatostatin disulfide bond

2.3.1 Synthesis

N-Fluorescein-dibromomaleimide **22** and *N*-biotin-dibromomaleimide **23** were kindly provided by Dr Christopher Ryan (Fig. 2.8). The protocols for their synthesis have been reported.[396, 397]

N-PEG300-dibromomaleimide **24** and *N*-PEG5000-dibromomaleimide **25** were synthesised in single step reactions following the protocol by Walker for a Mitsunobu reaction variant for maleimides (see Scheme. 1.4 A).[104, 106] Although reported differently,[105] the order of addition of compounds had an impact on the results and the pre-formation of the betaine and mixture of the reactants at –78 °C was vital to obtain product. Since the mPEG chains are soluble only in dichloromethane but dibromomaleimide is not, a final 1 : 1 mixture of THF with dichloromethane was employed with success, even though the application of the chlorinated solvent in Mitsunobu reactions is rarely reported.[398] Due to the hydrophilicity of the products, the separation of the phosphine oxide and the hydrazine by-products, which is often problematic,[399] was easy to accomplish. By careful flash chromatography it was possible to purify the modified PEG chain from the residual starting material but a significant amount of product was lost on the columns. An increased quantity of neopentyl alcohol (replacing an excess of mPEG) reduced the amount of unreacted mPEG in the mixture and increased the quantity of separable product. mPEG5000 was obtained from Sigma-Aldrich and contained, as most PEG polymers do,[148] a mixture of chains with different numbers of polymer repeats with an average molecular weight of 5 kDa (108 PEG units). This was reflected in the Mitsunobu reaction products.

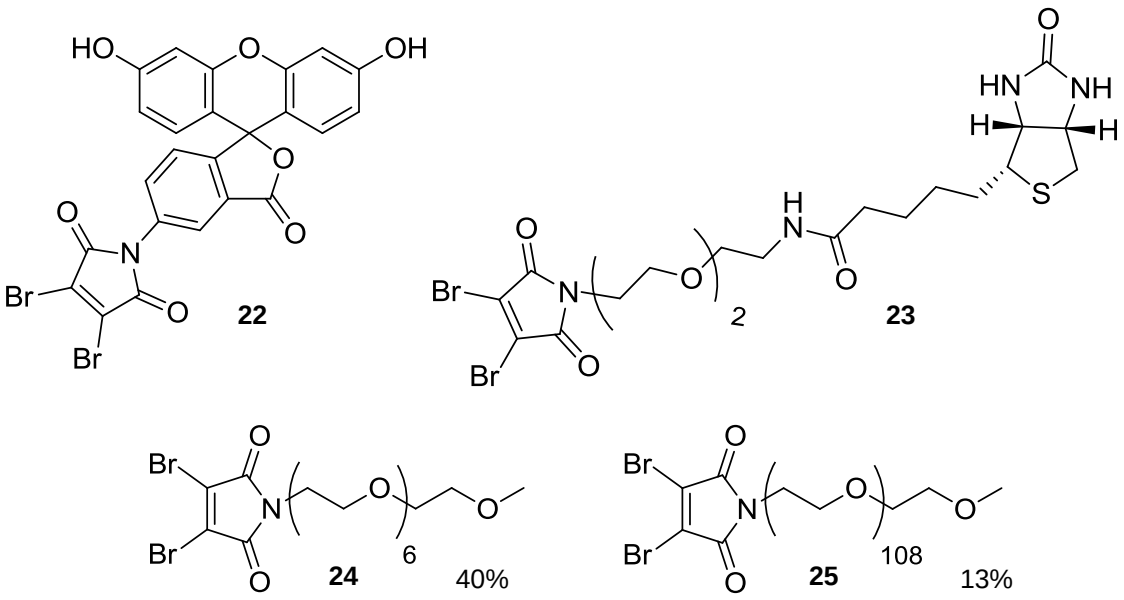
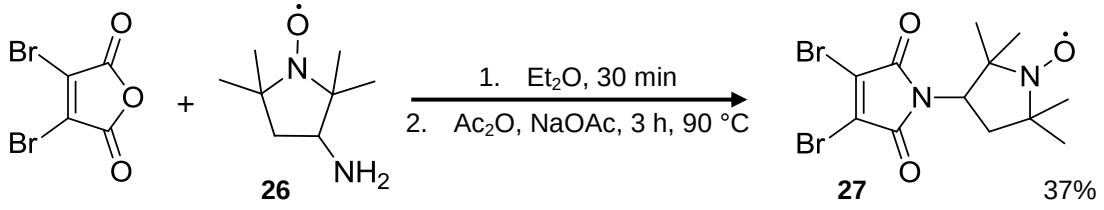


Fig. 2.8: *N*-functionalised dibromomaleimides. *N*-fluorescein-dibromomaleimide (**22**), *N*-biotin-dibromomaleimide (**23**), *N*-PEG300-dibromomaleimide (**24**) and *N*-PEG5000-dibromomaleimide (**25**).

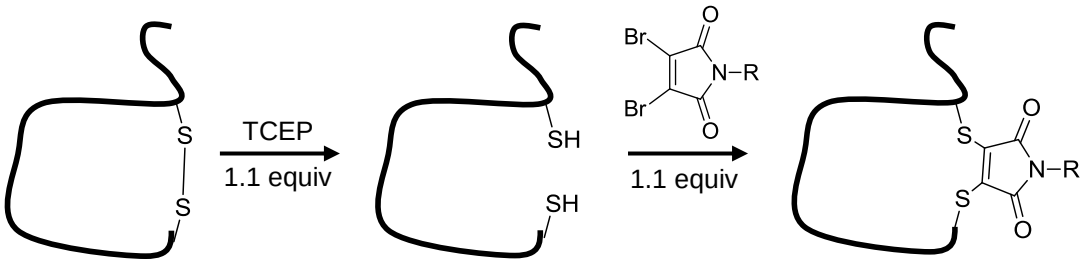
Dibromomaleic anhydride **5** was prepared following a literature method[400] in order to synthesise a dibromomaleimide spin label from the amine of the stable 2,2,5,5,-tetramethyl-1-pyrrolidinyloxy radical (TPO, **26**). Simple heating of both compounds in acetic acid gave the desired product but in low yields (around 4%). When the protocol shown (Scheme 2.9), adopted from Hideg and co-workers,[146] was used instead, the amount of product increased significantly. The direct connection between the maleimide ring and the spin label was chosen deliberately to minimise the translational freedom of the probe following the example of maleimide-TEMPO spin labels (Fig. 1.5 A). NMR data on *N*-TPO-dibromomaleimide **27** had to be recorded after reduction with hydrazobenzene.



Scheme 2.9: Reaction of dibromomaleic anhydride with TPO (**26**) to yield *N*-TPO-dibromomaleimide (**27**).

2.3.2 Insertion of functionalised dibromomaleimides into the somatostatin disulfide bond

Denicola-Seoane and Anderson have observed that the size of the substituent of *N*-alkylated maleimides can have an impact on the bioconjugation of these compounds.[53] In order to test this for the disulfide bridging reaction, SST was reduced and reacted with 1.1 equiv of *N*-fluorescein-, *N*-biotin-, spin labelled or *N*-PEGylated dibromomaleimides (Scheme 2.10). The reaction was monitored by LCMS (Fig 2.9).



R = Fluorescein, biotin, PEGs or TPO

Scheme 2.10: Modification of somatostatin with *N*-functionalised dibromomaleimides.

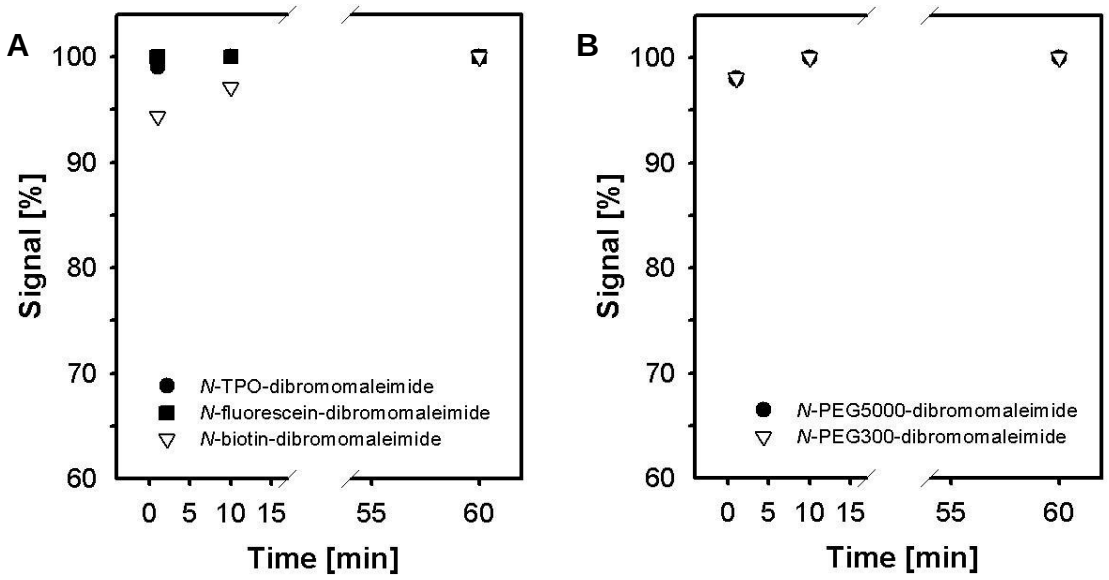


Fig. 2.9: Bromomaleimide mediated conjugation of functional molecules to somatostatin monitored by LCMS. The signal % refers to the functionalised SST. (A) Spin labelling, biotinylation and fluorescein-conjugation. (B) PEGylation.

In the case of *N*-PEG5000-dibromomaleimide the mass signals of the compound and the product were not detectable by MS but gave a good UV signal on the LC column which was used to determine the disappearance of the unmodified peptide (Fig 2.10). The identity of the compounds, which exceeded the detection limit of the LCMS (2,090 Da), were confirmed by MALDI-TOF MS (Fig 2.11).

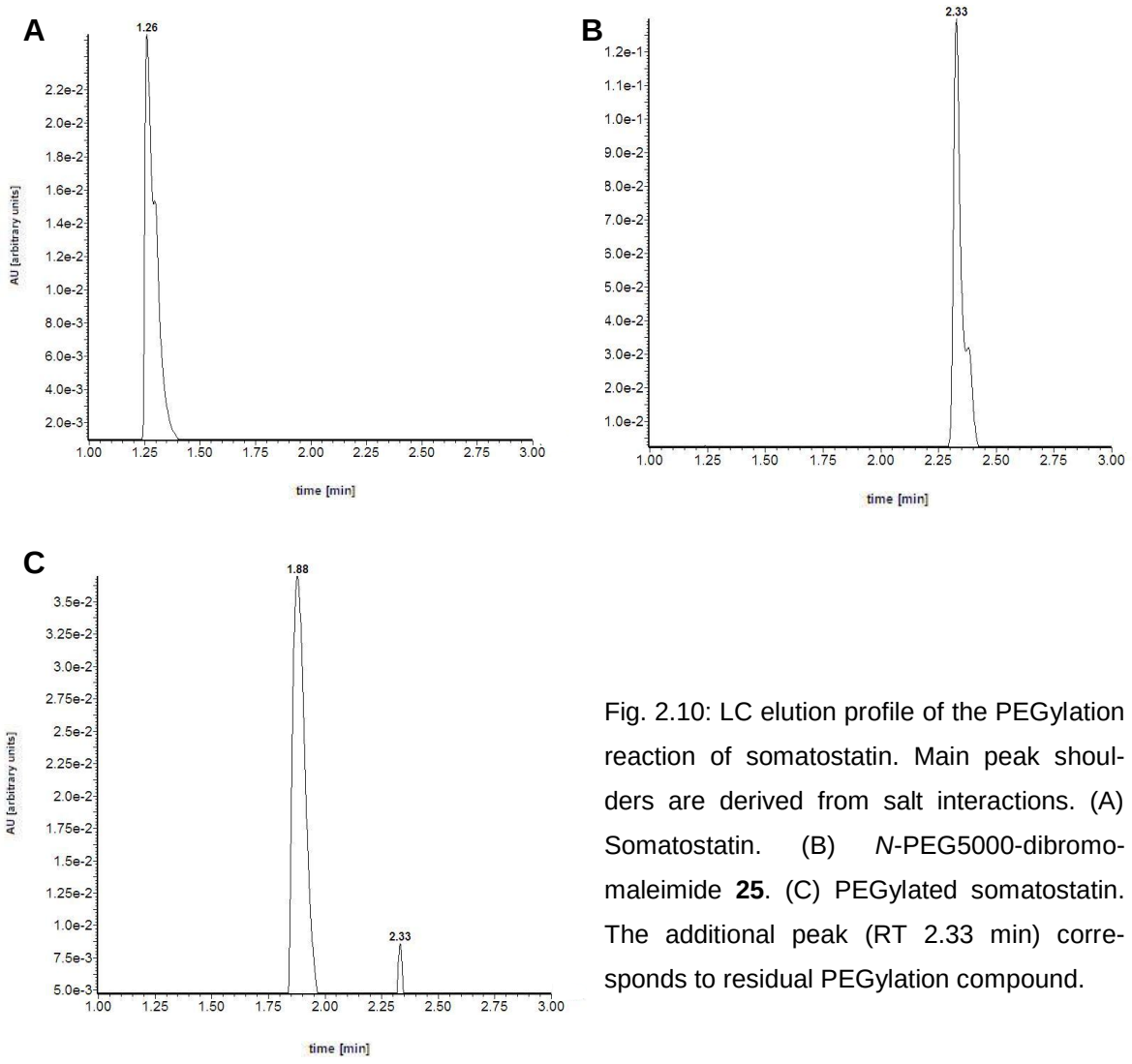


Fig. 2.10: LC elution profile of the PEGylation reaction of somatostatin. Main peak shoulders are derived from salt interactions. (A) Somatostatin. (B) *N*-PEG5000-dibromomaleimide **25**. (C) PEGylated somatostatin. The additional peak (RT 2.33 min) corresponds to residual PEGylation compound.

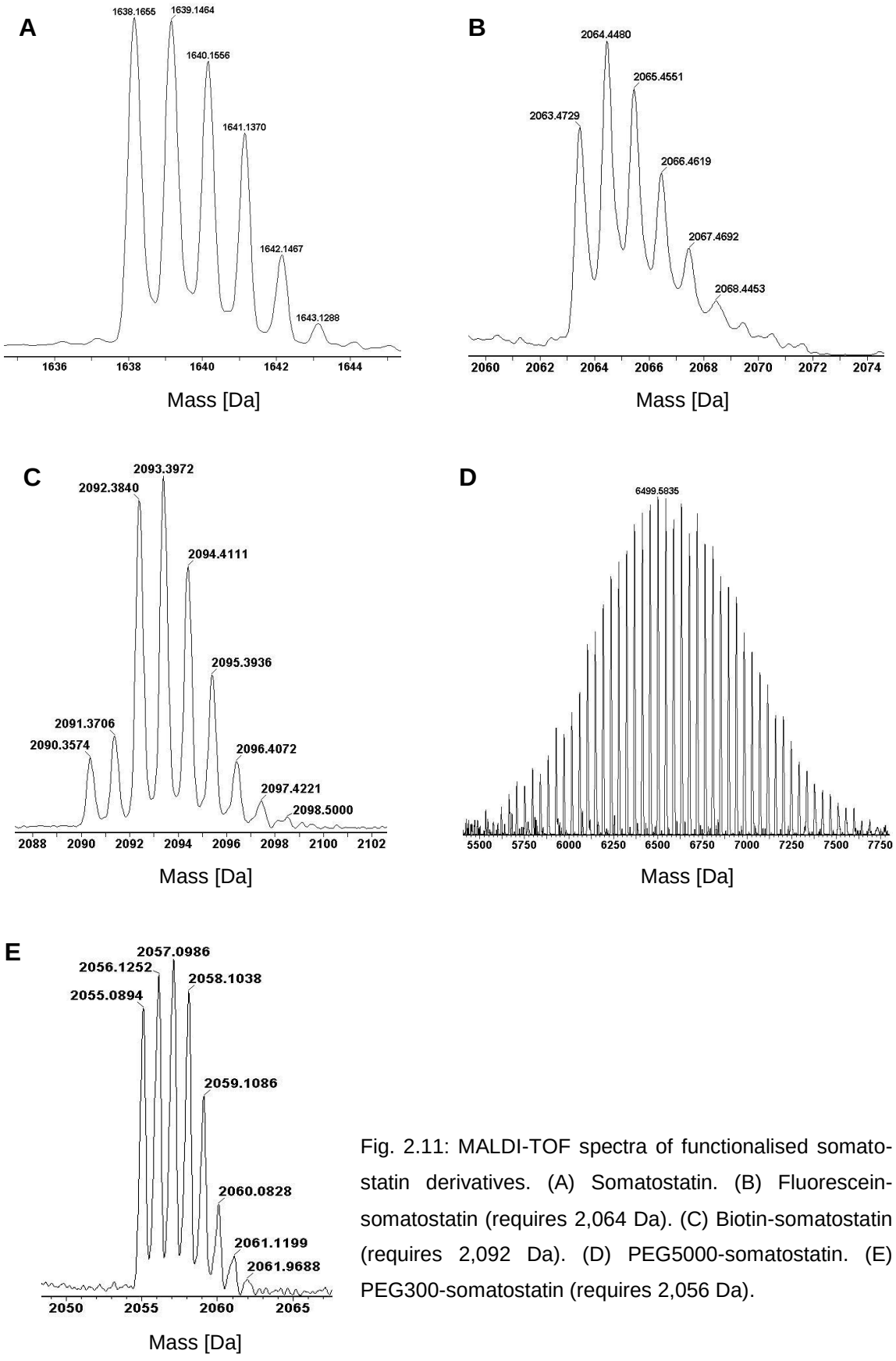


Fig. 2.11: MALDI-TOF spectra of functionalised somatostatin derivatives. (A) Somatostatin. (B) Fluorescein-somatostatin (requires 2,064 Da). (C) Biotin-somatostatin (requires 2,092 Da). (D) PEG5000-somatostatin. (E) PEG300-somatostatin (requires 2,056 Da).

When compared to non-functionalised dibromomaleimide almost no reduction in activity was observed despite the size of some of the attached molecules. The reactions with the fluorescein- and the spin labelled-compound even seemed a bit faster while the biotinylated maleimide exhibited a slightly lower reactivity. With exception of the last molecule, all reactions had reached completion after 10 min. MALDI-TOF analysis of the functionalised peptides confirmed the completeness of reaction. No signal for the free peptide was observed, with the exception of the sample conjugated to the large PEG chain. As the LCMS data indicated full conversion to the PEGylated somatostatin (Fig 2.10) and the cleavage of disulfide bonds by laser-induced ionisation (from a certain intensity on, which was necessary to ionise the PEGylated molecules) is a known phenomenon,[158, 401] it was assumed that this signal was an artefact of the MS technique.

Although not the first example for the modification of SST via its disulfide bond,[170] these reactions are certainly the fastest and most complete with the broadest range of functionality introduced. The high reactivity of all compounds suggests that the activity of dibromomaleimides will tolerate most modifications on its imide without significant loss of activity.

2.3.3 Reversibility of the functionalisation of somatostatin

In order to investigate if a more bulky substituent on the ring nitrogen of the maleimide bridge would inhibit the cleavage by a reducing agent, SST was modified according to the established protocol with *N*-methyl- **18**, *N*-fluorescein- **22**, *N*-biotin- **23**, *N*-PEG300- **24**, *N*-PEG5000- **25** or *N*-TPO-dibromomaleimide **26** and subjected to 100 equiv of 2-mercaptoethanol. The reaction was monitored at ambient temperature by LCMS (Fig. 2.12.)

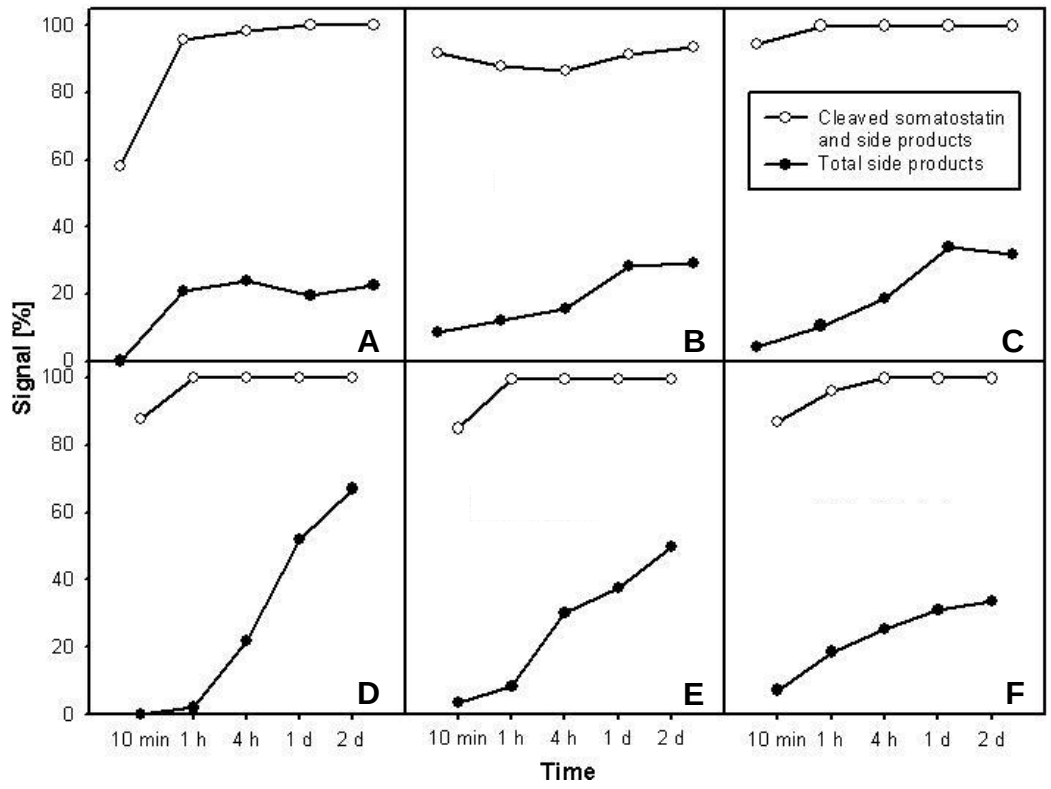
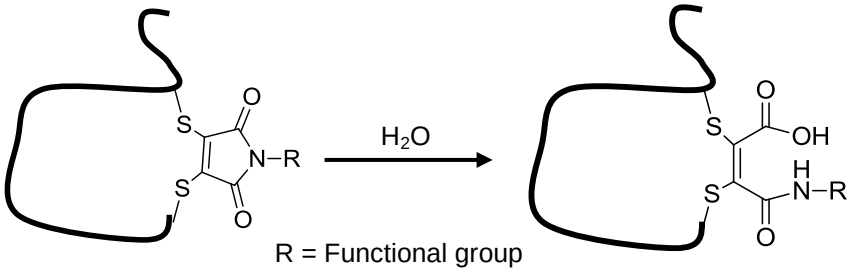


Fig 2.12: Reversion of the functionalisation of somatostatin with 100 equiv of 2-mercaptoethanol. (A) *N*-methylmaleimide-somatostatin. (B) Biotin-somatostatin. (C) Fluorescein-somatostatin. (D) PEG5000-somatostatin. (E) PEG300-somatostatin. (F) TPO-somatostatin.

All compounds were cleaved from the disulfide bond of SST to a large extent within 10 min. The reaction was complete within 1 to 4 h, with the exception of the biotinylated peptide. The usual pattern of side product formation of mixed disulfides was observed. This outcome shows that even the large 5,000 Da PEG chain did not inhibit the cleavage of the maleimide bridge.

2.3.4 Hydrolysis of the maleimide bridge

The hydrolysis of maleimides, where a hydroxide ion attacks one of the carbonyl groups to open the ring and to yield a carboxy- and an amide group, is a well known phenomenon (see Scheme 1.5).[102] The reaction is mainly pH- but also temperature-dependent.[115] As it has been shown that the substituents on the imide, particularly electron withdrawing groups, have an influence on this reaction[112, 114, 118] maleimide bridged and functionalised somatostatins were prepared and subjected to different conditions (Scheme 2.11 and Tables 2.5 – 2.10, the % hydrolysis have been rounded to the next 5% to reflect the limits of the MS based quantification method).



Scheme 2.11: Hydrolysis of a maleimide bridged somatostatin analogue.

Table 2.5: Hydrolysis of maleimide bridged somatostatin **20**. No signal = product degradation.

Time	1 d	2 d	3 d	6 d	9 d	14 d	21 d	28 d
hydrolysis [%] 4 °C, pH 6.2	0	0	0	0	0	0	0	0
hydrolysis [%] RT, pH 6.2	0	0	0	5	10	20	25	30
hydrolysis [%] 37 °C, pH 6.2	0	4	10	15	20	25	no signal	no signal
hydrolysis [%] 37 °C, pH 8.0	15	25	25	20	no signal	no signal	no signal	no signal

Table 2.6: Hydrolysis of fluorescein-somatostatin.

Time	After dialysis	60 min	2 h	4 h	6 h	1 d	2 d
hydrolysis [%] 4 °C, pH 6.2	5	5	10	10	10	20	45
hydrolysis [%] 4 °C, pH 8.0	0	5	10	10	20	50	50
hydrolysis [%] RT, pH 6.2	5	15	15	25	40	60	70
hydrolysis [%] 37 °C, pH 6.2	10	20	35	70	75	85	90
hydrolysis [%] 37 °C, pH 8.0	5	45	80	90	95	100	100

Table 2.7: Hydrolysis of biotin-somatostatin.

Time	1 min	10 min	60 min	2 h	4 h	6 h	1 d	2 d
hydrolysis [%] 4 °C, pH 6.2	0	0	0	0	5	5	5	10
hydrolysis [%] RT, pH 6.2	0	0	0	5	10	10	10	10
hydrolysis [%] 37 °C, pH 6.2	0	0	10	15	10	5	20	45

Table 2.8: Hydrolysis of PEG300-somatostatin. No signal = product degradation.

Time	1 d	2 d	3 d	6 d	9 d	14 d	21 d	28 d
hydrolysis [%] 4 °C, pH 6.2	0	0	5	5	5	15	20	15
hydrolysis [%] RT, pH 6.2	5	5	10	20	20	30	40	45
hydrolysis [%] 37 °C, pH 6.2	15	15	20	45	no signal	no signal	no signal	no signal
hydrolysis [%] 37 °C, pH 8.0	30	40	50	75	no signal	no signal	no signal	no signal

Table 2.9: Hydrolysis of PEG5000-somatostatin.

Week	1	2	3	4	5	6
hydrolysis [%] 4 °C, pH 6.2	0	0	5	0	5	5
hydrolysis [%] RT, pH 6.2	5	10	20	20	30	50

Table 2.10: Hydrolysis of TPO-somatostatin.

Time	1 min	10 min	60 min	2 h	4 h	6 h	1 d	2 d
hydrolysis [%] 4 °C, pH 6.2	0	0	0	0	0	0	0	5
hydrolysis [%] RT, pH 6.2	0	0	0	0	0	0	10	15
hydrolysis [%] 37 °C, pH 6.2	0	0	0	0	10	10	40	60

As all SST derivatives showed a different behaviour in this experiment, the time scales had to be adjusted for every compound individually. The extent of hydrolysis of PEG5000-somatostatin was estimated from MALDI-TOF samples and should be interpreted carefully. Due to the long time scale of the experiment only limited data is available on this compound.

Overall, every maleimide bridged peptide derivative exhibited hydrolysis. Variations in pH and temperature had the expected influence on this. The unmodified maleimide and the PEGylated versions of the compound were relatively stable against hydrolysis, especially if kept at low pH and low temperature. This suggests that short-term storage of maleimide modified proteins is possible if suitable conditions are chosen. Spin labelled and biotinylated peptides showed moderate levels of hydrolysis even at ambient temperature in time frames that are characteristic for biochemical assays. Thus, care has only to be taken if sample storage over prolonged periods of time is necessary.

Fluorescein-substituted maleimide demonstrated the greatest degree of hydrolysis, which is likely due to the electron withdrawing nature of the multiple ring

systems of the dye.[114] Combined elevation of both pH and temperature yielded fully hydrolysed compound within 24 h but no loss in the fluorescence intensity was observed (see 2.4.1). To test if the opening of the maleimide ring has an influence on the reversibility of the protein modification, completely hydrolysed fluorescein-somatostatin was prepared and treated with 100 equiv of 2-mercaptoethanol (Fig. 2.13). The reaction was monitored by LCMS at ambient temperature.

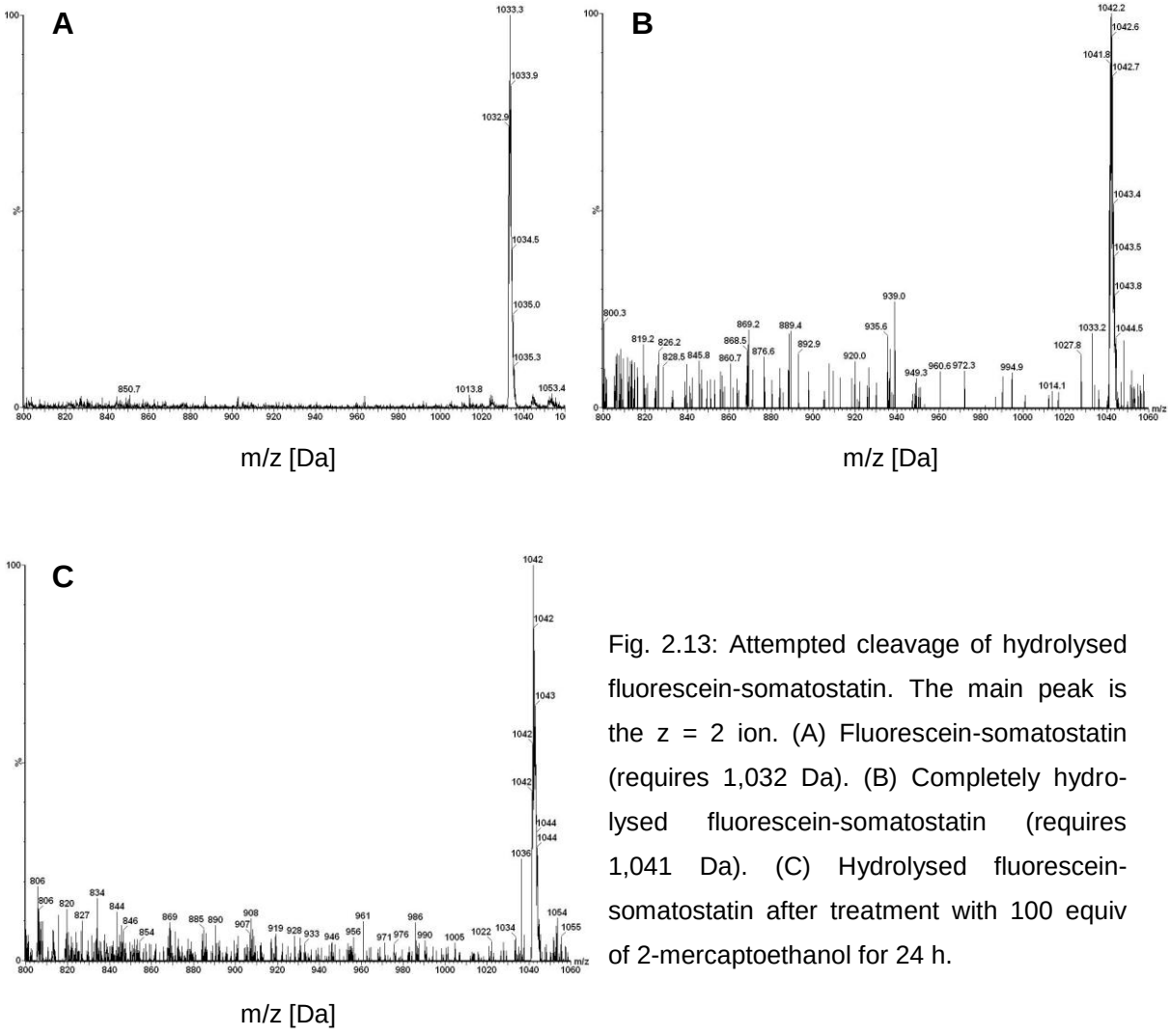


Fig. 2.13: Attempted cleavage of hydrolysed fluorescein-somatostatin. The main peak is the z = 2 ion. (A) Fluorescein-somatostatin (requires 1,032 Da). (B) Completely hydrolysed fluorescein-somatostatin (requires 1,041 Da). (C) Hydrolysed fluorescein-somatostatin after treatment with 100 equiv of 2-mercaptoethanol for 24 h.

No loss of the hydrolysed maleimide bridge was observed over 24 h of treatment with the reducing agent. Although this has negative implications for the use of maleimide modified proteins as drug delivery systems it also yields the chance to prepare non-cleavable conjugates which is desirable in some cases, such as radioactive labelling of biomolecules. Furthermore, the difference in hydrolysis rates of various maleimide compounds has proven that the imide

substituent can be used to adjust the behaviour of the molecule with respect to this effect. Finally, groups that either promote or inhibit hydrolysis can be inserted as linkers between the maleimide and the functional molecule, as demonstrated by the Baker group and others.[115, 397]

2.3.5 Stability of functionalised somatostatins

While analysing the synthesised peptide derivatives not only hydrolysis was observed but also other changes to the original product. As SST is known to have a very short half-life in the human body,[259] these effects were investigated by the exposure of all SST analogues to 37 °C for 48 h. The samples were analysed by LCMS (Table. 2.11).

Table 2.11: Fate of somatostatin derivatives after 48 h incubation at 37 °C (pH 6.2).

Derivative	Intact [%]	Cleaved [%]	Hydrolysed [%]	Reshaped [%]	Loss of label [%]
maleimide-SST	52	21	3	24	-
fluorescein-SST	0	0	100	0	0
biotin-SST	2	21	8	69	0
PEG300-SST	40	5	5	50	0
TPO-SST	10	30	15	0	45

Although the method used for ionisation (electron spray) is considered to be mild, the results have to be interpreted carefully as some effects were clearly technique derived artefacts. For example cleavage and loss of the label was observed for fluorescein-somatostatin but the corresponding signals disappeared when the cone voltage was lowered.

This analogue was also the most stable in terms of preservation of the whole conjugate as only hydrolysis could be observed. PEGylated and bridged somatostatin were also relatively stable. In contrast the biotinylated peptide was converted to a large extent into a product which exhibited the same mass ion distribution but at a slightly longer retention time on the LC column (data not shown). A more detailed identification was not attempted but a possible explanation might be the reaction of the maleimide bridge with one of the lysine side chains (Cys3 is directly adjacent to such a residue) resulting in a shape different from the usual cyclic peptide. The levels of hydrolysis differ from those previously shown as none of the other degradative effects had been taken into account for

the calculation of this phenomenon. Only the spin labelled hormone showed a direct loss of the label from the maleimide ring possibly induced by reactions of the nitroxide radical. The cleavage observed for all compounds but fluorescein-somatostatin was potentially induced by side products generated during the bridging reaction or by buffer components and should be avoidable by purification of the products after synthesis.

2.4 Activity of somatostatin analogues

2.4.1 Optical activity of fluorescein-somatostatin

To test if the connection of fluorescein to the maleimide ring and its conjugation to somatostatin yielded a functional fluorescent dye, fluorescence spectra were recorded for these molecules at 25 °C and at an excitation wavelength of 488 nm (Fig. 2.14). Fluorescein-amine which was used as starting material for the synthesis[396] was included as a control.

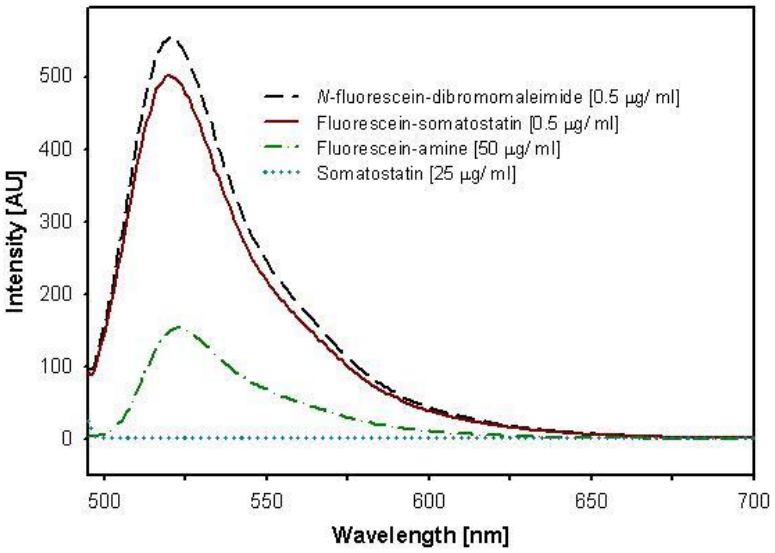


Fig. 2.14: Fluorescence of fluorescein-compounds and somatostatin. Excitation wavelength = 488 nm.

Unmodified somatostatin showed no fluorescence but exhibited a strong signal after maleimide mediated conjugation to fluorescein. This did not change upon hydrolysis of the bridged peptide analogue (see 2.3.4). No large change of the signal intensity of the reacted and unreacted *N*-fluorescein-dibromomaleimide was observed. The strong increase of the fluorescence of the fluorescein-amine in contrast is a reported effect[138] which is based on the conversion of the amine to an amide and accompanied loss of a quenching effect of the lone electron pair of the nitrogen.

2.4.2 Properties of the maleimide spin label

EPR spectra of both the spin label **27** and spin labelled somatostatin were recorded at ambient temperature on a continuous-wave (CW) spectrometer to investigate the activity and quality of the label (Fig 2.15).

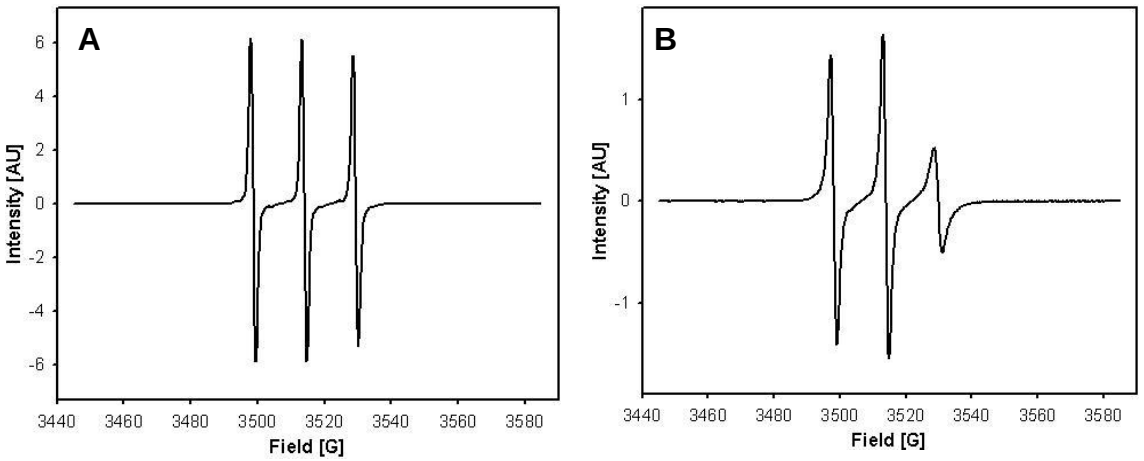


Fig. 2.15: Spectroscopic activity of the *N*-TPO-dibromomaleimide spin label. (A) *N*-TPO-dibromomaleimide spin label. (B) TPO-somatostatin.

Both spectra were recorded at a concentration of 150 µM. The maleimide spin label exhibited three sharp peaks, which is typical for nitroxide radicals. The signal to noise ratio was excellent (a single scan is shown); even the nitrogen isotopes were visible and the g-factor was comparable to other spin labels. When attached to somatostatin, line-broadening was observed as well as a decrease in the third radical peak indicating the loss of mobility of the spin label. Overall, the *N*-TPO-dibromomaleimide spin label showed a high spin density, a clear line shape and a high threshold in power saturation which are desirable features for an organic EPR label.[145]

2.4.3 Biological activity

To test the biological activity of the maleimide bridged SST as well as the PEGylated (PEG5000) and fluorescein-conjugated analogues, a collaboration with Prof Andy Tinker (UCL Medicine) was set up. Dr Muriel Nobles (UCL Medicine) performed patch-clamp assays with HEK293 cells, testing the three analogues and the wild type hormone to investigate if the modified peptide is still able to activate its receptor (Fig 2.16 and 2.17). A cell line expressing the G protein-coupled inwardly-rectifying potassium ion channel (GIRK), a natural down-

stream target of SST activity,[262] was transiently transfected with sst_2 receptor DNA, one of the SST receptors that is known to interact with this type of ion channel.[252] Successful transfection was visualised via epifluorescence and controls for the patch-clamp assay included pre-treatment of cells with pertussis toxin, an inhibitor of G-proteins[262] or tertiapin-Q, an inhibitor of the GIRK channel ion flow.

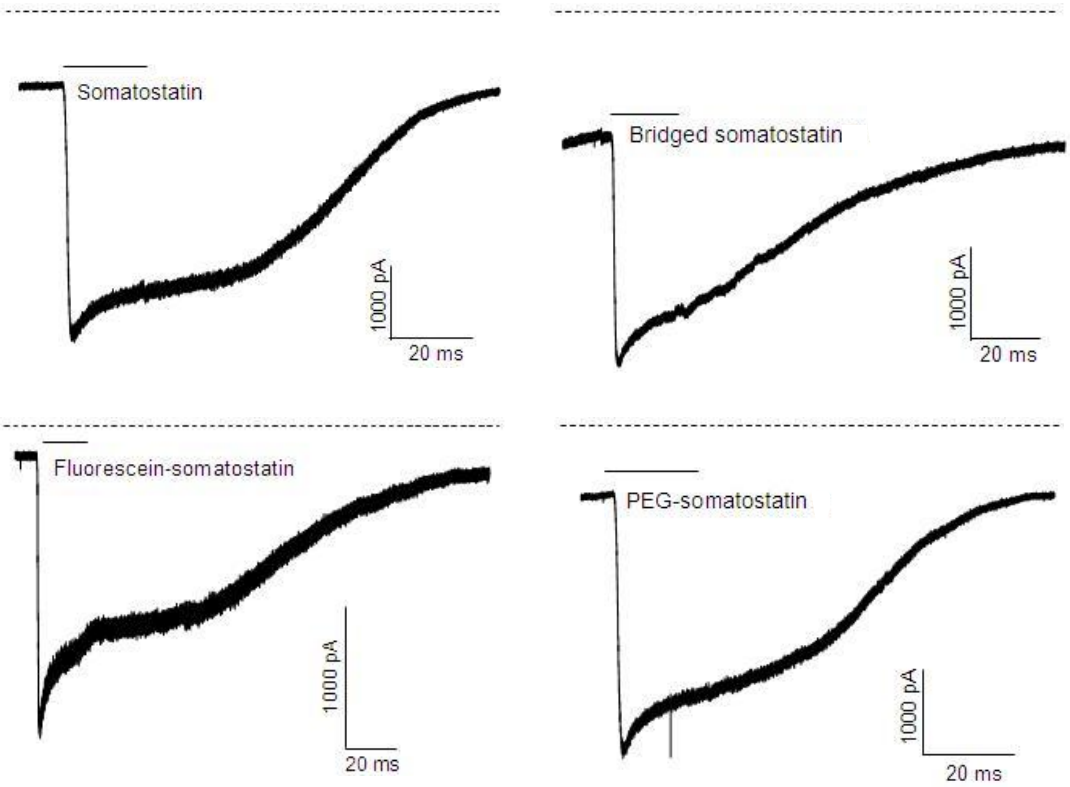


Fig. 2.16: Representative current traces recorded with the HEK293 cell line expressing sst_2 transiently. Cells were clamped at –60 mV and 20 µM of somatostatin or its derivatives were applied for 20 s. Dotted lines indicate zero current. Continuous lines indicate exposure time.

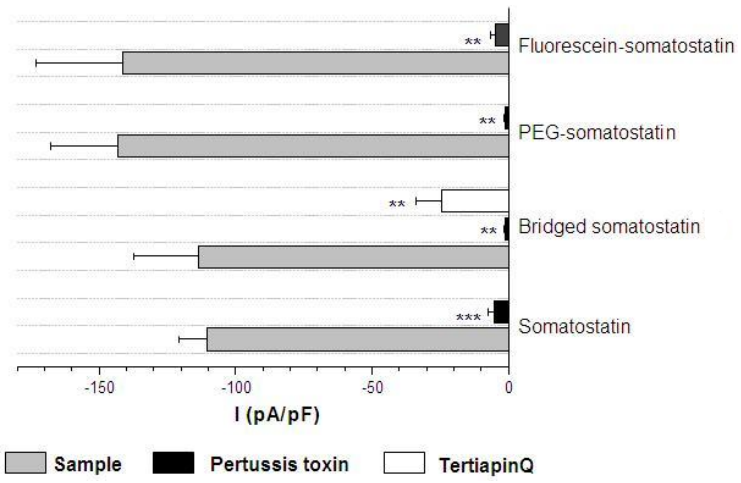


Fig. 2.17: Amplitude of currents activated by somatostatin and analogues. Peak values of currents are shown in sample conditions, after pretreatment of cells with pertussis toxin (for 24 h) or preincubation with tertiapin-Q (100 nM for 5 min). A t-test was used to evaluate the observed effects. ** $P<0.01$, *** $p<0.001$.

All three analogues tested were able to activate the SST induced signal cascade in a comparable manner to the unmodified hormone. The regeneration of the membrane current was slightly slower in the case of the maleimide modified peptide but this effect was not significant. The strength of activation expressed in the peak current was similar for all four compounds tested and inhibited by pertussis toxin and tertiapin-Q showing the channel- and receptor-specificity of the treatment. Overall, the three tested SST analogues were biological active.

In summary, all somatostatin analogues synthesised by modification of the hormone's disulfide bond via maleimide chemistry exhibited their functional purpose (e.g. fluorescence) and were bioactive. The robustness and versatility of the method indicates great potential for the methodology when applied to functionalise accessible disulfide bridges in peptides and proteins.

2.5 Development of *in situ* protocols for the modification of protein disulfide bonds

2.5.1 Synthesis

Analogous to dicysteinemaleimide **8** (see 2.1.2), di-2-mercaptoethanolmaleimide **28**, di-2-mercaptopyridinemaleimide **29** and dithiophenolmaleimide **30** were synthesised (Fig. 2.18).

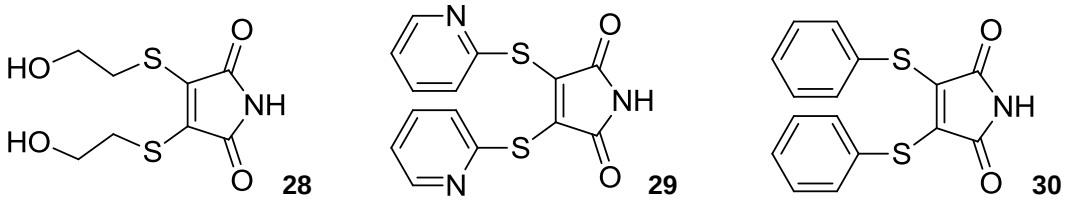


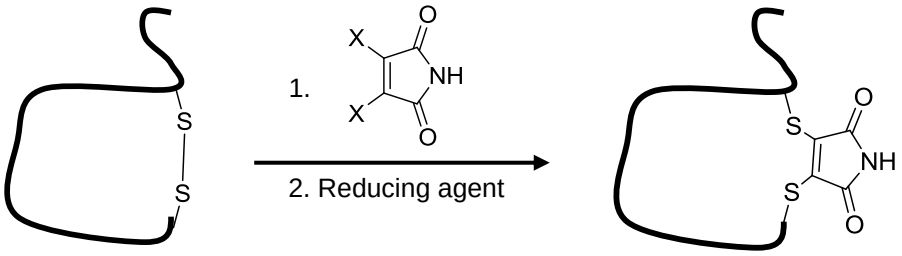
Fig. 2.18: Synthesised dithiomaleimides. Di-2-mercaptoethanolmaleimide (**28**), di-2-mercaptopyridinemaleimide (**29**) and dithiophenolmaleimide (**30**).

Slow addition of 2.2 equiv of the thiol-substituent to dibromomaleimide **6** in the presence of a base furnished the compounds in yields ranging between 51% – 75%. This compares well with the reported procedures for dithiomaleimides.[244-246] Di-2-mercaptoethanolmaleimide **28** is highly water soluble in contrast to di-2-mercaptopyridinemaleimide and dithiophenolmaleimide. Thus, solutions of the

maleimides **29** and **30** were always prepared immediately before the experiment to avoid precipitation. Di-2-mercaptopyridinemaleimide **29** was found to be very unstable and was susceptible to degradation by light and temperature.

2.5.2 Dibromomaleimide cross-reacts with TCEP

All protocols developed so far, as well as those which have been previously published,[170, 202, 240] rely on the fact that the reduced form of the protein is stable. In reality proteins can start to unfold or disulfide scrambling is induced upon opening of a disulfide bridge. In addition, protein complexes that are only connected by disulfide bonds cannot tolerate a sequential methodology of reduction and re-connection. Thus, an ideal bridging strategy has to minimise the time a disulfide exists in its reduced form. This could be accomplished by addition of the bridging reagent prior to the reducing agent so that any opened disulfide bond can immediately be re-bridged (Scheme 2.12).



Scheme 2.12: Theoretical sequence of the *in situ* modification of a disulfide bond.

This requires that i) the re-bridging reaction is rapid and ii) the bridging compound is unreactive towards the reducing agent and vice versa, such that they can be present in the solution at the same time but do not interfere with each other.

Halomaleimides have exhibited excellent reactivity, fulfilling the first criteria. To test if they could be used in tandem with a reducing agent, dibromomaleimide was added to oxidised SST, followed by *tris*(2-carboxyethyl)phosphine (any thiol reducing agent would react directly with dibromomaleimide **6** and an excess would be necessary for complete reduction) and the reaction analysed by LCMS at ambient temperature (Fig. 2.19). The experiment was repeated with varying amounts of bridging and reducing agent.

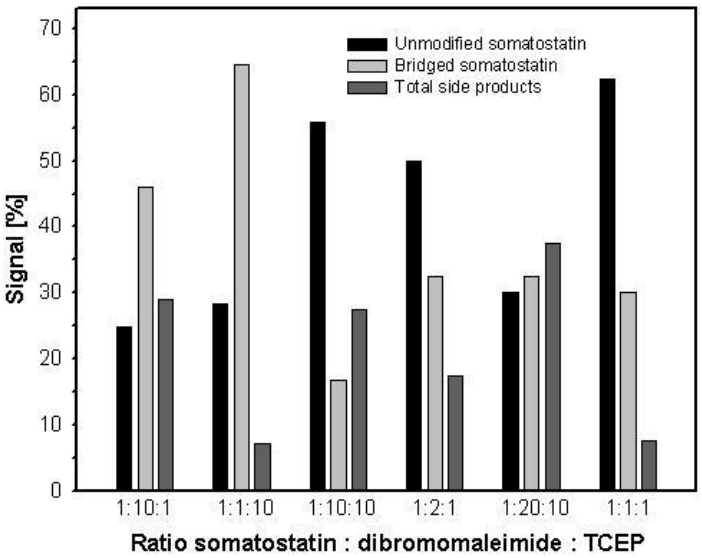


Fig. 2.19: Simultaneous addition of different amounts of dibromomaleimide and TCEP to oxidised somatostatin.

Despite the formation of some modified peptide, a full conversion was not observed in any experiments. This implies that the reaction of TCEP with somatostatin (reduction) as well as the reaction of the free cysteines with dibromomaleimide (bridging) was faster than a consumptive side-reaction of these compounds with each other. However, a high excess of reducing and re-bridging agent over the protein did not increase the formation of maleimide bridged SST. The best yield of modified peptide (65%) was obtained with a ratio of 1 : 1 : 10 of SST : dibromomaleimide : TCEP. This again suggests that the reduction of the peptide takes place faster than the reaction between the maleimide and TCEP.

Since some of the observed side products (Fig 2.20) were derived from addition reactions of the phosphine as indicated by MS, attempts were made to minimise the presence of the reducing agent. Thus, oxidised SST was mixed with 10 equiv of dibromomaleimide and single equivalents of TCEP were added at intervals of 10 min (Fig. 2.20).

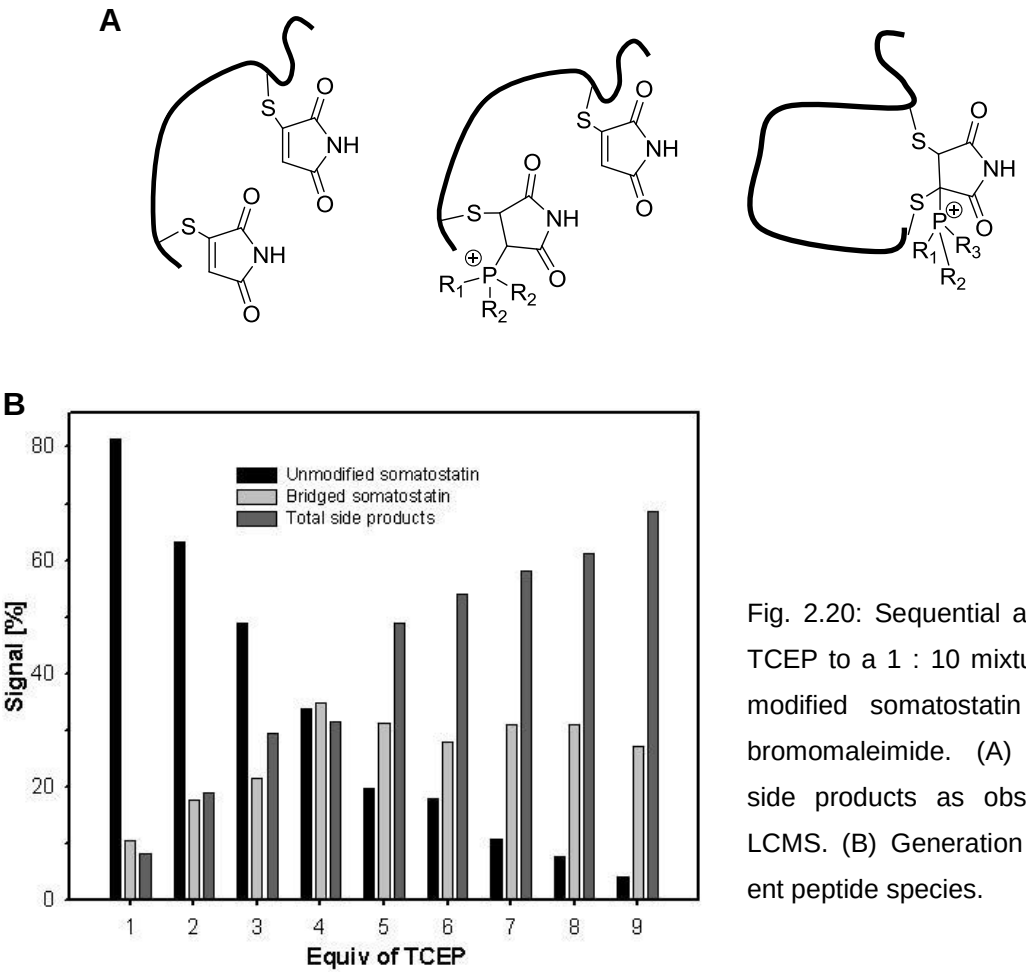


Fig. 2.20: Sequential addition of TCEP to a 1 : 10 mixture of unmodified somatostatin and dibromomaleimide. (A) Potential side products as observed by LCMS. (B) Generation of different peptide species.

The stepwise addition of the reducing agent was also inefficient. Significant amounts of side products were formed even in the initial stages of the reaction and no more product synthesis was observed with 5 or more portions of TCEP. The nature of the undesired SST products suggests two major problems: First, over-labelling, i.e. individual reaction of each cysteine with a maleimide, followed potentially by a reduction step (see 2.1.5), that occurs in the presence of an excess of dibromomaleimide **6**. Second, both the double-alkylated somatostatin as well as the bridged product was found with a phosphine attached. As this last by-product was not formed simultaneously to a loss of the desired SST **20** in the later stage of the experiment, TCEP-attack on the dibromomaleimide[392, 393] had probably taken place before the bridging event.

Together this showed that the requirement for tolerance of reducing and bridging reagent was not fulfilled with the combination of dibromomaleimide and TCEP and thus is not suitable for a high yielding *in situ* protocol for the modifi-

cation of disulfide bonds. Although a yield of 65% was obtained, the separation from the observed side products is likely to be difficult.

2.5.3 Reactivity of dithiomaleimides

Thiol exchange between maleimide bridged SST and thiol reducing agents had been observed during the successful cleavage reactions of the modified peptide hormone (see 2.2.5). To assess if the products of these exchange reactions, dithiomaleimides, would be suitable reagents for disulfide bond bridging a range of these compounds (**8**, **28**, **29** and **30**) were synthesised and reacted with reduced SST. As a decreased efficiency in bridging of the open cystine was expected, 1, 5 or 10 equiv of the dithiomaleimides were added. The reaction was monitored by LCMS (Fig. 2.21).

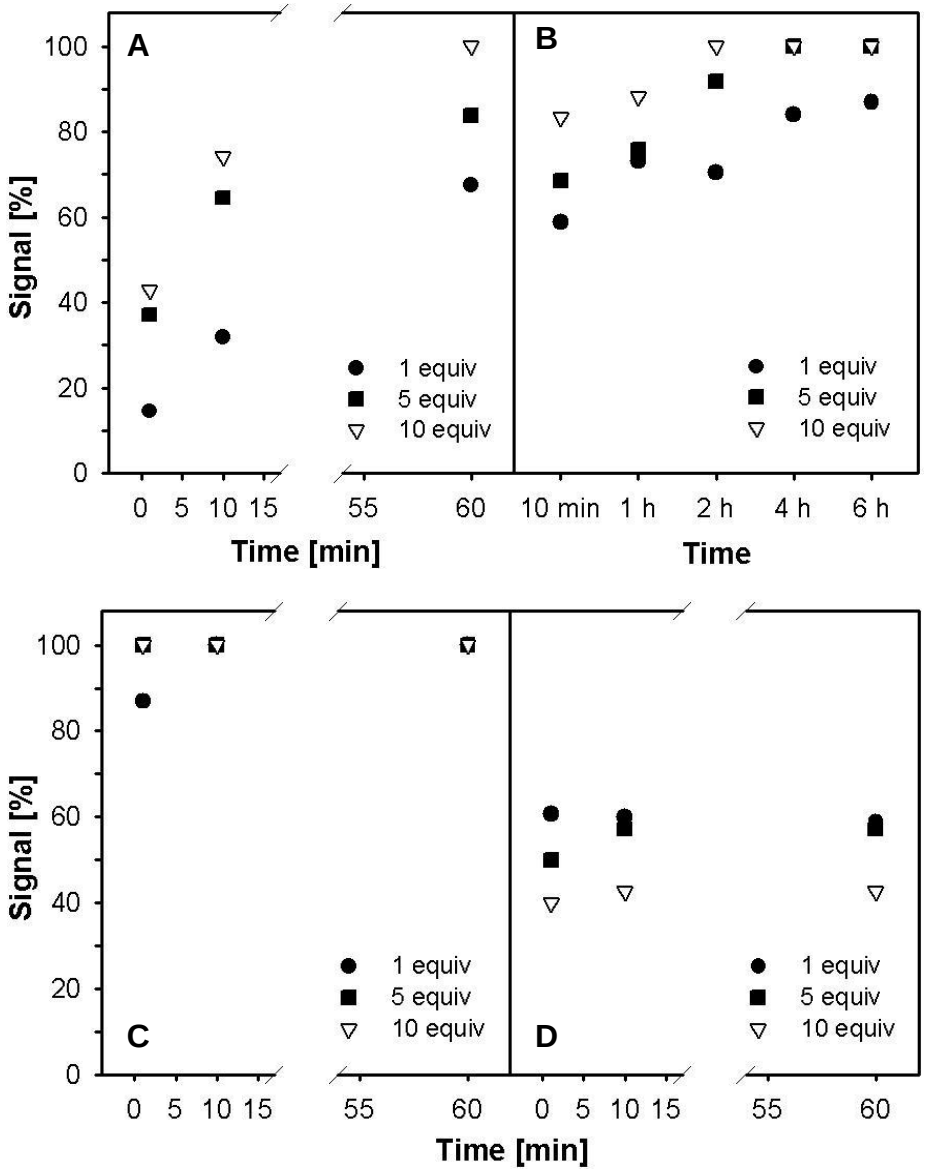


Fig. 2.21: Bridging of reduced somatostatin with dithiomaleimides. The signal % corresponds to the relative population of the maleimide bridged somatostatin **20**. (A) Di-2-mercapto-ethanolmaleimide **28**. (B) Dicysteinemaleimide **8**. (C) Dithiophenolmaleimide **30**. (D) Di-2-mercapto-pyridinemaleimide **29**.

The four dithiomaleimides tested exhibited very different activities. The most rapid conversion of open to bridged somatostatin was achieved with dithiophenolmaleimide **30**. As little as 1 equiv of the compound yielded 87% of bridged peptide after 1 min. The second fastest dithiomaleimide was di-2-mercaptoethanolmaleimide **28**. Here 10 equiv were necessary to obtain complete conversion within 1 h. When the reaction with 1 equiv was monitored over 24 h no further increase in product yield could be observed, only an increased amount of side products. This suggests that in this reaction a dynamic equilibri-

um is established between the maleimide moiety within the cyclic structure of the peptide and the free small molecule dithiol. This would be based on the release of free thiols upon the insertion of the 5-membered ring into somatostatin, which can attack the product and open the modified disulfide bond again. The addition of more equivalents of dithiomaleimide shifts the equilibrium towards the bridged peptide. Interestingly, this also suggests that by using thiomaleimides instead of halomaleimides the quality of the substituents, not only as a leaving group but also as a nucleophile for the attack back on the product, will play an important role. Since the nucleophilicity of thiols depends on the pH, different thiomaleimides might exhibit different adaptability at various proton concentrations.

The reaction with dicysteinemaleimide **8** reached equilibrium slowly but eventually yielded completely bridged peptide **20**. Two different reactions were observed in the case of di-2-mercaptopyridinemaleimide **29**. With 1 equiv of this compound equilibrium was reached within 1 min but with only 60% of the bridged peptide. When more bridging reagent was added a prominent side product peak was detected by MS with a mass corresponding to the peptide plus two molecules of mono-2-mercaptopyridinemaleimide. This observation suggests that this compound is highly reactive with the first attack of a peptide cysteine occurring faster than the ring closure. In summary, dithiophenol- **30** and di-2-mercaptoethanolmaleimide **28** were found to be the most promising dithiomaleimide bridging reagents.

2.5.4 Cross-reactivity of dithiomaleimides with TCEP

After the dithiomaleimides had exhibited good reactivity, it was necessary to test for possible cross-reactivity with TCEP. In order to obtain comparable results the halomaleimides were included in this experiment. All compounds were mixed with 1 equiv of TCEP in an aqueous buffer system (50 mM sodium phosphate, pH 6.2, 40% MeCN, 7.5% DMF) and stirred for 10 min at ambient temperature. The maleimides were isolated and purified to calculate the loss of material which should have occurred due to reaction with TCEP. Outcomes were normalised by the results of a control reaction without reducing agent to account for purification related losses (Table 2.12).

Table 2.12: Cross-reactivity of dihalo- and dithiomaleimides with TCEP.

	Sample reaction		Control reaction
Compound	Yield [%]	Normalised yield [%]	Yield [%]
dichloromaleimide	61	75	82
diiodomaleimide	64	77	83
di-2-mercapto-pyridinemaleimide	72	79	91
dibromomaleimide	67	84	80
dithiophenolmaleimide	88	88	100
dicysteinemaleimide	97	97	100
di-2-mercapto-ethanolmaleimide	100	100	100

The reactions showed a clear trend; halomaleimides have a higher reactivity towards the phosphine in an aqueous system than the thiomaleimides. As reported before[392] dichloromaleimide exhibited the highest cross-reactivity with a loss of around 25% of the material within 10 minutes. Approximately 20% of reactant was lost in the case of diiodo- and dibromomaleimide as well as with the most reactive dithiol-compound, di-2-mercaptopyridinemaleimide. The remaining dithiomaleimides showed only small cross-reactivity with no loss of material in the case of di-2-mercaptoethanolmaleimide.

2.5.5 *In situ* reactions with dithiomaleimides

As di-2-mercaptoethanol **28** and dithiophenolmaleimide **30** fulfilled both requirements for potential *in situ* bridging reagents – fast re-connection of the reduced disulfide bond and good resistance against TCEP – these two compounds were investigated for applicability in the *in situ* bridging protocol. 1 equiv of somatostatin was mixed with either 10 equiv of di-2-mercaptoethanolmaleimide or 5 equiv of dithiophenolmaleimide. After 5 min various amounts of TCEP were added and, following incubation at ambient temperature for 1 and 2 h, all samples were analysed by LCMS (Fig. 2.22).

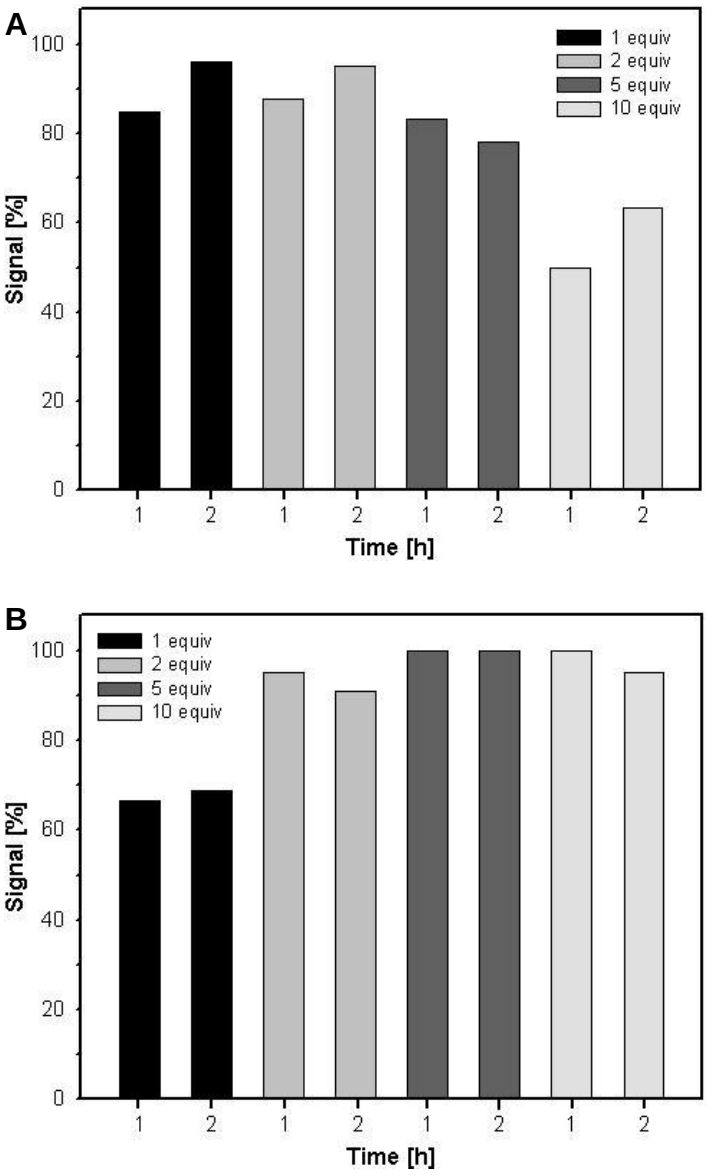


Fig. 2.22: Activity of dithiomaleimides in *in situ* bridging reactions of SST. The signal % corresponds to the relative population of the maleimide bridged somatostatin **20**. (A) Addition of various amounts of TCEP to a 1 : 10 mixture of somatostatin : di-2-mercaptoethanolmaleimide **28**. (B) Addition of various amounts of TCEP to a 1 : 5 mixture of somatostatin : dithiophenolmaleimide **30**.

Both reagents bridged somatostatin with good efficiency. In the presence of 1 or 2 equiv of TCEP di-2-mercaptoethanolmaleimide **28** reached 95% conversion of the model peptide in the analysed time frame. Increased amounts of reducing agent either interfered with the bridging reaction or started to attack the product. As implied by the cross-reactivity experiments an increased amount of TCEP was necessary to fully reduce somatostatin in the presence of dithiophenolmaleimide **30**. 100% conversion was achieved within 1 h at ratios of 1 : 5 : 5 and 1 : 5 : 10 of peptide : dithiophenolmaleimide : TCEP.

To investigate if the orthogonal reaction leads to the insertion of the maleimide bridge into the disulfide bond of SST as fast as observed when the peptide was reduced before the bridging reaction (see 2.5.3), 1 equiv of the hormone was mixed with 5 equiv of dithiophenolmaleimide and 3 equiv of TCEP were added.

The reaction was monitored at short intervals by LCMS at ambient temperature (Fig. 2.23).

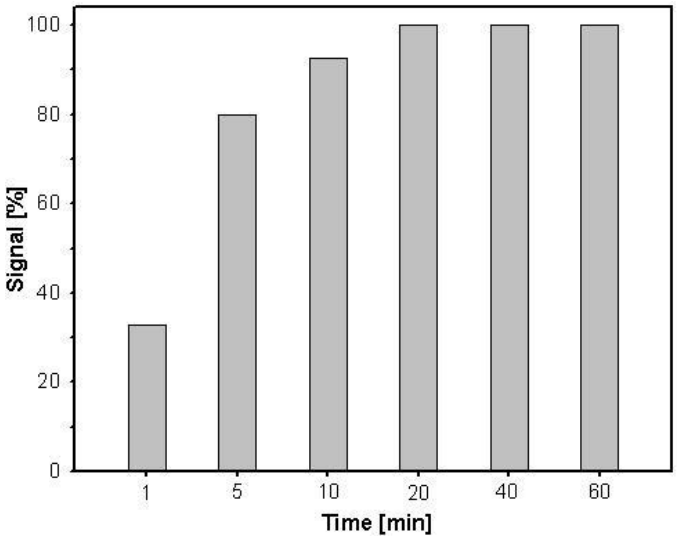


Fig. 2.23: Rapid modification of the somatostatin disulfide bond by orthogonal activity of dithiophenolmaleimide **30** and TCEP. The signal % corresponds to the relative population of the maleimide bridged somatostatin **20**.

Although not as fast as when the disulfide bond had been reduced before, dithiophenolmaleimide yielded fully bridged somatostatin in tandem with TCEP within 10 to 20 min. To measure the speed at which reduction alone takes place, SST was treated with the phosphine and aliquots subsequently quenched with a large excess of dibromomaleimide. 50% reduction of the disulfide bond was observed after around 1 min and 95% after 10 min (data not shown). Together this indicates that, in the *in situ* reaction, the bridging happens roughly as fast as reduction takes place.

2.5.6 Bridging of somatostatin in the presence of 2-mercaptoethanol

In some cases TCEP might interfere with other chemical groups in a protein target or exhibit a reducing activity that is too strong when only mild conditions are needed, such as in the case of the opening of a single specific disulfide bond in the presence of other cystines. To account for the possibility that conventional reducing agents have to be used, a sequential experiment with the combination of 2-mercaptoethanol and dibromomaleimide was performed. Somatostatin was reduced with TCEP and mixed with 10 equiv of 2-mercaptoethanol. After 5 min various amounts of dibromomaleimide were added and the presence of bridged somatostatin and side products were monitored by LCMS for 24 h (Fig. 2.24).

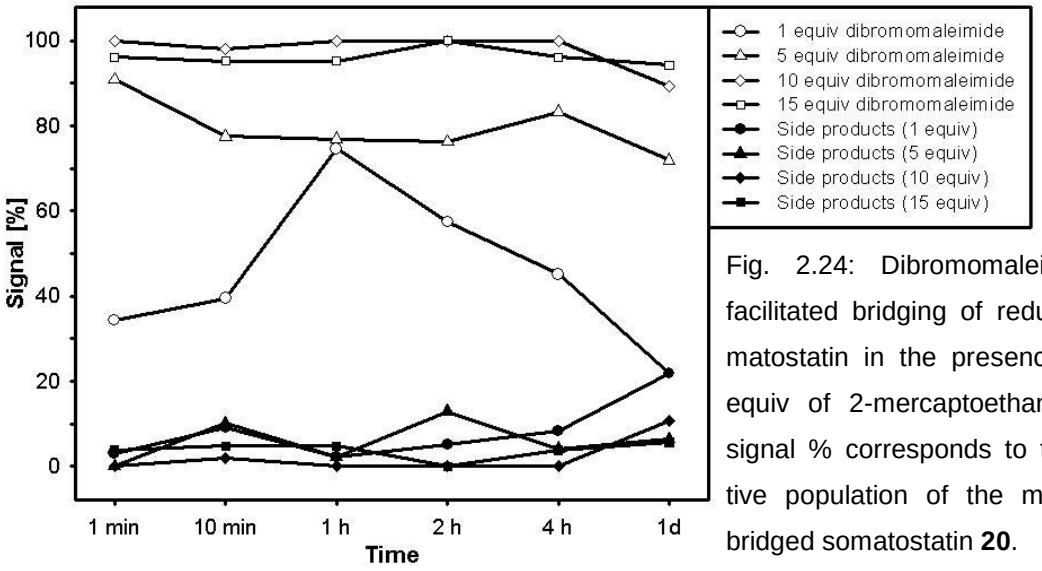


Fig. 2.24: Dibromomaleimide **6** facilitated bridging of reduced somatostatin in the presence of 10 equiv of 2-mercaptoethanol. The signal % corresponds to the relative population of the maleimide bridged somatostatin **20**.

As expected, the addition of the dibromomaleimide **6** to the 2-mercaptoethanol containing solution led immediately to the formation of di-2-mercaptoethanolmaleimide **28** as indicated by the strong yellow colour. This was further confirmed by the absorbance trace of the LC-column which showed a prominent signal corresponding to this compound. In concordance with the bridging reactions using dithiomaleimides, an equilibrium was established for the maleimide moiety between bridged peptide **20** and small molecule dithiol **28**. The 37% conversion after 1 min, compared to 16% conversion in the case of 1 equiv of di-2-mercaptoethanolmaleimide (see Fig. 2.21 A), suggests that dibromomaleimide favoured the reaction with the peptide over that with 2-mercaptoethanol which was present in 10x excess. This difference in reactivity had disappeared after 10 min and 73% conversion after 1 h compared well with the previous observation for di-2-mercaptoethanolmaleimide. A possible preference to form the cyclic SST product **20** with somatostatin became more evident when 5 equiv of dibromomaleimide were used. Here more than 90% conversion was observed after 1 min. It is important to note that 1 equiv of the halomaleimide reacts with 2 equiv of 2-mercaptoethanol resulting in an approximately 1 : 1 ratio between thiol and bridging reagent in this sample. A further increase of the amount of dibromomaleimide yielded complete conversion and reduced the amount of side products observed.

In conclusion, although no orthogonal reaction is possible between 2-mercaptoethanol and dibromomaleimide **6**, the fact that the initially undesirable side reaction results rapidly in the formation of another disulfide bridging rea-

gent opens the opportunity to use both reagents sequentially, with the maleimide in excess, to neutralise the remaining reducing agent.

2.5.7 Selenol supported orthogonal modification of disulfide bonds

The possibility to use benzeneselenol as a catalyst to remove the maleimide bridge from the modified somatostatin had been tested (see 2.2.6). The fact that selenols also supports the thiol-mediated cleavage of disulfide bonds[215, 402] could perhaps be explored to develop an alternative *in situ* protocol. In this scenario, the selenium compound could potentially act as a reducing agent or as a nucleophile to replace thiol molecules from the dithiomaleimide. In the second case the released sulphur groups could facilitate, supported by the selenol, the cleavage of a disulfide bond whose free cysteines would react immediately with the substituted maleimide. This last reaction would liberate more thiol driving the reaction towards completion.

To investigate this theory somatostatin was first treated with 10 equiv of benzeneselenol and cleavage was analysed by addition of an excess of dibromomaleimide. No maleimide bridged peptide was observed, proving no reduction had taken place (Table 2.13). If, however, 2-mercaptoethanol was added to the sample as well, formation of the modified hormone was observed. Furthermore, if 10 equiv of 2-mercaptoethanol were mixed with SST followed by only 1 equiv of the selenol, an increased amount of disulfide bond reduction was found than with the thiol alone (data not shown). Together this verifies that benzeneselenol itself does not cleave the SST cystine.

Table 2.13: Selenol-mediated cleavage of the somatostatin disulfide bond.

Amount of benzeneselenol	Amount of 2-mercaptoethanol	Cleavage after 1 h [%]	Cleavage after 3 d [%]
10 equiv	0 equiv	0	0
10 equiv	2 equiv	0	31
1 equiv	10 equiv	30	22

Next it was necessary to establish if benzeneselenol is able to replace one of the thiol-substituents from the dithiomaleimide compounds. To this end dithio-

phenolmaleimide was mixed with a 5x excess of benzeneselenol in MeOH. No reaction was observed after 3 h at ambient temperature (thiophenol and selenol are too similar to be distinguished by TLC). But when a large excess of maleimide was added to trap any free thiol or selenol both thiophenol-succinimide and benzeneselenol-succinimide were found after purification of the reaction mixture (Fig. 2.25). The yields were 4% and 17% respectively and the identity of the compound was confirmed by NMR as well as MS and was in good agreement with published data on these molecules.[403, 404] This experiment showed that benzeneselenol is able to replace the substituents of dithiomaleimides.

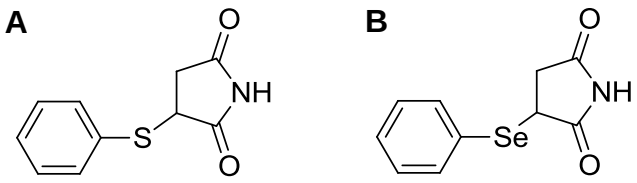


Fig. 2.25: Products of the reaction of dithiophenolmaleimide with benzeneselenol after quenching with maleimide. (A) Thiophenol-succinimide. (B) Benzeneselenol-succinimide.

To finally test the hypothesis of the selenol-mediated *in situ* modification of disulfide bonds, both dithiomaleimides that had been successfully used in combination with TCEP (di-2-mercaptoethanolmaleimide **28** and dithiophenolmaleimide **30**) were added in various amounts to oxidised SST followed, after short incubation, by different quantities of a freshly prepared benzeneselenol solution (Fig. 2.26).

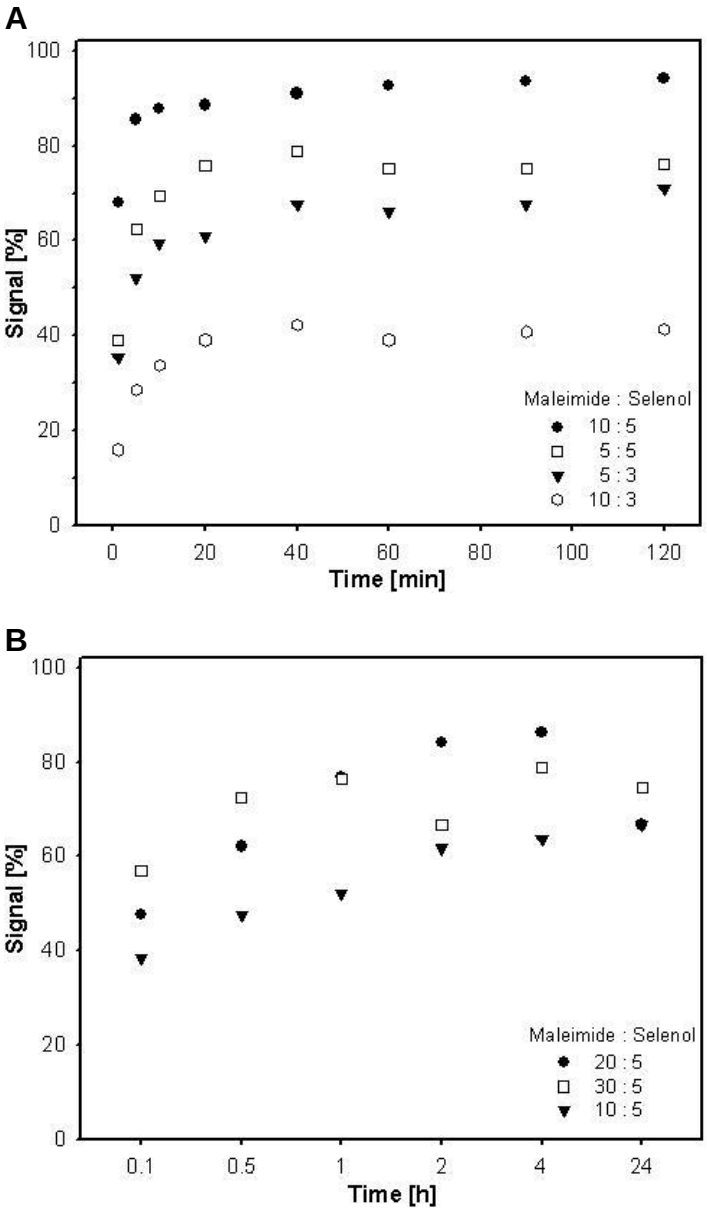


Fig. 2.26: Selenol-mediated *in situ* bridging of the disulfide bond of somatostatin. The signal % corresponds to the relative population of the maleimide bridged somatostatin **20**. (A) Dithiophenolmaleimide **30**. (B) Di-2-mercaptoethanolmaleimide **28**.

The results revealed that the ratio between bridging compound and selenol was more important than observed with TCEP. Also an increased amount of reagents was necessary both when compared to the phosphine-based protocol as well as when di-2-mercaptoethanolmaleimide was compared to dithiophenolmaleimide. Furthermore, the first compound was much slower in its reaction, reaching the maximum of product formation after 4 h while the same result was observed with dithiophenolmaleimide after 10–20 min. Although effective in its bridging reactivity, neither compound yielded full conversion of the peptide. The reason for this might be that the combination of thiol and selenol is strong enough to cleave the maleimide-bridged disulfide under these conditions or that

air-oxidation of the selenol to the unreactive diselenide compound occurs too quickly.[71]

Although not as efficient as the *in situ* modification of SST with TCEP, the selenol-mediated protocol can be considered an alternative where the reducing strength of the phosphine would be problematic. Furthermore the selenol-system differs from the TCEP-mediated *in situ* bridging in that it does not consume its reducing equivalents (conversion to the oxide in the case of TCEP) but continuously generates more thiol molecules. Such reactivity might be useful to modify proteins with more than one disulfide bond or those where additional cystines become accessible after the reduction of solvent exposed ones.[182, 405]

2.5.8 Limited efficiency of thiol/ thiomaleimides *in situ* protocols

After TCEP and benzeneselenol had been successfully used for the *in situ* modification of the disulfide bond of SST, it was envisaged that the pairing of a thiol reducing agent and its corresponding dithiomaleimide compound could also possibly exhibit such an activity. Therefore, oxidised peptide was mixed with 10 equiv of di-2-mercaptoethanolmaleimide **28** and different amounts of 2-mercaptoethanol were added after a short incubation. The reaction was monitored by LCMS at ambient temperature (Fig. 2.27).

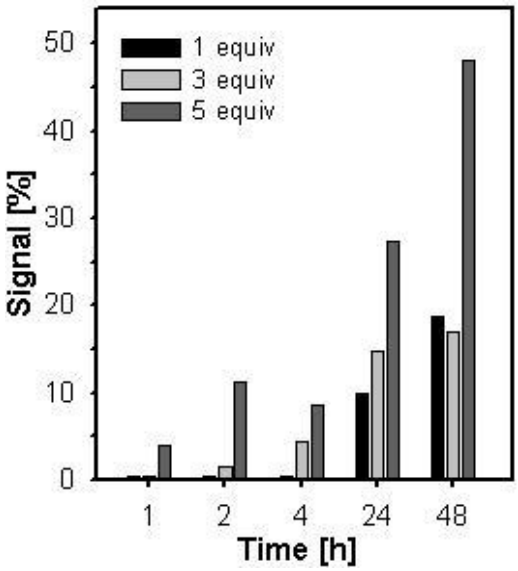


Fig. 2.27: *In situ* bridging of the somatostatin disulfide bond by a combination of 2-mercaptoethanol (amounts indicated in the legend) and 10 equiv of di-2-mercaptoethanolmaleimide. The signal % corresponds to the relative population of the maleimide bridged somatostatin **20**.

Overall, the reaction was very slow. Only increased amounts of free thiol led to the formation of an acceptable yield of the bridged somatostatin **20**, but not

comparable to the other established methods. Up to 25% of side product formation (mixed disulfides of the hormone with the reducing agent) was observed suggesting that disulfide bond cleavage occurred but did not result in product formation. Increasing the quantity of maleimide **28** up to 40 equiv had no positive influence on the yield (data not shown). Increasing the amount of 2-mercaptoethanol was not attempted, as the efficiency of such a protocol had been shown by the combination of the small thiol with dibromomaleimide (see 2.5.6).

2.5.9 Summary of *in situ* methods

In conclusion, it was possible to establish three highly effective *in situ* methods for the modification of the disulfide bridge of somatostatin, exploring the orthogonal activity of dithiomaleimides with different reducing agents. This type of reaction has not been reported in the literature before and yields great potential for the functionalisation of proteins.

By direct comparison, the combination of TCEP with dithiophenolmaleimide was the only method that resulted in 100% conversion of the starting material to the product (Fig. 2.28).

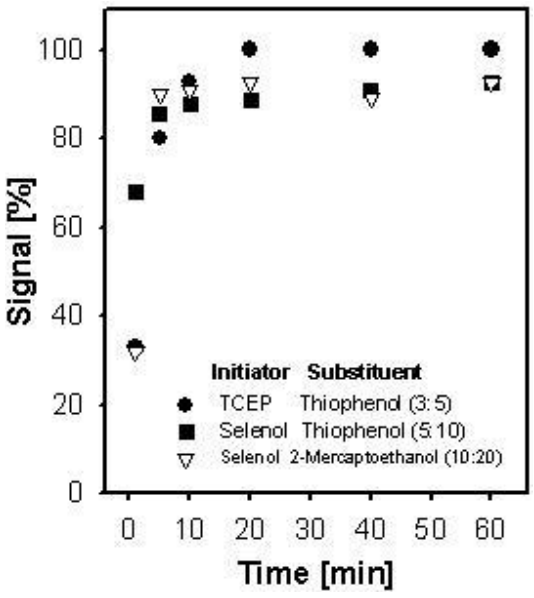


Fig. 2.28: Comparison of the developed methods for the *in situ* modification of the somatostatin disulfide bond. The signal % corresponds to the relative population of the maleimide bridged somatostatin **20**.

Both protocols utilising a selenol were dependent on larger amounts of reagents. The combination of dithiophenolmaleimide with the selenium compound instead of the phosphine yielded the fastest reaction. If thiophenol would inter-

fere with a certain application or assay, di-2-mercaptoethanolmaleimide could be used as an alternative.

The variety of compounds and protocols implies that a suitable method for functionalisation could be established for most proteins and the investigation of additional phosphines, selenols and thiol-substituents might yield an even greater range of methodology.

2.6 *In situ* functionalisation of the somatostatin disulfide bond

2.6.1 Synthesis

In order to use the developed protocols for the *in situ* modification of disulfide bonds it was necessary to synthesise the dithiophenol-versions of the functionalised dihalomaleimides. This was done for *N*-PEG5000-dibromomaleimide **25** and *N*-TPO-dibromomaleimide **27** (Fig. 2.29).

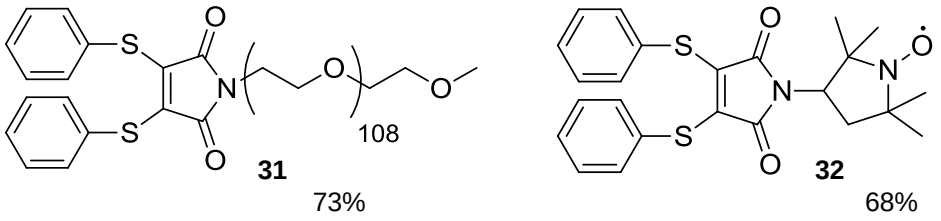


Fig. 2.29: Functionalised dithiophenolmaleimides. *N*-PEG5000-dithiophenolmaleimide (**31**) and *N*-TPO-dithiophenolmaleimide (**32**).

N-PEG5000-dithiophenolmaleimide **31** was not synthesised from the similar bromomaleimide **25** by addition of thiophenol as it was expected to be difficult to purify the product. Instead dithiophenolmaleimide **30** was used in place of dibromomaleimide **6** in the established Mitsunobu protocol (see 2.3.1). The two phenol groups increased the hydrophobicity of the molecule substantially. This improved the separation of the product **31** from the residual free mPEG chains, increasing the yield greatly (from 13% for **25** to 73%).

To prepare *N*-TPO-dithiophenolmaleimide **32**, the successfully established route to the dibromomaleimide spin label **27** was taken (Scheme 2.9) followed by very slowly addition of 2 equiv of thiophenol as a very dilute solution. Active

spin label (see below) was obtained by this method in an acceptable yield (68%).

2.6.2 *In situ* PEGylation of somatostatin

SST was PEGylated, but instead of using the two-step sequential process of reduction followed by re-bridging with the bromomaleimide **25**, the established *in situ* dithiophenolmaleimide-based protocols (see Scheme 2.12) were employed in conjunction with *N*-PEG5000-dithiophenolmaleimide (Fig. 2.30).

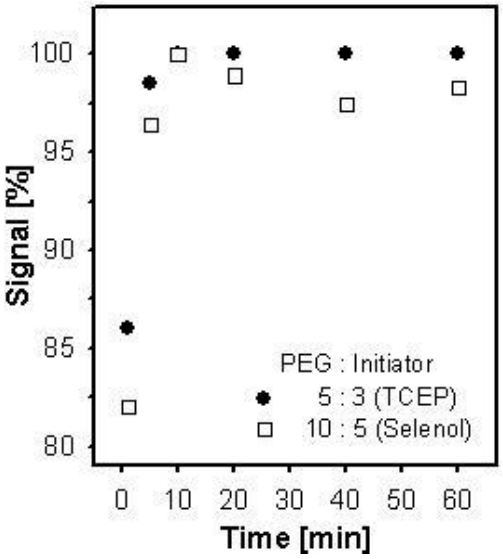


Fig. 2.30: *In situ* PEGylation of somatostatin. The signal % refers to the relative population of the PEGylated peptide.

When TCEP was used as the reducing agent quantitative conversion of the peptide to PEGylated hormone occurred within 10 minutes at ambient temperature. In the case of benzeneselenol, the expected 90% – 95% conjugation was detected almost as fast as seen in the phosphine-based protocol. As described before, the reaction could not be monitored by the detection of the mass species of the product but by disappearance of the unmodified peptide from the UV trace (Fig. 2.31). The identity of the generated products was confirmed by MALDI-TOF MS (Fig. 2.32).

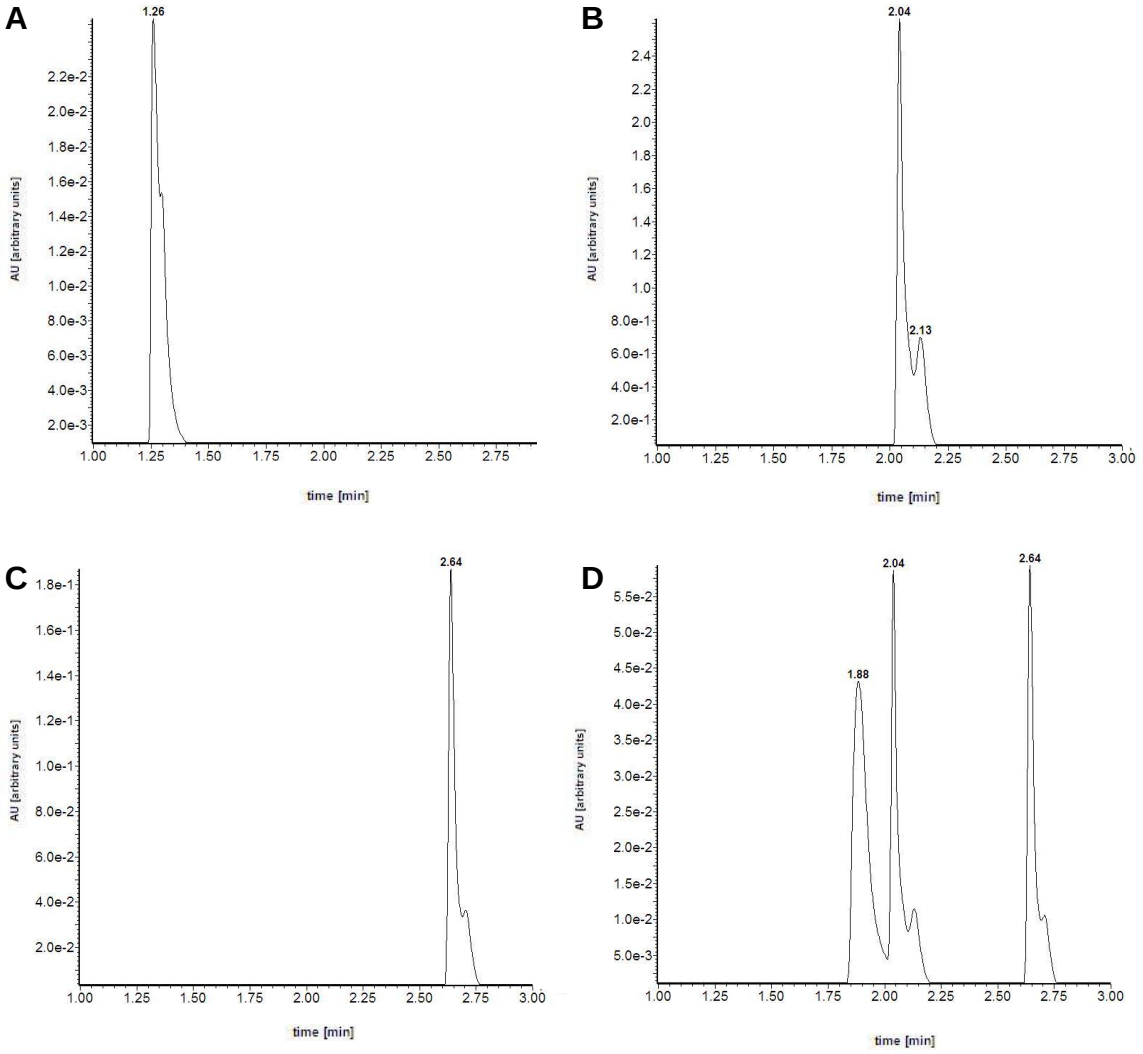


Fig. 2.31: LC elution profile of the *in situ* PEGylation reaction of somatostatin. Main peak shoulders are derived from salt interactions. (A) Somatostatin. (B) Thiophenol. (C) *N*-PEG5000-dithiophenolmaleimide **31**. (D) PEGylated somatostatin after *in situ* reaction with TCEP and **31**. The additional peaks correspond to generated free thiophenol (RT 2.04 min) and residual compound **31** (RT 2.64 min) respectively.

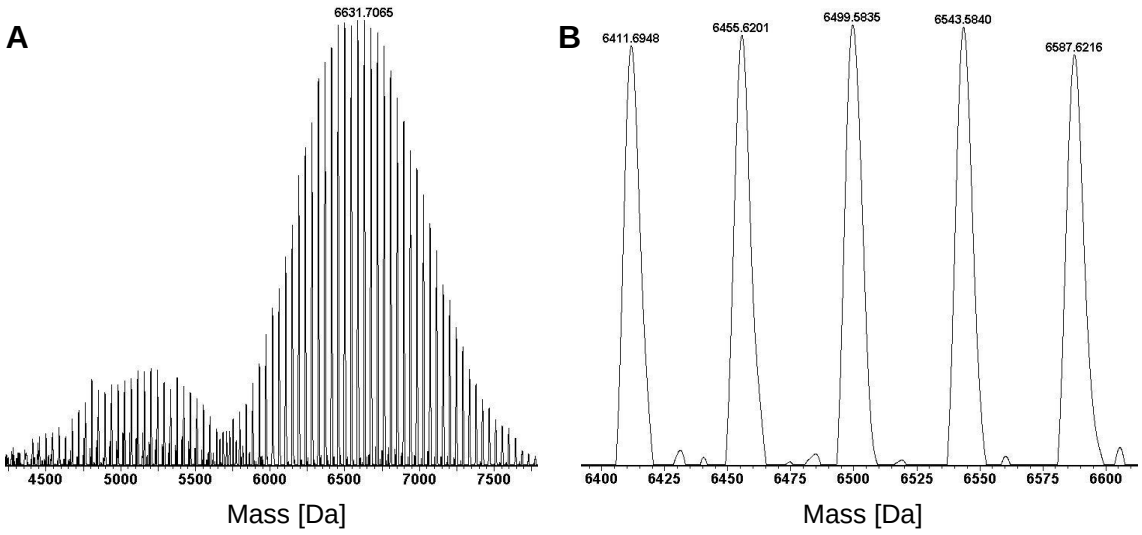


Fig. 2.32: MALDI-TOF spectra of *in situ* generated PEGylated somatostatin. (A) PEGylated peptide and residual PEG-maleimide. (B) Magnification of (A) shows the 44 Da mass each additional ethylene glycol unit adds to the overall weight of the molecule.

In summary, conjugation to a bulky molecule such as a 5,000 Da PEG chain with the *in situ* protocols did not diminish the efficiency of the reaction.

As all components of the PEGylation reaction were fully soluble in buffer, the experiment with TCEP as a reducing agent was repeated without DMF and MeCN (Fig. 2.33).

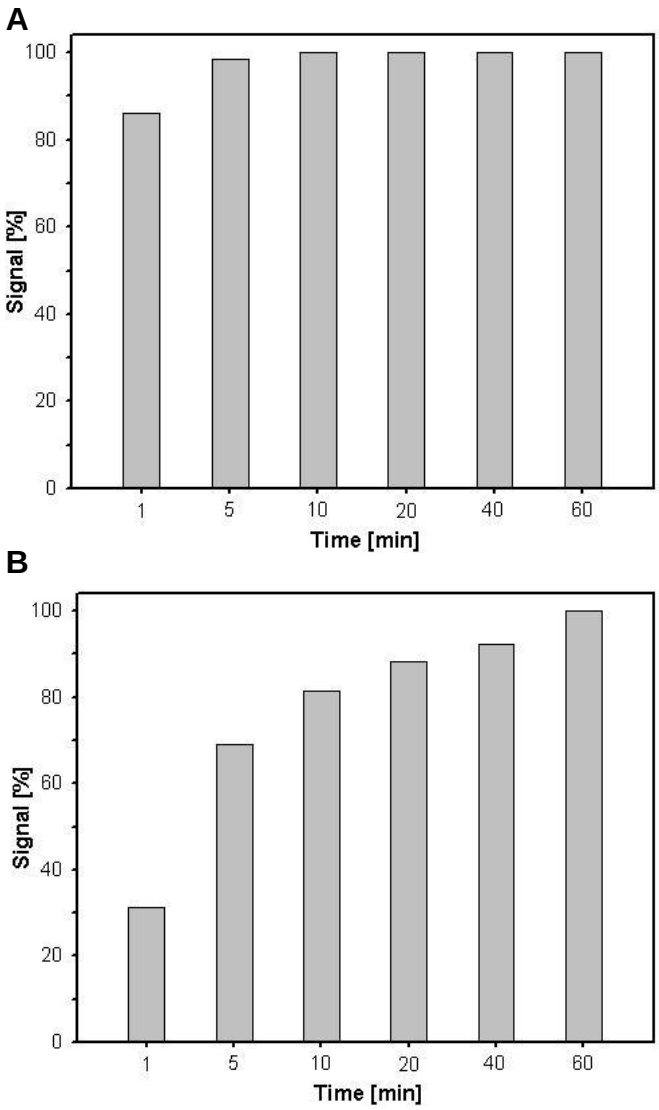


Fig. 2.33: *In situ* PEGylation of somatostatin with and without organic solvents. The signal % refers to the PEGylated peptide. (A) Reaction in the presence of 40% MeCN and 2.5% DMF. (B) Reaction in buffer only.

Surprisingly the absence of organic solvents had a negative impact on the speed with which the reaction took place. Although complete conversion was reached eventually, it took 60 min instead of 10 min. Still 80% conjugation of SST to the PEG chain was observed within the shorter time frame. A possible explanation might be that the presence of MeCN and DMF disrupts the water shell around the disulfide bond making it more accessible. A similar influence is imaginable on the whole structure of the peptide hormone. Overall the addition of a small amount of organic solvent might be beneficial for the reaction but the tolerance of any individual protein, especially in its reduced form, to such a treatment should be tested carefully before the modification is carried out.

2.6.3 *In situ* spin labelling of somatostatin

Before the *N*-TPO-dithiophenolmaleimide spin label **32** could be used it was necessary to confirm that the nitroxide radical was still present. As a first proof a NMR spectra was recorded to test if it was still broadened by the unpaired electron.[146] When this was observed an EPR spectrum was acquired which showed a signal basically identical to the dibromomaleimide spin label (see Fig. 2.15 A).
As all *in situ* protocols require the presence of an excess of reducing agents and generate additional free thiols the resistance of compound **32** against these treatments was investigated by addition of 5 equiv of TCEP, thiophenol or benzeneselenol. A time-course experiment was set up and EPR spectra were recorded constantly over 2 h at ambient temperature (data not shown). Except for a small decrease in the third signal peak when mixed with TCEP, which occurred within the first 5 min, no loss of signal was observed. This suggests that the spin label **32** is relatively robust against the reducing equivalents necessary for the *in situ* modification of proteins.
Next somatostatin was spin labelled *in situ* using both dithiophenolmaleimide-based protocols (Fig. 2.34).

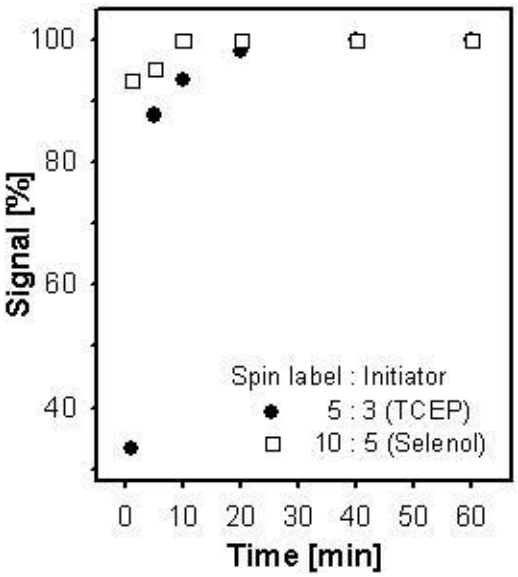


Fig. 2.34: *In situ* spin labelling of somatostatin. The indicated signal refers to the TPO-peptide conjugate.

As observed for the *in situ* PEGylation, the conjugation reactions proceeded rapidly and full conversion of the peptide to its spin labelled version was achieved. This was true also in the case of benzeneselenol mediated disulfide modification which had not been observed before. *N*-TPO-dibromomaleimide had exhibited an increased activity in the sequential disulfide bridging reactions

(see Fig. 2.9 A) which suggests that this effect could be an intrinsic property of the *N*-TPO-maleimide compound. Rabenstein and Weaver[251] have observed that the disulfide bond of somatostatin is quickly reformed after reduction. They rationalised that this might be the consequence of the peptide not forming a random coil upon loss of the cyclising cystine but instead adopting an ordered conformation which supports the re-oxidation by keeping the free cysteines close together. Similar findings have been reported by Martin-Gago[406] and co-workers, who found that somatostatin retains a native-like structure upon reduction, stabilised by π-π interactions of the peptides' phenylalanine residues. Thus a possible theory to explain the good reactivity of the *N*-TPO-maleimide spin labels might be that the rigidity of the molecule keeps it in a shape which fits the non-random conformation of reduced SST very well and hence making a fast reaction possible. Additional data from crystallography and kinetic experiments would be necessary to obtain further confirmation of this theory.

Due to the small size of SST, the complete removal of the excess of spin label could not be achieved. Thus all recorded EPR spectra of *in situ* labelled peptide are dominated by the signal of free label (Fig. 2.35). Still some line broadening and decrease in the third line signal was observed. This should not be a problem if larger proteins were modified.

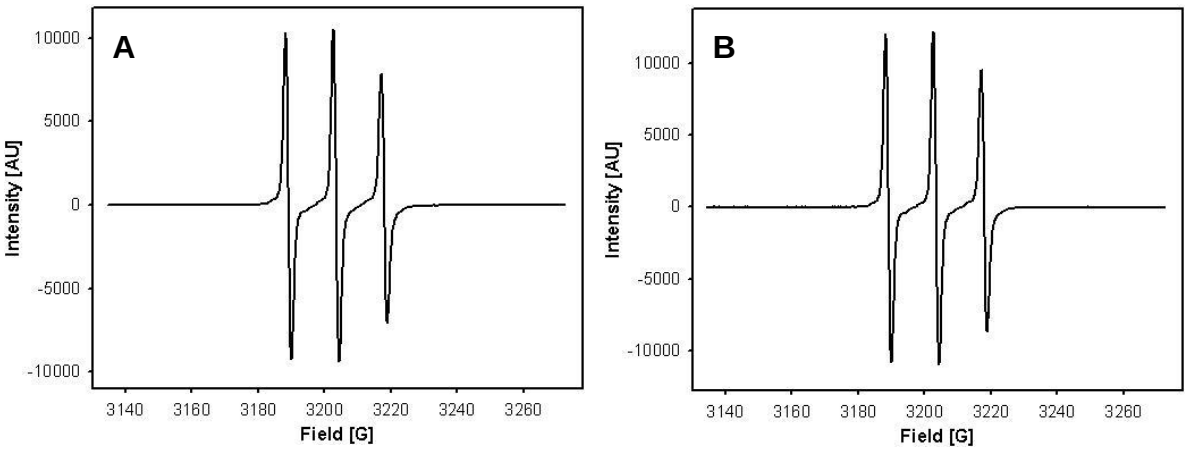


Fig. 2.35: EPR spectra of *in situ* spin labelled somatostatin. Both are dominated by free spin label. (A) Labelling via the TCEP protocol. (B) Labelling via the benzeneselenol protocol.

In summary, the established *in situ* protocols were used to functionalise the model peptide somatostatin with high efficiency and good reactivity. High toler-

ance of the maleimide based methodology with regard to modification of the protocol was observed. The simplicity and efficient one-pot character of the reactions yield good potential for use in the daily routine of various labs.

2.7 Modification of insulin

2.7.1 Synthesis

As it became apparent while experimenting with insulin that the dibenzeneselenol derivatives of maleimide would be more efficient in the modification reaction these compounds (**33**, **34** and **35**) were synthesised.

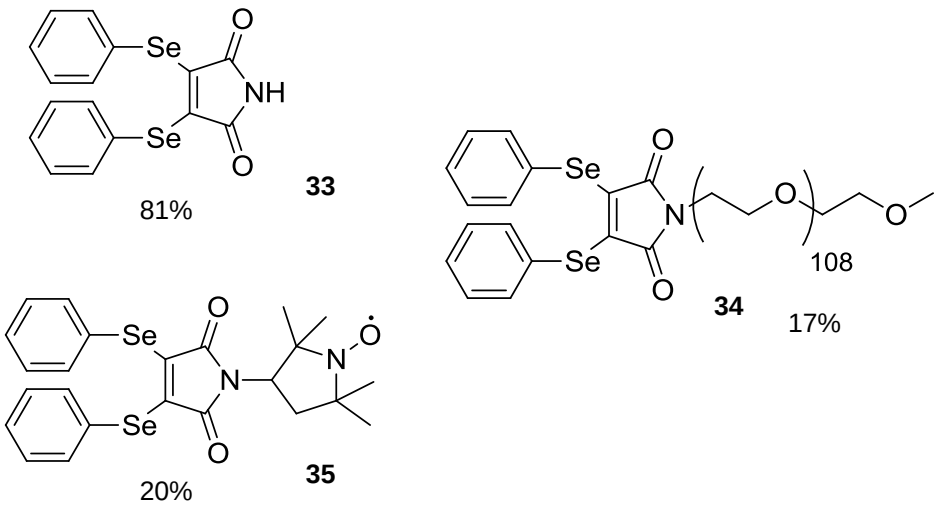


Fig. 2.36: Dibenzeneselenolmaleimide derivatives. Dibenzeneselenolmaleimide (**33**), *N*-PEG5000-dibenzeneselenolmaleimide (**34**) and *N*-TPO-dibenzeneselenolmaleimide (**35**).

Dibenzeneselenolmaleimide **33** and *N*-TPO-dibenzeneselenolmaleimide **35** were prepared in a similar way to their dithiophenolmaleimide counterparts by the slow addition of diluted benzeneselenol to the dibromomaleimide version of the molecules. *N*-PEG5000-dibenzeneselenolmaleimide **34** was synthesised from dibenzeneselenolmaleimide via the described Mitsunobu reaction (see 2.3.1).

2.7.2 Accessibility of the insulin disulfide bonds

Insulin is an interesting target for modification via its disulfide bonds both due to its challenging network of cystines, which are partially buried and partially solvent exposed (see 1.6.2), as well as its biological importance.

Firstly the accessibility of the disulfide bonds was tested. Solubilised insulin was treated with 1 to 50 equiv of TCEP for 1 h. The analysis of all insulin samples was done by MALDI-TOF MS as the maleimide derivatives of the hormone were not visible by LCMS. With increasing amounts of reducing agent, increasing signals of the A and B chains were detected and nearly complete separation of the two peptide chains was obtained with 20 equiv of TCEP (Fig 2.37). To test for the oxidation status of the disulfide bonds in the released chains, an excess of monobromomaleimide was also added to the reduced samples.

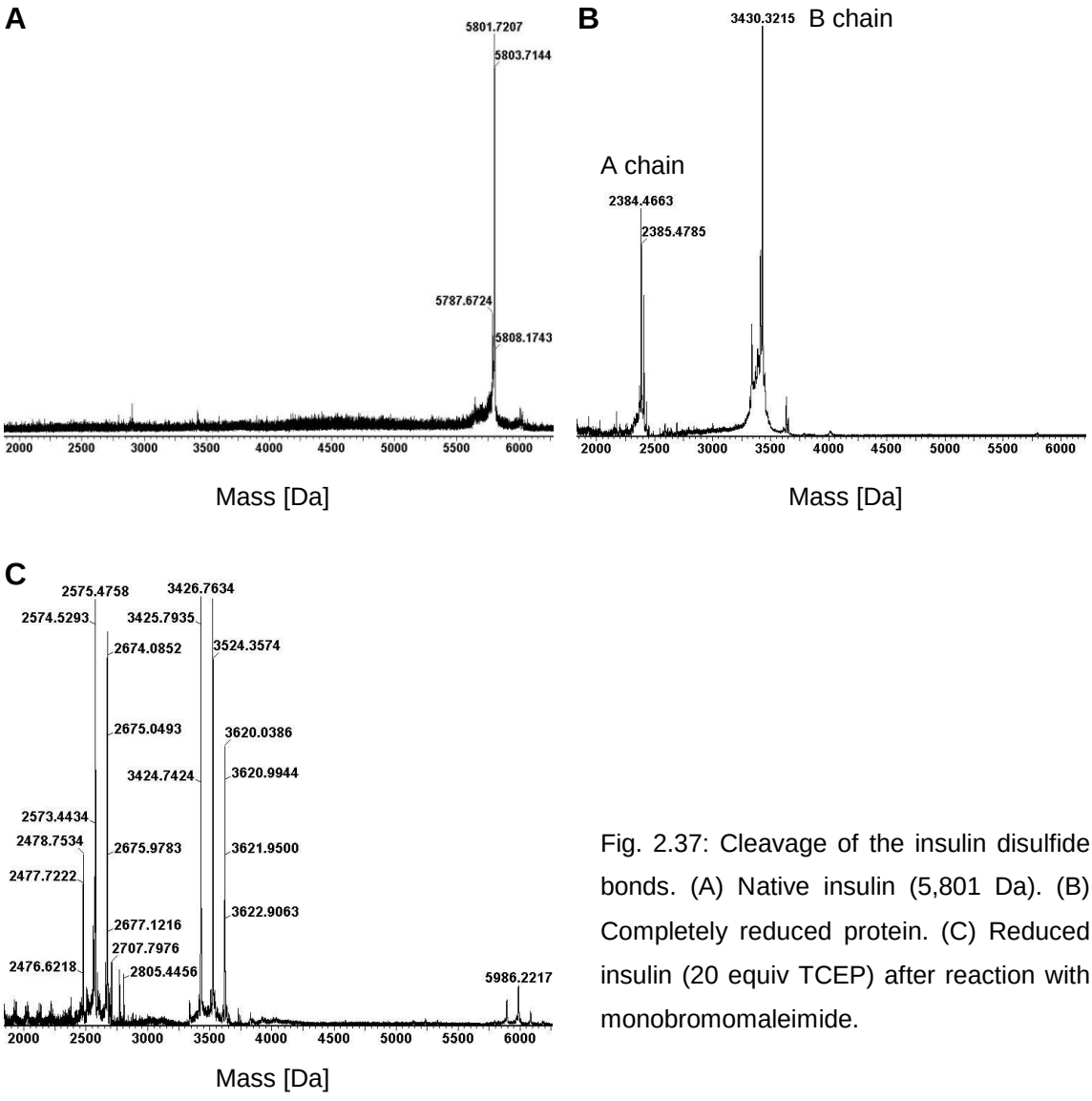


Fig. 2.37: Cleavage of the insulin disulfide bonds. (A) Native insulin (5,801 Da). (B) Completely reduced protein. (C) Reduced insulin (20 equiv TCEP) after reaction with monobromomaleimide.

Interestingly the B chain peptide (peak at 3,427 Da) could be detected without any maleimide attached despite the excess of the compound over the 6 potential available cysteines. This was not the case for the A chain, however, which was found to have reacted with either 1, 2 or 3 maleimide units. The tendency

of the B chain to aggregate upon cleavage of the disulfide bonds has been described[305] and might offer an explanation as this could shield the free thiols, making them inaccessible.

2.7.3 Sequential modification of the insulin disulfide bonds

The high amounts of TCEP that were necessary to fully reduce insulin implied that it should be possible to selectively reduce only one of the disulfide bonds to modify the protein. A sequential strategy was therefore adopted; insulin was treated with 1, 2 or 3 equiv of the phosphine for 1 h at 4 °C followed by addition of 5 equiv of dibromomaleimide. Such a sequence should yield the mono- or bis-modified peptide, depending on how much access to the partially buried cystine A20 – B19 the cleavage of the fully solvent exposed disulfide bond A7 – B7 would grant to the reducing and bridging reagents (Fig 2.38). Since the remaining intramolecular disulfide bridge is normally not solvent accessible, it is very unlikely that a triple-modified insulin molecule would be formed.

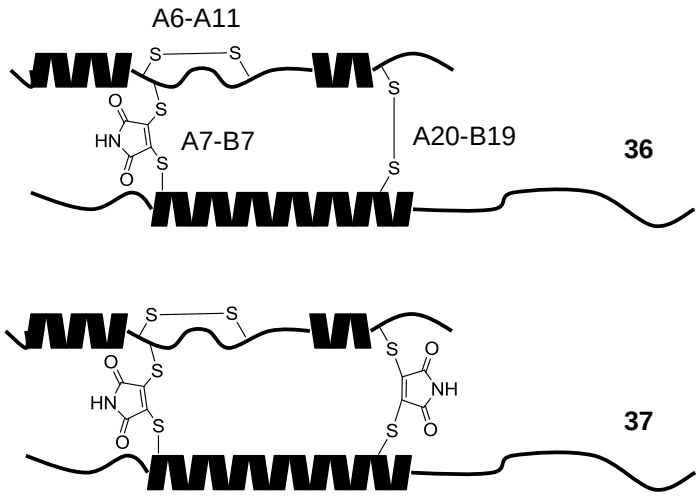


Fig. 2.38: Proposed products of the modification of insulin via its accessible disulfide bonds. Mono-addition product (**36**) and bis-addition product (**37**). The positions of the native disulfide bonds have been highlighted in product **36**.

Surprisingly none of the assumed products were found (data not shown). Similar to the reduction studies, only modified A and B chains (with and without bromine atoms attached) were observed. It has been reported, that liberation of the cysteines of the B chain quickly leads to disulfide scrambling and auto-reduction of insulin.[303] Thus it is likely that in this experiment the reduction of only a single disulfide bond with the small amounts of TCEP generated very reactive thiols which acted also as reducing agents and were responsible for the

complete loss of native protein. This led to the conclusion that partially reduced insulin is not stable.

2.7.4 *In situ* modification of the insulin disulfide bonds

As the sequential modification of the insulin disulfide bonds was not possible the established *in situ* bridging strategy utilising TCEP was employed. When a combination of 5 equiv of dithiophenolmaleimide **30** and 3 equiv TCEP was used the desired mono-product **36** and, to a smaller extent, the bis-product **37** were detected by MS but in very small amounts and the remaining protein had separated into its two peptide chains (data not shown). This showed that an *in situ* protocol had the potential to modify insulin but needed optimisation.

To this end the quantities of both TCEP (1–20 equiv) and dithiophenolmaleimide (1–10 equiv) were varied as well as time (5 min – 24 h) and temperature (4° C – 50 °C) (see 4.6.3). Urea (1–7 M) was included in some experiment to increase the accessibility of the cystines as well as a small amount of SDS (sodium dodecyl sulphate) to increase the solubility of the B chain.[305] Lastly, an experiment was tried whereby TCEP was added in small portions over prolonged periods of time.

Although the yields increased slightly (data not shown) a general trend became obvious that with higher quantitites of dithiophenolmaleimide and TCEP more products were obtained but this was also accompanied by an increased loss of native insulin. Overall, TCEP induced cleavage of the insulin disulfide bonds, accelerated by the aggregation and auto-reductive behaviour of the B chain, seemed to occur too fast for an efficient bridging reaction. This was supported by the detection of multiple monothiophenolmaleimide adducts of the B chain in the presence of higher amounts of dithiophenolmaleimide (data not shown).

To decrease the reducing strength in the disulfide bond modification system the *in situ* protocol with benzeneselenol and dithiophenolmaleimide was tested. The first series of experiments (1–5 equiv selenol, 3–10 equiv maleimide **30**, see 4.6.4) revealed that by using these compounds the formation of free A and B peptide chains was greatly reduced and product formation was comparable to the best results with the TCEP system (data not shown). Small amounts of rea-

gents yielded predominantly mono-product **36,** while higher quantities lead to more bis-product **37**.

Prolonged reaction times were explored to optimise the yield but the increased presence of free A and B chains was detected instead. Thus, a series of experiments were conducted with the best yielding protocols for the mono-addition product (1 equiv selenol/ 3 equiv dithiophenolmaleimide) and the bis-addition product (5 equiv selenol/ 5 equiv dithiophenolmaleimide) thus far. Also, only short reaction times were allowed before subsequent dialysis which was used to remove the benzeneselenol in order to stop further reduction of the disulfide bonds. The results were estimated by MALDI-TOF MS and should be interpreted carefully as this method yields only limited quantitative information (Fig. 2.39).

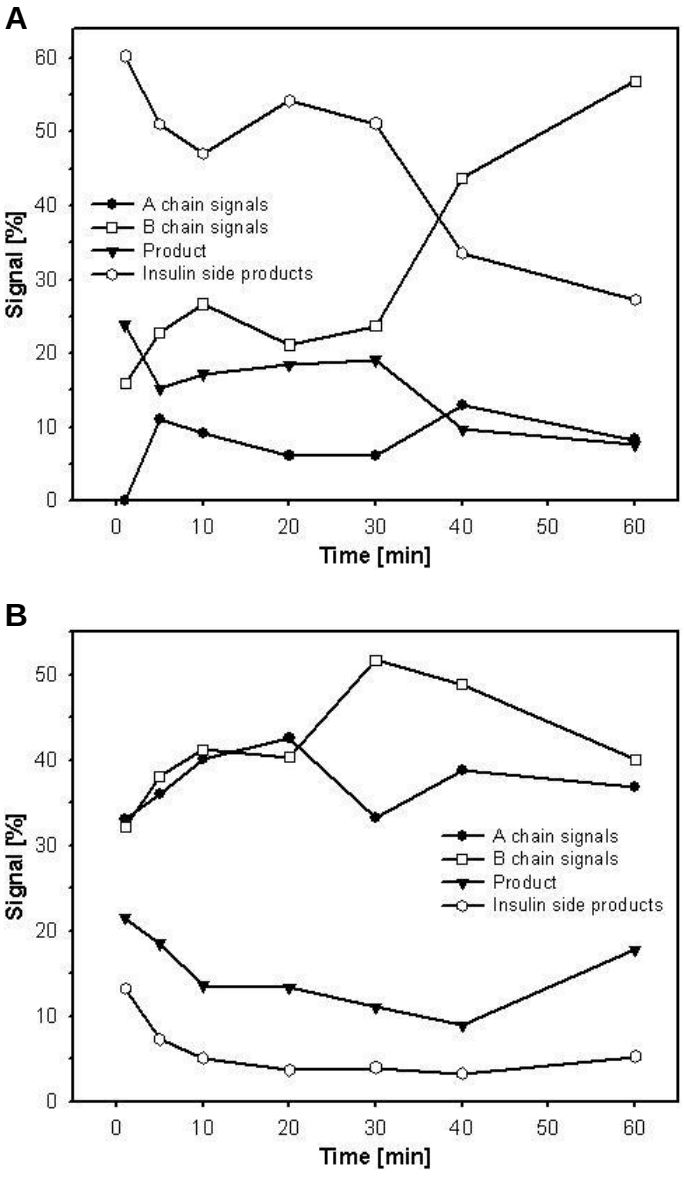


Fig. 2.39: Rapid modification of insulin disulfide bonds with benzeneselenol and dithiophenolmaleimide **30** as observed by MALDI-TOF MS. Insulin side products contain mainly native insulin but also the not indicated product. (A) 1 equiv selenol/ 5 equiv **30**. Product = mono-product **36**. (B) 5 equiv selenol/ 5 equiv **30**. Product = bis-product **37**.

In both reactions the amount of product formation was maximal after 1 min. In the conditions optimised to obtain the mono-product **36** the native protein was very prominent at the beginning and started to disappear over time. In contrast the signal for unmodified insulin was very weak already after 1 min in conditions that favour the formation of the bis-product **37**. Interestingly, a loss of the desired products was observed over time in both cases.

Focusing on short reaction times, protocols were developed that yielded 27% of the mono-product **36** (0.5 equiv selenol/ 2 equiv maleimide **30**) and 46% of the bis-product **37** (4 equiv selenol/ 18 equiv maleimide **30**) (Fig. 2.40).

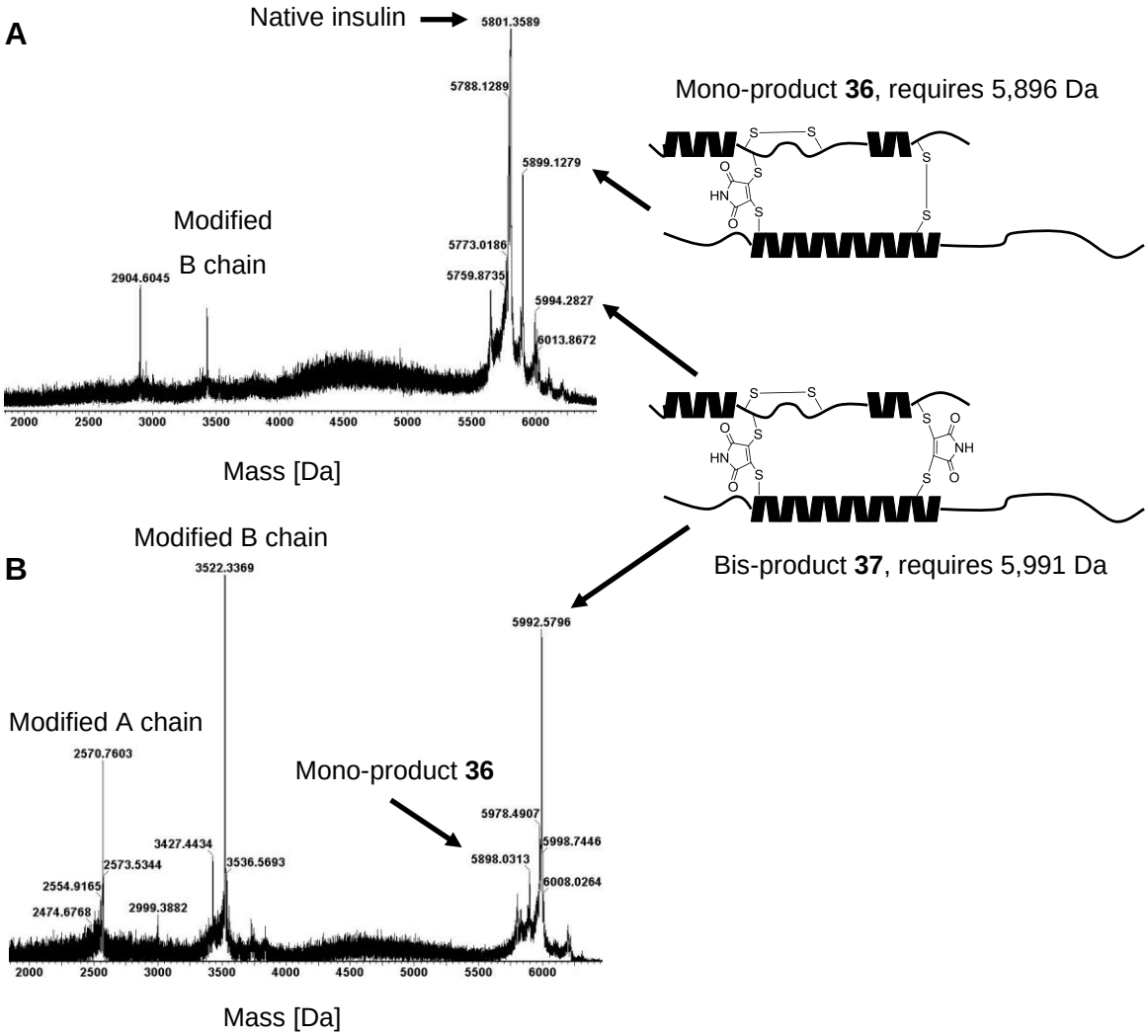


Fig. 2.40: *In situ* modification of the insulin double bonds under optimised conditions (after 1 min). (A) 0.5 equiv selenol/ 2 equiv dithiophenolmaleimide. (B) 4 equiv selenol/ 18 equiv dithiophenolmaleimide.

The yields were calculated very simplified using only the relative signal intensities of the MS spectra (see 4.2) and thus served only as an approximate quantification for the protocol development. Although not ideal, even at these quantities the reaction protocol might be useful for the large scale modification of insulin as the peptide can be produced relatively inexpensively in large quantities but further optimisation was clearly desirable.

2.7.5 Mixtures of halo- and thiomaleimides allow control of the selenol-meditated *in situ* modification of insulin

Repetitions of the optimised reactions showed that the reproducibility was not satisfying. The poor control of the reaction-stop by dialysis was potentially one of the main reasons. To halt the reaction in a more regulated and rapid manner it would be necessary to neutralise free benzeneselenol more efficiently to stop the reduction. As maleimide reacts with the selenol (see 2.5.7) reaction-stop was initiated by addition of 50 equiv of maleimide **1** at different time points to the optimised reaction, but resulted mainly in formation of maleimide-adducts of free A and B chains (data not shown).

To avoid random non-productive modification of the target protein (in terms of cystine modification) and to obtain bridged insulin as a “by-product”, dibromomaleimide **6** was used instead of maleimide under the same conditions and the reaction examined by MALDI-TOF MS (Fig. 2.41 and 2.42).

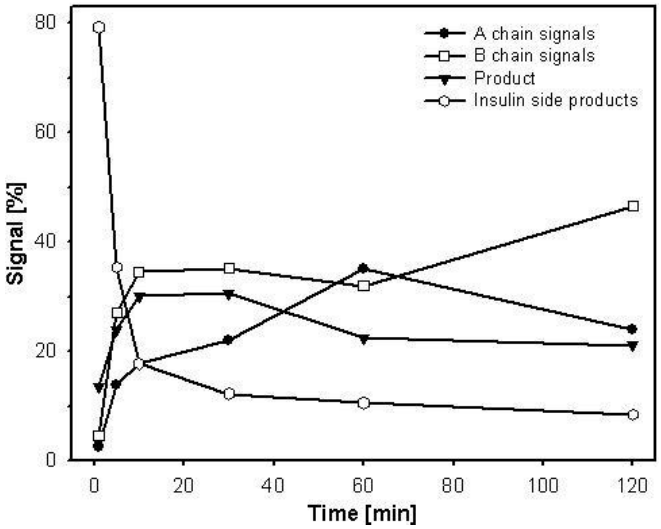


Fig. 2.41: Dibromomaleimide stopped *in situ* modification of insulin. Conditions: 4 equiv selenol/ 18 equiv dithiophenolmaleimide, then 20 equiv dibromomaleimide. Product = bis-product **37**. Insulin side products contain mainly native insulin but also mono-product **36**.

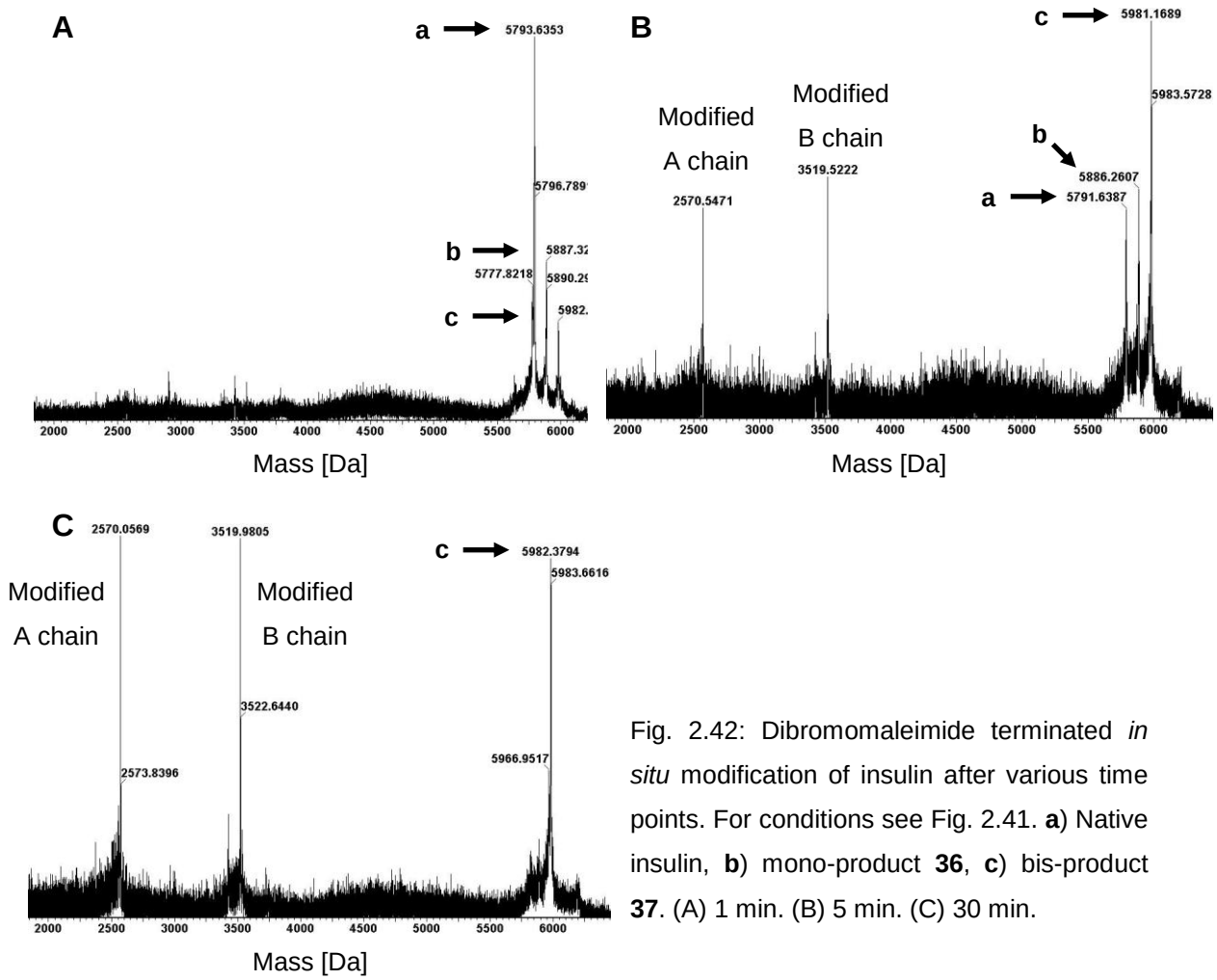


Fig. 2.42: Dibromomaleimide terminated *in situ* modification of insulin after various time points. For conditions see Fig. 2.41. **a**) Native insulin, **b**) mono-product **36**, **c**) bis-product **37**. (A) 1 min. (B) 5 min. (C) 30 min.

Addition of dibromomaleimide **6** after 1 min clearly stopped the reaction as the product mix of this time point contained less modified insulin than observed in dialysed samples. When the ongoing conjugation was halted after 5 min the spectrum very closely resembles that obtained after dialysis, indicating that the buffer exchange is also able to stop the reaction but that it acts more slowly. Three interesting observations were made if dibromomaleimide was added at 30 min and later: First mainly the bis-modified hormone **37** was generated; this implies that the insertion of a first maleimide bridge is a critical point in the modification process, as the two peptide chains either fall apart after loss of a single cystine or are re-connected by the maleimide. In this case also the second disulfide bond is modified if given enough time and reactants. This also suggests that re-bridging a single disulfide bond allows for increased access of the reagents to a second disulfide bond. Finally, this underlines that dithiophenolmaleimide is fast enough to bridge the two cysteines before auto-reduction occurs and that the selenol system is milder compared to TCEP.

The second observation was the fact that both free peptide chains were generated in their highest possible status of modification (two maleimide units per A chain and one per B chain) and only small amounts of unconjugated B chain were found. This again indicates that the cysteines, once released from the disulfide bridges, quickly attack one of the maleimide compounds and thus the reconnection to the other peptide chain is the critical step that has to happen before a second intermolecular cystine is cleaved. The double modification of the A chain further supports the general assumption that this peptide unfolds upon reduction. Finally, the presence of mainly fully bridged free chains implies that the reaction products **36** and **37** are not formed from randomly re-connected A and B chain after they have been separated as these would have cyclised too rapidly after release from the native protein via a reactive maleimide species.

The third observation was the change of the observed rate of product formation over time. Two competing reactions seem to take place; one that results in the synthesis of modified insulin and one that yields modified free peptide chains. Although MALDI-TOF MS is not quantitative, Fig. 2.42 clearly shows that in the beginning the desired products are predominantly formed (1 and 5 min) while later on the modified A and B chains are the main product. This potentially indicates that bridged insulin is the kinetically favoured product, and is therefore formed quickly in the beginning, but that the free peptide chains are favoured by thermodynamics and thus their amount increases over time, perhaps driven by aggregation of the B chain. It is also possible that the released thiophenol attacks the maleimide bridges of the products and thus the overall signal of the bis-product **37** decreases over prolonged periods of time.

2.7.6 Stepwise modification of insulin via repeated start-stop reactions

The observations made so far suggested that the re-bridging of reduced disulfide bonds in insulin is only efficient in the early phase of the reaction. It was rationalised that by stopping the ongoing modification at the right moment and then re-starting it by addition of more reducing agent could possibly overcome this limitation. To understand the role of the released maleimide substituent thiophenol was tested to examine whether it is able to reduce the disulfide bonds of insulin. Various amounts of the thiol were added to the hormone and the ef-

fect analysed by MALDI-TOF MS. For comparison the same experiment was carried out with benzeneselenol instead of thiophenol (Table 2.14).

Table 2.14: Reduction of insulin with benzeneselenol and thiophenol after 1 h.

Amount of benzeneselenol	Intact insulin left [%]	Amount of thiophenol	Intact insulin left [%]
2 equiv	31	2 equiv	12
5 equiv	22	5 equiv	5
10 equiv	14	10 equiv	3
20 equiv	7	20 equiv	1

Both compounds acted surprisingly well as reducing agents. Thiophenol was almost as active as TCEP (see 2.7.2) and, in contrast to the experiments with somatostatin (see 2.5.7), benzeneselenol was able to reduce the disulfide bonds of insulin in the absence of additional thiols. This suggests that the cystines in insulin are less stable than in SST and/ or more reactive facilitating auto-cleavage. Interestingly even higher amounts of benzeneselenol did not yield completely reduced insulin indicating the formation of the diselenide over time thereby “inactivating” the reducing agent.[407] This assumption was supported by an increased clouding of the samples which contained benzeneselenol over time, possibly the result of formation of the insoluble diselenide, as well as the loss of the characteristic smell of the compound. Overall these findings show that the choice of the substituent of the maleimide reagent is important for the development of efficient modification protocols for insulin.

For further optimisation, the amount of dibromomaleimide **6** necessary to stop the reduction and the ideal moment at which to do so were determined. This was accomplished by reacting insulin with 18 equiv of dithiophenolmaleimide **30** and 4 equiv of benzeneselenol followed by addition of either various amounts of dibromomaleimide after 30 s or 20 equiv of this compound after various times. The experiment was analysed by MALDI-TOF MS (Fig. 2.43).

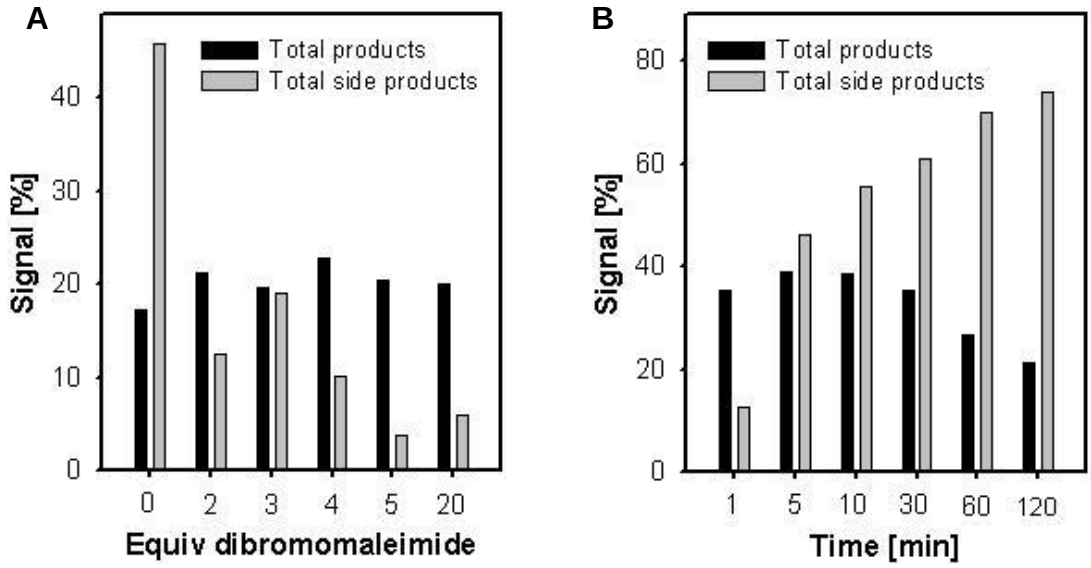


Fig. 2.43: Determination of ideal parameters for the start-stop modification of insulin. Products include mono-product **36** and bis-product **37**, the remaining signal corresponds to native insulin. (A) Amount of dibromomaleimide necessary for reaction-stop. (B) Timing of the reaction.

2 Equiv of dibromomaleimide **6** were enough to stop the reaction; more of this compound did not change the observed pattern of products and side products significantly. Thus each molecule of maleimide **6** neutralises two molecules of selenol or thiol as expected from the synthesis of the dithiophenolmaleimides. With regard to the timing, efficient synthesis of products took place only in the first minutes of the reaction, an observation which compared well to the results obtained during optimisation of the *in situ* protocol.

Utilising this knowledge, start-stop reactions were performed. A typical reaction involved the addition of dithiophenolmaleimide **30** to insulin followed by benzeneselenol. Dibromomaleimide **6** was added to stop the reaction, the mixture was incubated at ambient temperature and the reaction re-started by addition of more benzeneselenol. Aliquots were removed after certain cycles, dialysed and analysed by MALDI-TOF MS. Parameters varied included the quantity of compounds used, the time of incubation and time before reaction-stop as well as the addition of more dithiophenolmaleimide after a certain number of cycles (see 4.6.12). A summary of the best result obtained is shown in Fig. 2.44.

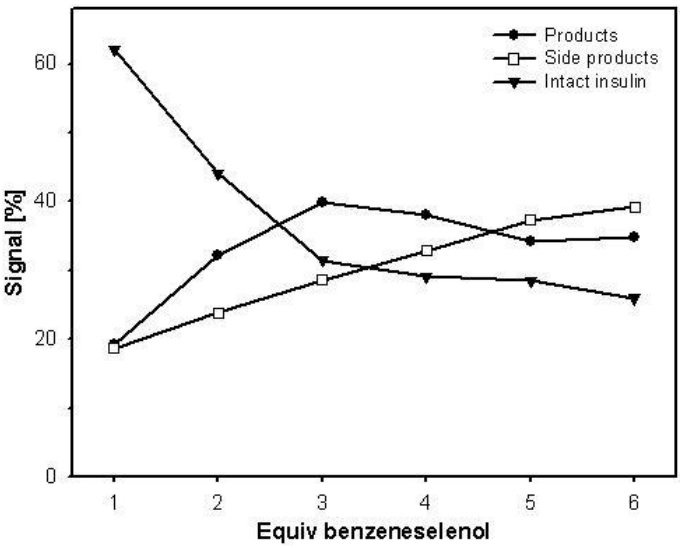
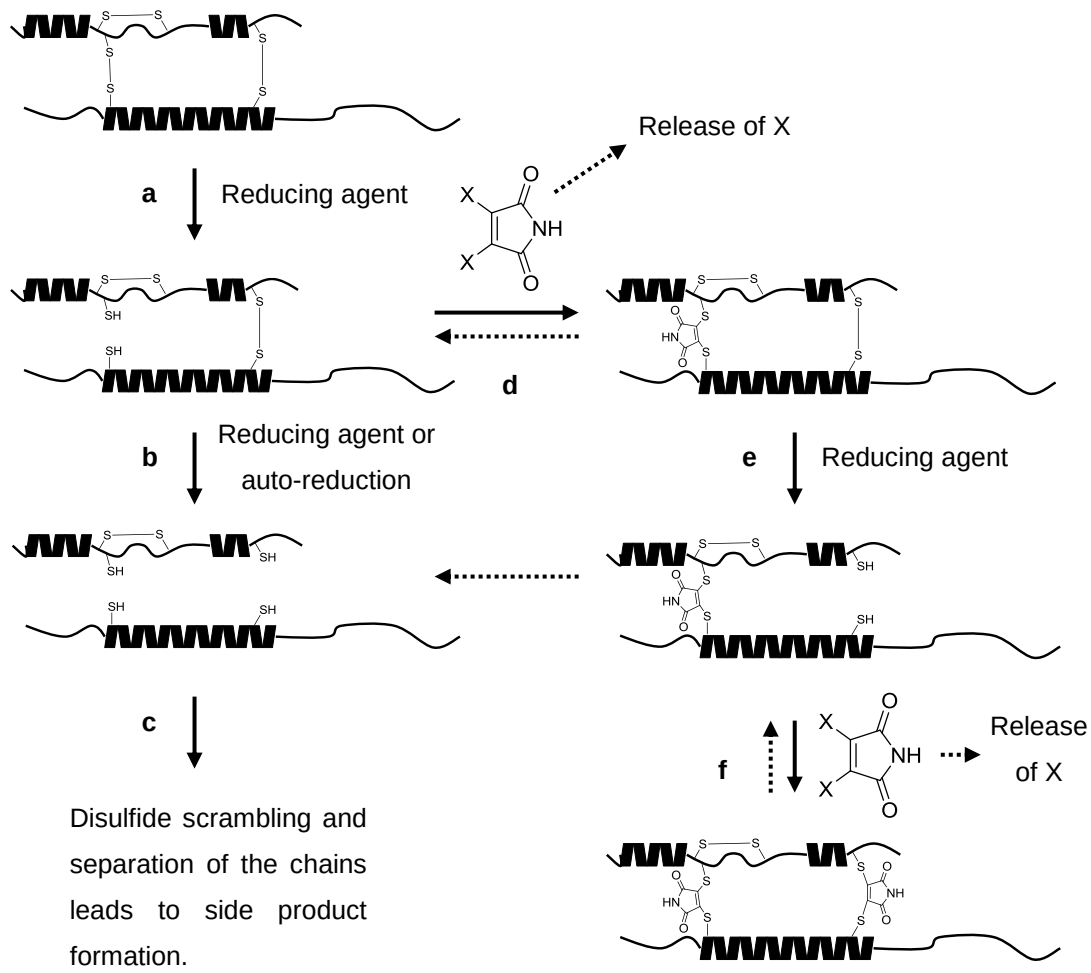


Fig. 2.44: Start-stop modification of insulin. Products include mono-product **36** and bis-product **37**. Conditions: 10 equiv of dithiophenolmaleimide **30**, 0.5 equiv benzeneselenol, reaction-stop with 0.25 equiv dibromomaleimide, incubation for 5 min each, 12 cycles, addition of 2 equiv of compound **30** after cycle 4 and 8.

The highest yields were obtained when the reducing agent was added in small amounts and the reaction left for 5 min before stopping it. Although it was possible to increase the quantity of mono- and bis-products **36** and **37** formed, the overall yield could not be raised substantially (see Fig. 2.41). Also the effect that should have been eliminated by this elaborate protocol was observed again; in the later cycles the formation of more side products than desired products took place despite the fact that native insulin was still left. Clearly another parameter than the reaction timing must be more important for the modification of the peptide hormones' cystines.

2.7.7 Discussion of *in situ* reactions of substituted maleimides with insulin

Insulin offers without doubt a more complex system than somatostatin. The presence of 3 disulfide bonds which vary in their accessibility, the loss of the native structure upon reduction of the two interchain cystines and the obvious high reactivity of one or more cysteines in the hormone make its (high yielding) modification challenging. Scheme 2.13 is an attempt to compile and compare possible reaction pathways and was used to rationalise potential strategies for the development of a more successful modification protocol. For simplification some potential side reactions and products have not been included.



Scheme 2.13: Theoretical pathways of the *in situ* modification of insulin with substituted maleimides. See text for discussion.

The reaction is started by the addition of the reducing agent and cleavage of one of the insulin disulfide bonds (a). The results obtained so far have shown that the next step is an important point of differentiation. Either reduction of a second cystine occurs (b), by the action of more reducing agent or auto-reduction, and the result is chain separation and side product formation (c). Alternatively the free cysteines react with the bridging reagent (d) to yield the mono-addition product. The next steps from this compound, reduction of a second cystine (e) and its bridging (f), seemed to be very robust as the formation of mainly the bis-product has been observed throughout the *in situ* reactions.

Besides this obvious sequence of reactions two more processes have to be taken into consideration: First the release of the maleimide substituent during the bridging reactions, which is a nucleophile and thus could act as a reducing agent itself, cleaving the still intact disulfide bonds of insulin. Second, the reduc-

ing agent, but also the released leaving groups and the free cysteines in the separated A and B chains, can possibly attack the maleimide bridges in the product and thus reverse the reactions (dotted arrows) in the product forming pathway. In contrast the formation of side products is irreversible and this might explain the observed loss of mono- and bis-product over time.

Overall only pathway (a) – (d) – (e) – (f) in Scheme 2.13 leads to the desired insulin product(s). In order to improve the yields, the reagents and conditions of the reaction have to be adjusted to favour these steps and ideally disfavour the others. Following rationales can be drawn from this idea:

1) The reactivity and amounts of all nucleophiles present (including the released insulin cysteines and maleimide substituents) should be lowered to slow down undesired processes such as auto-reduction and attack on the already formed maleimide bridges.
2) The leaving groups on the maleimide should be chosen to react only slowly (or ideally not at all) with insulin to allow for control of the reaction.
3) The reactivity of the bridging reagent should be as high as possible to favour steps d) and f). Also enough of the maleimide should to be present at any time to immediately react with and thus block the free cysteines of the A and B chains.

Not all of these rationales can be fulfilled at the same time as some of them are somewhat contradictive (such as 2) and 3) for example). A set of reactions was performed to test these ideas separately or in combination.

2.7.8 Control of the modification of insulin by pH

The idea from the above list that was tested first was a general attempt to reduce the reactivity of all present nucleophiles as this would have an impact on all the other rationales as well. Since the targeted groups are mainly thiols, the pH was chosen as a variable parameter to control the presence of the reactive species – the thiolate anion. To this end insulin was prepared in pH 5.0, 6.5, 7.4 and 8.0 phosphate buffers (containing the usual amounts of organic solvents) and reacted for different times under the so far established best conditions for the formation of the bis-product **37** (4 equiv benzeneselenol/ 18 equiv dithio-

phenolmaleimide) (Fig. 2.45). The results for pH 5.0 are not shown as the hormone did not dissolve under these conditions (the apparent pI of insulin is 6.4).[291]

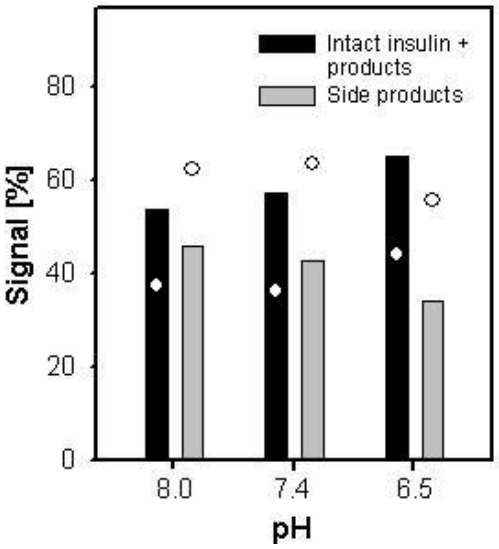


Fig. 2.45: pH controlled modification of insulin. Products include mono- and bis-product **36** and **37**. Bars indicate signals after 5 min reaction; empty circles indicate signals after 30 min. Reactions were stopped with dibromomaleimide after these times.

At pH 7.4 the usual reaction was observed: nearly complete conversion was achieved after 30 min but this was accompanied by the formation of a large amount of side products. A shift towards pH 8.0 did not change this behaviour substantially. In contrast, lowering the pH to 6.5 slowed the reaction down considerably. Fewer products were formed but also less side products were observed by MS and the main signal even after 30 min was the unmodified insulin. This again emphasises the difference between the multi-disulfide containing insulin and somatostatin, where the reactions proceeded rapidly at pH 6.2.

2.7.9 *In situ* generation of, and reaction with, dibenzeneselenolmaleimide

Next the focus was put on the choice of the maleimide substituent. Rationales 2) and 3) demand that it is a good leaving group but does not act as a reducing agent once released. Although none of the compounds synthesised so far fulfilled both requirements the reduction study of insulin (see 2.7.6) had revealed some interesting properties of benzeneselenol: it was less active in the cleavage of the hormone's disulfide bonds than thiophenol and quickly forms the diselenide which effectively removes the compound from the reaction.[407, 408] Furthermore, if mixed with dibromomaleimide it would react in a similar way to thiophenol and displace the bromides to form dibenzeneselenolmaleimide **33**.

This *in situ* formation of **33** was tested first; dibromomaleimide **6** (10 equiv) was added to insulin (at pH 7.4) followed by twice the amount of benzeneselenol. After a short incubation period more selenol was added as a mild reducing agent either in one portion or repeatedly and the reaction monitored over time (see 4.6.14). An immediate yellow colouring of all samples upon addition of benzeneselenol suggested that dibenzeneselenolmaleimide had indeed been formed. Samples treated with higher amounts of selenol exhibited full conversion to the bis-product **37** with acceptable amounts of the side products formed (Fig. 2.46).

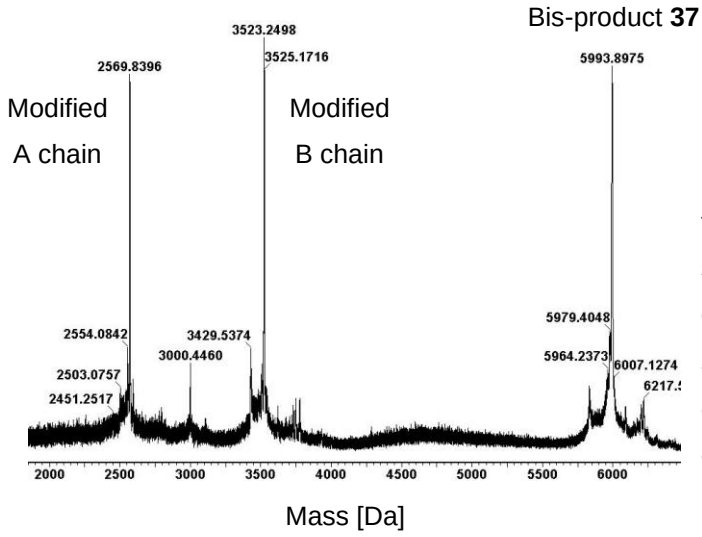


Fig. 2.46: Sample of the *in situ* generation and reaction of dibenzeneselenolmaleimide. Conditions: 10 equiv dibromomaleimide and 20 equiv benzeneselenol for 10 min, followed by addition of 1.0 equiv benzeneselenol and reaction-stop after 30 min.

While encouraging, additional experimentation and analysis made clear that this type of reaction was difficult to reproduce due to the poor control over the oxidation of the selenol compound and the large amounts of precipitate which were observed. To avoid these complications, dibenzeneselenolmaleimide **33** was synthesised (see Fig. 2.36), using the same protocol as for dithiophenolmaleimide **30** from dibromomaleimide **6** in good yield (81%).

2.7.10 Rationale based condition screening

To determine the ideal conditions for the bridging reaction with dibenzeneselenol, a screening experiment was carried out; to insulin in buffers at pH 7.4 or 6.5 were added 10 equiv of dithiophenolmaleimide **30** or dibenzeneselenolmaleimide **33** followed by addition of 1 equiv of reducing agent and subsequent reaction-stop with dibromomaleimide after 10 min. As a logical choice of a reducing agent with low reactivity in the insulin setting was not possible,

due to the lack of experimental data, the established or commonly used reducing agents (thiophenol, benzeneselenol, TCEP, DTT, 2-mercaptoethanol and L-cysteine) were tested under all conditions (Fig. 2.47 and 2.48).

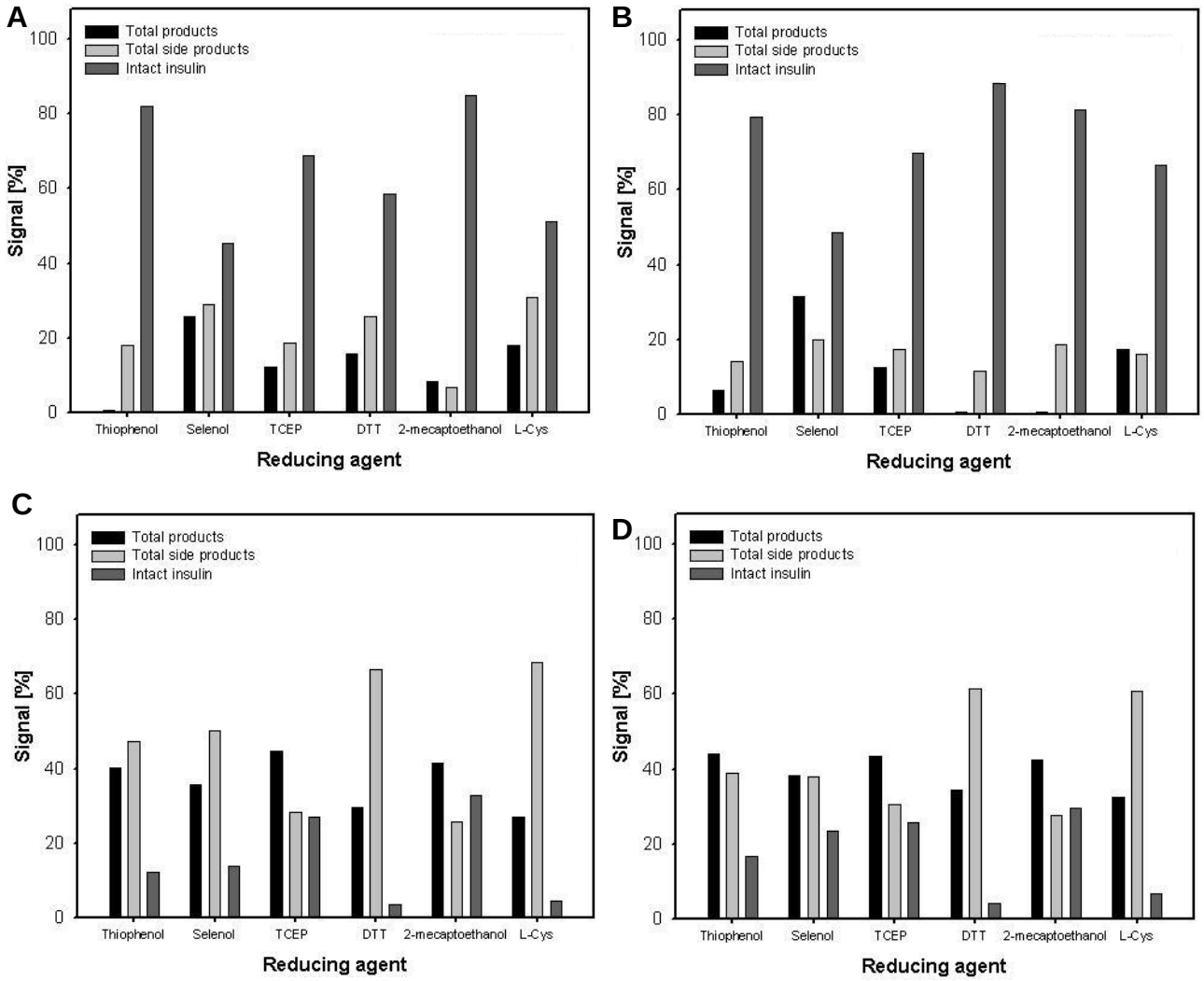


Fig. 2.47: Screening of conditions for the *in situ* modification of insulin. Products include mono-product **36** and bis-product **37**. (A) Dithiophenolmaleimide at pH 7.4. (B) Dithiophenolmaleimide at pH 6.5. (C) Dibenzeneselenolmaleimide at pH 7.4. (D) Dibenzeneselenolmaleimide at pH 6.5.

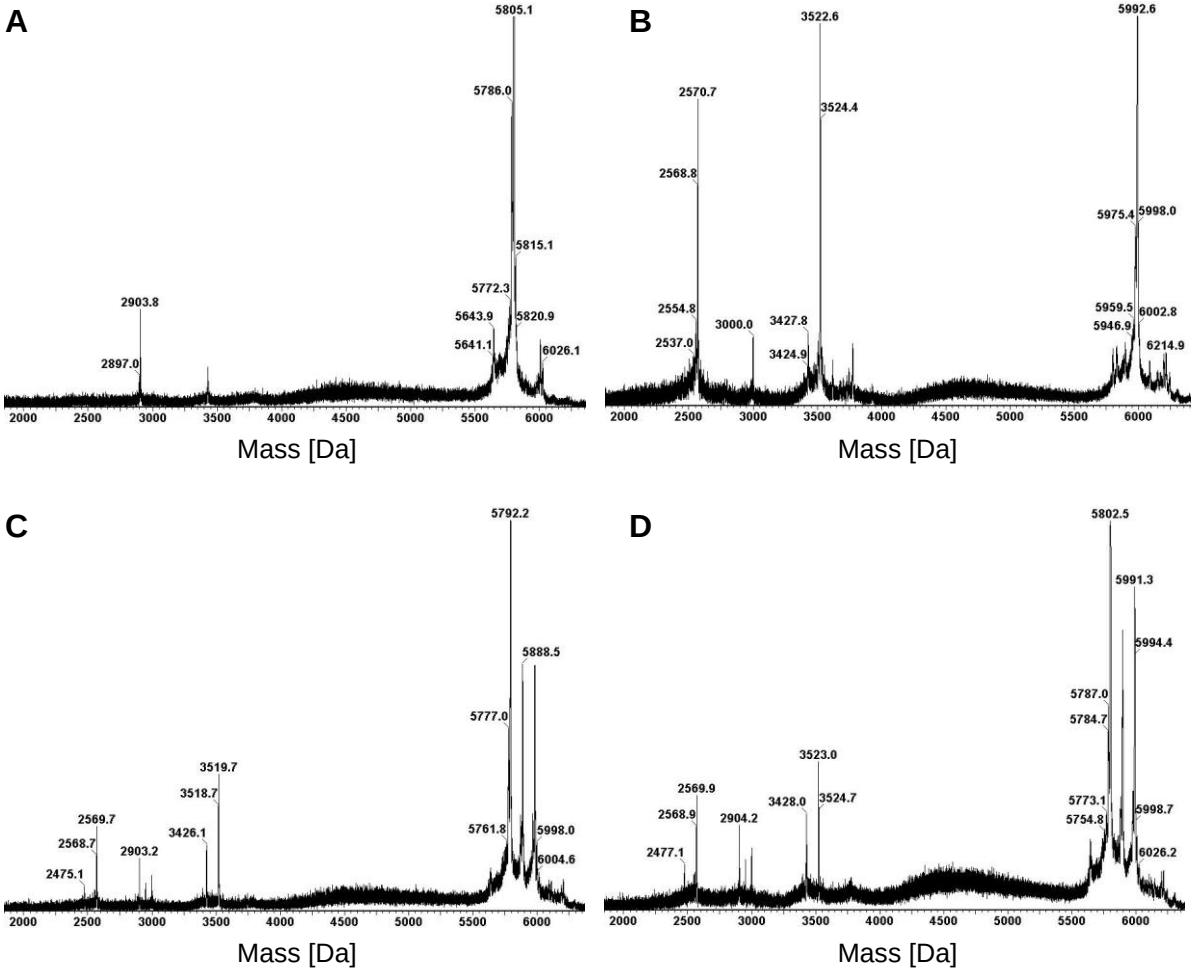


Fig. 2.48: Selected samples of the rational based condition screen. (A) Dithiophenolmaleimide + DTT at pH 6.5. (B) Dibenzeneselenolmaleimide + DTT at pH 6.5. (C) Dibenzeneselenolmaleimide + 2-mercaptoethanol at pH 7.4. (D) Dibenzeneselenolmaleimide + 2-mercaptoethanol at pH 6.5.

The experiment revealed a large difference in the reactivity of dithiophenolmaleimide and dibenzeneselenolmaleimide. In the presence of the small amounts of reducing agents used here, the dithiol exhibited a very low activity, which was further diminished when the reactions were conducted at a higher proton concentration. Even the strong reducing agent DTT produced only a small amount of products and side products when combined with dithiophenolmaleimide **30** (Fig. 2.48 A). In contrast, good conversion was observed in the presence of maleimide **33** (Fig. 2.48 B). The effect could be clearly attributed to the maleimide substituent as it was observed with all 6 reducing agents. This suggests, surprisingly, that the main driving force of the reaction under these conditions is not the reducing agent but the nucleophile released from the bridging reagent. The observation that benzeneselenol fulfils this role much better than thiophenol

somewhat contradicts the findings from the reduction study (see 2.7.6) but have to be seen in context. In the absence of maleimides the cleaved insulin cystines can re-oxidise, which might be more efficient in the presence of benzeneselenol or from mixed seleno-sulphur bonds. In addition, the reducing agent is also slowly inactivated by the formation of the diselenide. In the presence of dibenzeneselenolmaleimide however the revealed cysteines quickly attack the bridging agent and release more of the selenol to continue the cycle.

The best results were obtained when TCEP or 2-mercaptoethanol was used in tandem with dibenzeneselenolmaleimide **33** and the reaction at pH 6.5 yielded slightly more products than at pH 7.4 (Fig. 2.48 C and D). The combination of compound **33** and 2-mercaptoethanol at pH 6.5 was chosen for further development. This was due to the fact that even 20 equiv of 2-mercaptoethanol cleaved only 22% of insulin when used as a reducing agent in a reduction study as described in 2.7.6 (data not shown). In addition, the use of TCEP was avoided as the phosphine had previously exhibited certain reactivity towards the products of disulfide bridging reactions (see Fig. 2.20).

2.7.11 Optimisation of the *in situ* modification of insulin

Since only small amounts of the reducing agent seemed to be necessary to initiate the bridging reaction, an experiment was performed to determined the ideal amount of 2-mercaptoethanol to employ in conjunction with 10 equiv of dibenzeneselenolmaleimide (Fig. 2.49).

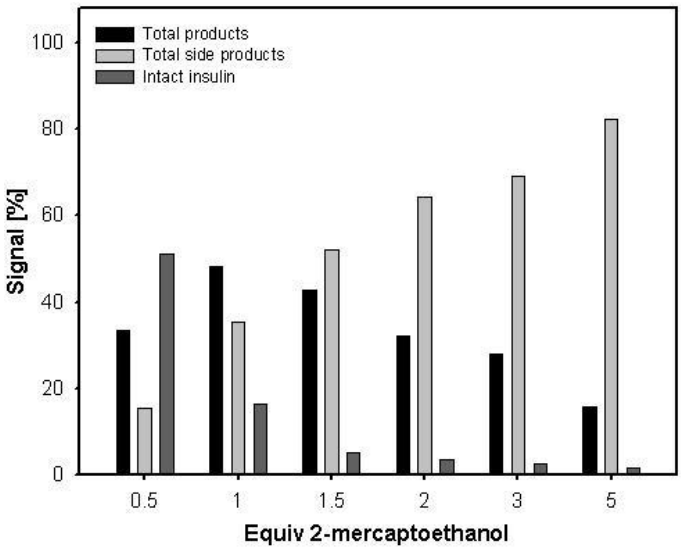


Fig. 2.49: Optimisation of the amount of reducing agent. Products include mono-product **36** and bis-product **37**. Conditions: 10 equiv dibenzeneselenolmaleimide, pH 6.5, reaction-stop after 10 min.

More than 2 equiv of 2-mercaptoethanol were detrimental to the efficiency of the reaction. This further supports the idea that the reducing agent should be used only in small amounts to start the modification process which then continues self-sufficiently. Although 1 equiv of 2-mercaptoethanol produced less side products, 1.5 equiv were used in the following experiments to promote maximal conversion.

The next logical step was to determine the ideal time to stop the reaction using dibromomaleimide (Fig. 2.50).

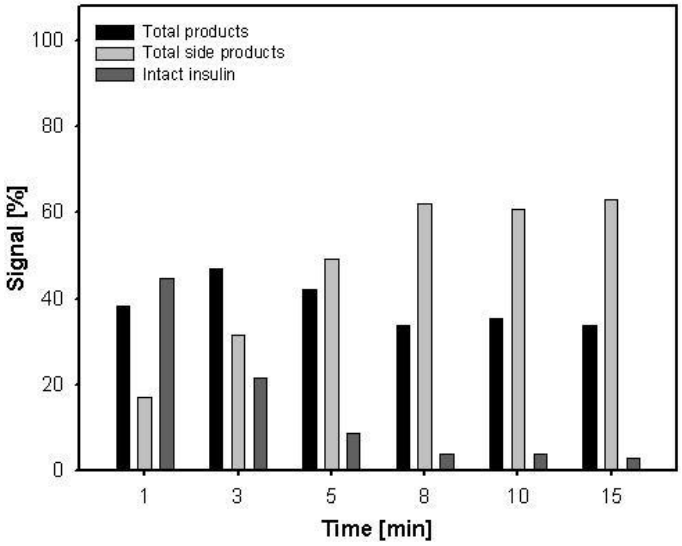


Fig. 2.50: Optimisation of the timing of the reaction-stop. Products include mono-product **36** and bis-product **37**. Conditions: 10 equiv dibenzene-selenolmaleimide, pH 6.5, 1.5 equiv 2-mercaptoethanol.

The maximal amount of products was formed after 3 min. From there the main reaction was the conversion of mono-addition product **36** to the bis-addition product **37** while most of the still intact insulin reacted to yield only modified side chains. Some of the bridged hormone was lost between 3 and 8 min, possibly due to the reversibility of the bridging reaction. The process stopped completely after 8 min where either all disulfides had reacted or no free nucleophile was left. A 10 min reaction time was chosen again with the focus on the best conversion possible to enable purification of a single product.

With optimal reaction time and reducing agent determined, the amount of bridging reagent was varied in the next experiment from 2 equiv to 25 equiv dibenzeneselenolmaleimide (Fig. 2.51)

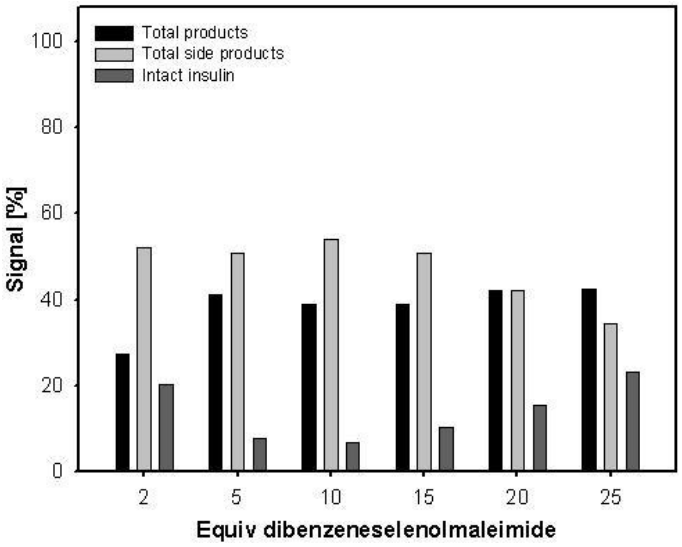


Fig. 2.51: Optimisation of the amount of bridging reagent. Products include mono-product **36** and bis-product **37**. Conditions: pH 6.5, 1.5 equiv 2-mercaptoethanol, reaction-stop after 10 min.

As expected, the addition of less dibenzeneselenolmaleimide yielded more side products and fewer products as the bridging reaction became less efficient. Interestingly the product formation was not dependent on the amounts of maleimide **33** above 5 equiv of this compound, but the appearance of modified side chains was suppressed and more native insulin was still left. This observation possibly reflects an increased tendency of the reducing agent as well as the released selenol to react with the maleimide, which now is present in a large excess, instead of the insulin cystines.

To further test this hypothesis multiples of the established 1.5 equiv 2-mercaptoethanol and 10 equiv dibenzeneselenolmaleimide were added to insulin (Fig. 2.52). If the efficiency of the reaction is retained (or increased) no preference of the reducing agent (or the benzeneselenol) for either the maleimide or the peptide hormone should exist.

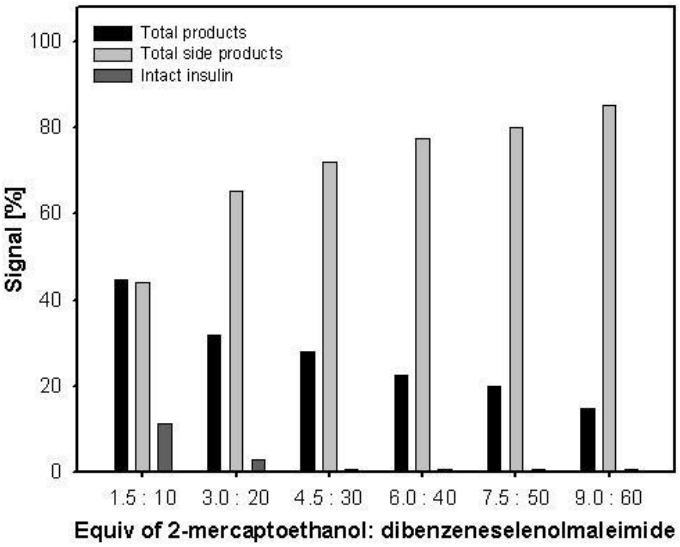


Fig. 2.52: Optimisation of the ratio of reactants and hormone. Products include mono-product **36** and bis-product **37**. Conditions: pH 6.5, reaction-stop after 10 min.

The experiment revealed that the presence of more reagents did not increase the reaction speed but promoted the formation of larger amounts of the side products. The reducing agent seems to favour the attack on the insulin cystines over a reaction with the maleimide reagent and thus only small amounts of the thiol can be used in the modification of the peptide hormone.

Since the use of more maleimide **33** seemed promising (Fig. 2.51), the timing of the reaction in the presence of more bridging reagent was tested, as described before, and completeness of the reaction after extended periods of time was also examined (Fig. 2.53).

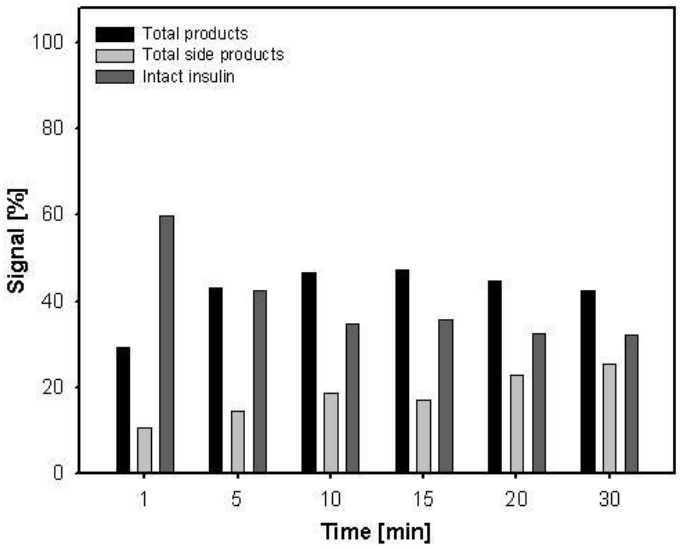


Fig. 2.53: Optimisation of the timing with more compound **33**. Products include mono-product **36** and bis-product **37**. Conditions: 25 equiv dibenzeneselenolmaleimide, pH 6.5, 1.5 equiv 2-mercaptoethanol, reaction-stop with 5 equiv dibromomaleimide.

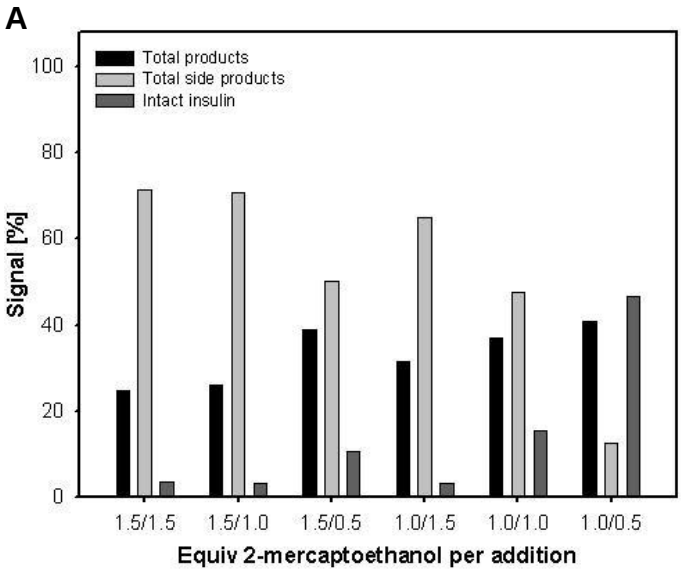
As observed earlier, the reaction came to a halt between 5 and 10 min with a significant amount of unmodified hormone left. This adds to the hypothesis that,

after this time, no free reducing agent remained. Since the goal was the preparation of a single homogeneous product (in good yield) further optimisation was necessary to achieve full conversion. Previous experiments had shown that the addition of more reducing agent at the start does not result in higher conversion (see 2.7.4 and this chapter). Thus, the protocol of repeated addition of small amounts of reducing agent to the mixture of insulin and the bridging reagent was revived. This time it would have the advantage that the reaction would stop automatically, due to the formation of the diselenide, removing the need for the addition of a reagent.

2.7.12 Self-terminating stepwise modification of insulin

To test this idea a mixture of insulin (in pH 6.5 buffer) and 25 equiv dibenzeneselenolmaleimide was treated with small amounts (0.3–2.0 equiv) 2-mercaptoethanol for 10 min and analysed (data not shown). This experiment showed that less than 1.0 equiv of the reducing agent was not sufficient to start the reaction in the presence of this large amount of bridging reagent.

Thus, the same reagent mixture was either treated with 1.0 or 1.5 equiv of the reducing agent, incubated for 10 min and another portion (0.5–1.5 equiv) of 2-mercaptoethanol was added. Alternatively 1.0 equiv of the reducing agent was added repeatedly every 10 min (data not shown). The best result was obtained with 1.0 equiv nucleophile followed by another 1.5 equiv (Fig. 2.54).



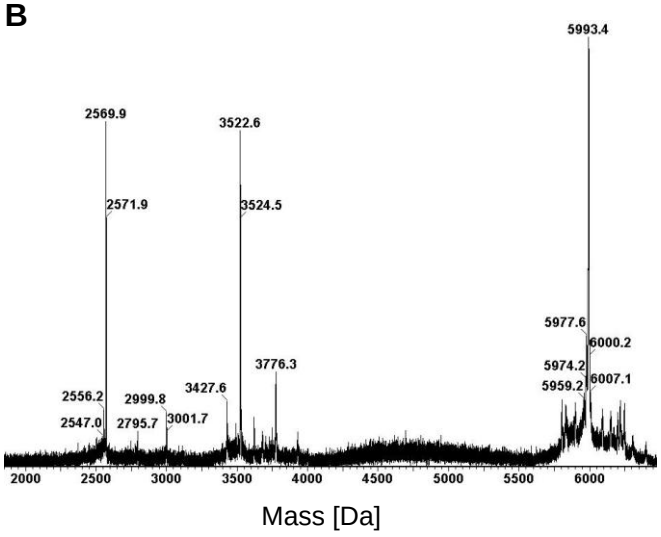
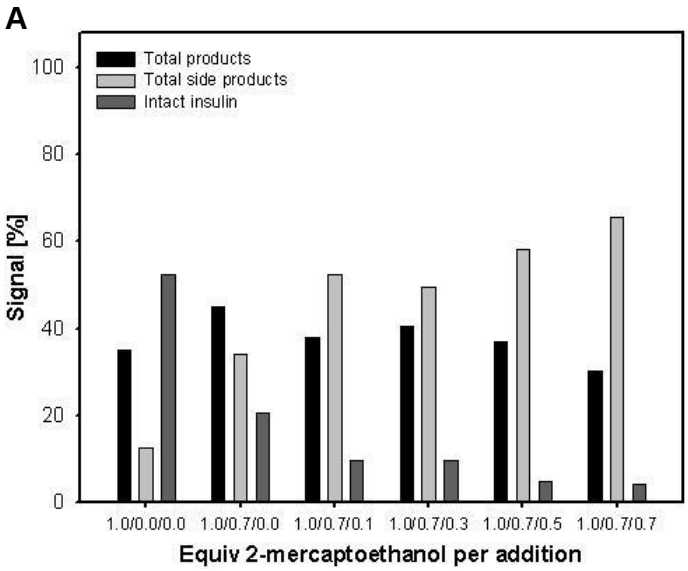


Fig. 2.54: Two-step modification of insulin. Products include mono-product **36** and bis-product **37**. Conditions: 25 equiv dibenzeneselenolmaleimide, pH 6.5, two portions of 2-mercaptoethanol for 10 min each, reaction-stop with 5 equiv dibromomaleimide. (A) Experiment summary. (B) MS of the addition of 1.0 equiv followed by 1.5 equiv of 2-mercaptoethanol.

As observed before, the amount of reducing agent employed had to be chosen carefully. Too much reagent produced a large amount of side products, while too little was not able to activate the reaction sufficiently to obtain good conversion.

A significant amount of modified side chains was still produced during these experiments. Therefore, splitting of the second portion of 2-mercaptoethanol into two smaller portions was attempted. The previous experiment was repeated with combinations of 1.0 equiv of the reducing agent with smaller amounts (0.2–0.7) in the second step (data not shown). This revealed that 1.0 equiv + 0.7 equiv would be a good starting point for a three-step process, which was carried out next (Fig. 2.55).



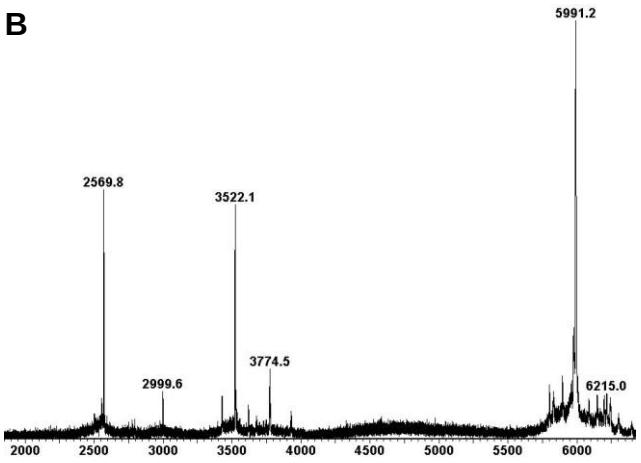


Fig. 2.55: Three-step modification of insulin. Products include mono-product **36** and bis-product **37**. Conditions: 25 equiv dibenzeneselenolmaleimide, pH 6.5, three portions of 2-mercapto-ethanol for 10 min each, reaction-stop with 5 equiv dibromomaleimide. (A) Experiment summary. (B) MS of the addition of 1.0 equiv followed by 0.7 equiv and 0.5 equiv of 2-mercapto-ethanol.

The MS analysis showed a clear advantage of a three-step process over a single or two-step addition of the reducing agent; the signal of the bis-product **37**, obtained as the almost only complete insulin signal left, dominated the MS spectra while the modified free A and B chain products both reached now less than 50% signal intensity. It should be stressed again that MALDI-TOF MS analysis is not quantitative and was used only to guide the process development. Interestingly, small amounts of 2-mercaptoethanol, which were not sufficient to start the single-step reaction, became enough to (re-)activate the process in the multi-step reaction. This again underlines the fact that a complex mixture of reactants is formed during the modification process.

Although there was still room for additional fine tuning and parameter optimisation (such as temperature or solvents) it was decided that the reaction efficiency as shown above was sufficient to produce separable products. Only purification of the products would confirm the reaction yield and reveal if the modification of the disulfide bonds of insulin had disrupted its structure. Optimisation of the protocol to obtain the mono-product **36** was not carried out at this point; it seemed unlikely that this analogue could be prepared without the presence of the bis-product **37**. Separation of both insulin analogues would only be possible if the group attached via the maleimide would be sufficiently large (such as a PEG chain) or hydrophobic (such as a fluorescent dye or spin label).

2.7.13 *In situ* spin labelling of insulin

The ultimate goal of the modification of insulin was its functionalisation both to prepare novel analogues for clinical applications but also to create useful tools to obtain deeper insights into the hormones' biological activity and dynamic behaviour. A double spin labelled analogue could yield structural insights as the distance between these labels can be monitored and could be used to characterise the reaction product as theoretical estimates exist for this parameter.[305]

To this end *N*-TPO-dibenzeneselenolmaleimide **35** was synthesised from its dibromomaleimide precursor as before and employed in the established three-step *in situ* modification protocol. Surprisingly, the MS analysis showed almost only the unmodified hormone with very small signals for the free peptide chains (data not shown). *In situ* reactions of spin labelled maleimide analogues had proceeded similar to the non-functionalised bridging reagent (see 2.6.3) suggesting no large change in reactivity of these compounds. Potentially the new spin label was contaminated with small amounts of *N*-TPO-dibromomaleimide, which would neutralise the thiol reducing agent. As the bridging reagent is present in a 25x excess in the reaction, less than 4% of the bromomaleimide analogue would stop the modification process (it should be added that the analysis of the chemical compounds by MS and NMR were complicated by the presence of the free radical of the spin label). To test this hypothesis, larger amounts of reducing agent were added to the usual insulin/ dibenzeneselenolmaleimide mix (Fig. 2.56).

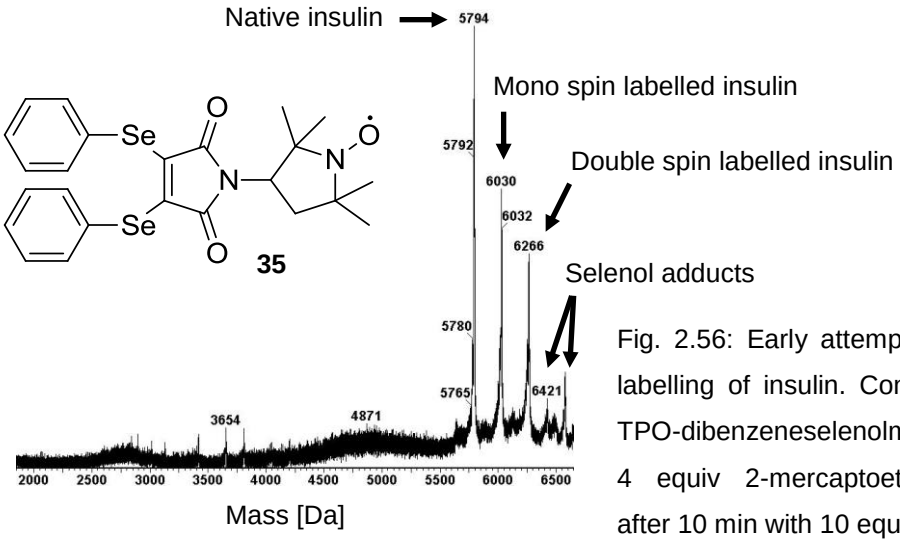
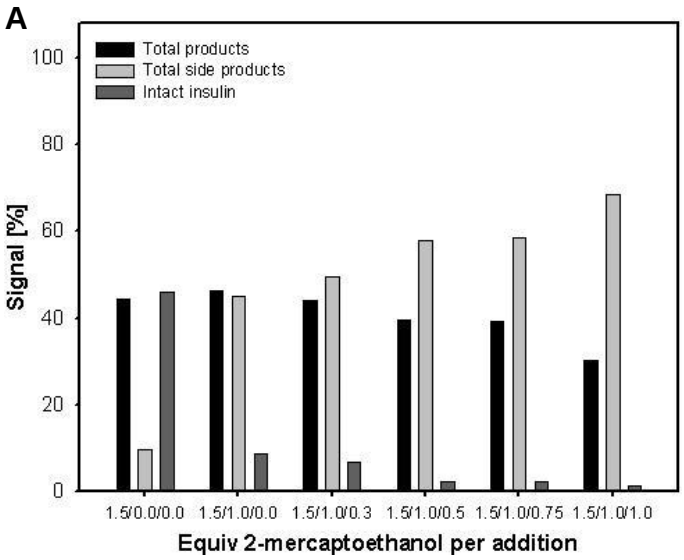


Fig. 2.56: Early attempt for the *in situ* spin labelling of insulin. Conditions: 25 equiv *N*-TPO-dibenzeneselenolmaleimide **35**, pH 6.5, 4 equiv 2-mercaptoethanol, reaction-stop after 10 min with 10 equiv dibromomaleimide.

Although it was possible to obtain product with more 2-mercaptoethanol the reaction was not efficient and produced adducts of benzeneselenol to the double spin labelled insulin.

It was decided that the synthesis or re-purification of the spin label should not be repeated since the starting material is costly and the process was not high yielding. Instead attempts were made to establish a step-wise modification protocol in the presence of less *N*-TPO-dibenzeneselenolmaleimide which would reduce the potential amount of the bromomaleimide analogue in the reaction mixture. This was accomplished as described in the previous chapter by experimental determination of good starting mixtures and appropriate follow up tests. A reproducible and high yielding process with full conversion was found using 10 equiv of the spin label **35** and 3x 10 min long addition steps with 1.5 equiv, 1.0 equiv and 0.75 equiv of 2-mercaptoethanol (Fig. 2.57).



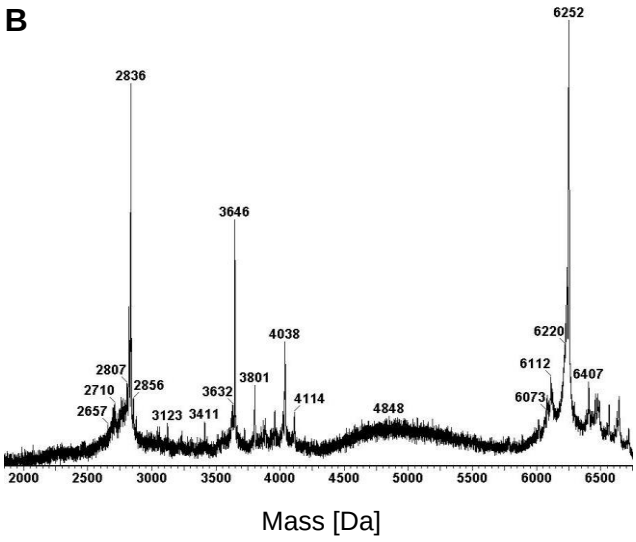


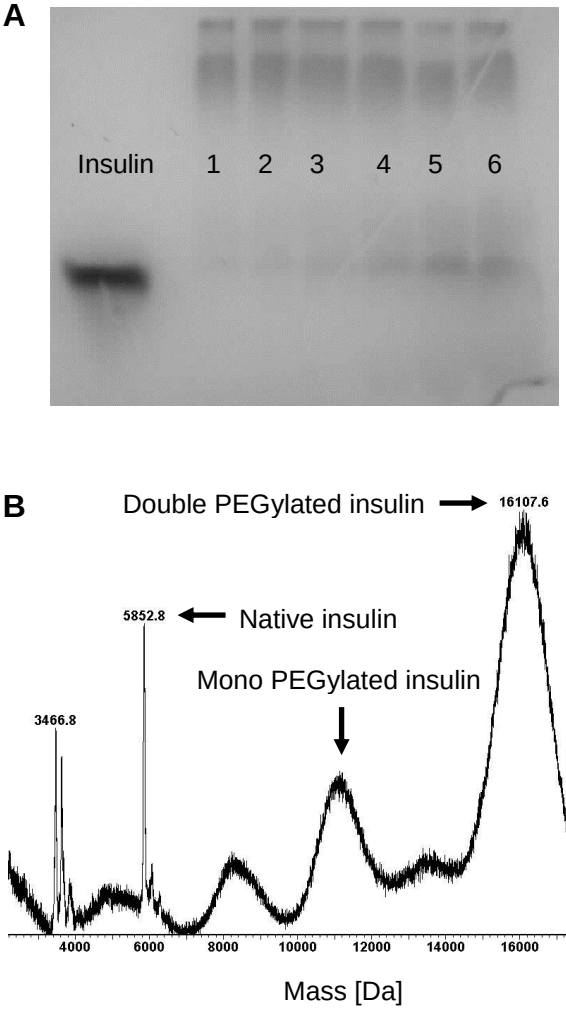
Fig. 2.57: Three-step *in situ* spin labelling of insulin. Products include mono- and bis-labelled hormone. Conditions: 10 equiv *N*-TPO-dibenzeneselenolmaleimide **35**, pH 6.5, three portions of 2-mercaptoethanol for 10 min each, reaction-stop with 10 equiv dibromomaleimide. (A) Experiment summary. (B) MS of the addition of 1.5 equiv followed by 1.0 equiv and 0.75 equiv of 2-mercaptoethanol.

The formation of fewer side products was observed when the third addition step contained less reducing agent, however, when preparing material for distance measurements in EPR the purity of the product is more important than the yield. Interestingly although the reaction was stopped with dibromomaleimide no insulin products with a maleimide bridged cystine (rather than a *N*-TPO-maleimide bridge) were observed. This showed that after the 3 reactions all disulfide bonds had reacted with a maleimide and no unmodified hormone was left.

2.7.14 *In situ* PEGylation of insulin

Numerous attempts have been made to synthesise PEGylated insulin in order to create stabilised or long acting analogues of the hormone.[39] Thus, the benzeneselenol version **34** of *N*-PEG5000-dibromomaleimide was prepared via the

established Mitsunobu protocol and a method for the modification of insulin developed in an analogous manner to the described spin labelling reaction. Tricine SDS-PAGE[409] was utilised to confirm the complete consumption of the peptide starting material (Fig. 2.58). The best results were again obtained with 3 steps – 1.5, 1.5 and 1.0 equiv 2-mercaptoethanol – in the presence of 25 equiv of *N*-PEG5000-dibenzeneselenolmaleimide **34**.



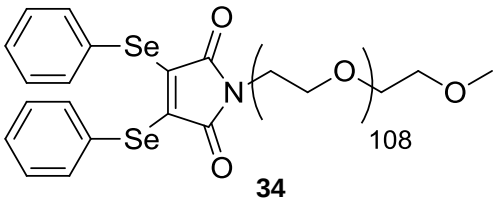


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Fig. 2.58: Three-step *in situ* PEGylation of insulin. Conditions: 25 equiv *N*-PEG5000-dibenzeneselenolmaleimide **34**, pH 6.5, three portions of 2-mercaptoethanol for 10 min each, reaction-stop with 10 equiv *N*-PEG5000-dibromomaleimide. (A) Tricine SDS-PAGE of the 3-step optimisation (equiv of 2-mercaptoethanol). 1) 1.5/1.5/1.0. 2) 1.5/1.5/0.75. 3) 1.5/1.0/0.75/ 4) 1.5/1.0/0.5. 5) 1.0/0.75/0.75 and 6) 1.0/0.75/0.5. (B) MS of the addition of 1.5 equiv followed by 1.5 equiv and 1.0 equiv of 2-mercaptoethanol.

2.7.15 Purification and characterisation of insulin analogues

To facilitate further characterisation, maleimide bridged and PEGylated insulin was purified. This was easily achieved in the case of the PEG-analogue as the increase in mass and the water-solubility of the majority of the reaction components allowed the direct separation via SEC (size exclusion chromatography). The desired product eluted close to the void volume of the Sepharcryl S-100 column indicating a greatly increased hydrodynamic radius, as usually observed for PEGylated proteins.[40] This also allowed for further purification by ultrafiltra-

tion with a high MWCO (molecular weight cut-off) filter. This and the baseline-separation observed during SEC indicate that the lower molecular weight species visible in MALDI-TOF spectra of the product (Fig. 2.59 A) are artefacts of the MS technique.[401] The overall yield was 24%. Although not optimal this could still be acceptable for an industrial process if the product exhibits a favourable pharmacokinetic behaviour as insulin is produced inexpensively in large amounts. It also compares relatively well to the yields obtained with "traditional" methods for the PEGylation of insulin (see 1.6.2).[285, 288, 294, 296, 300]

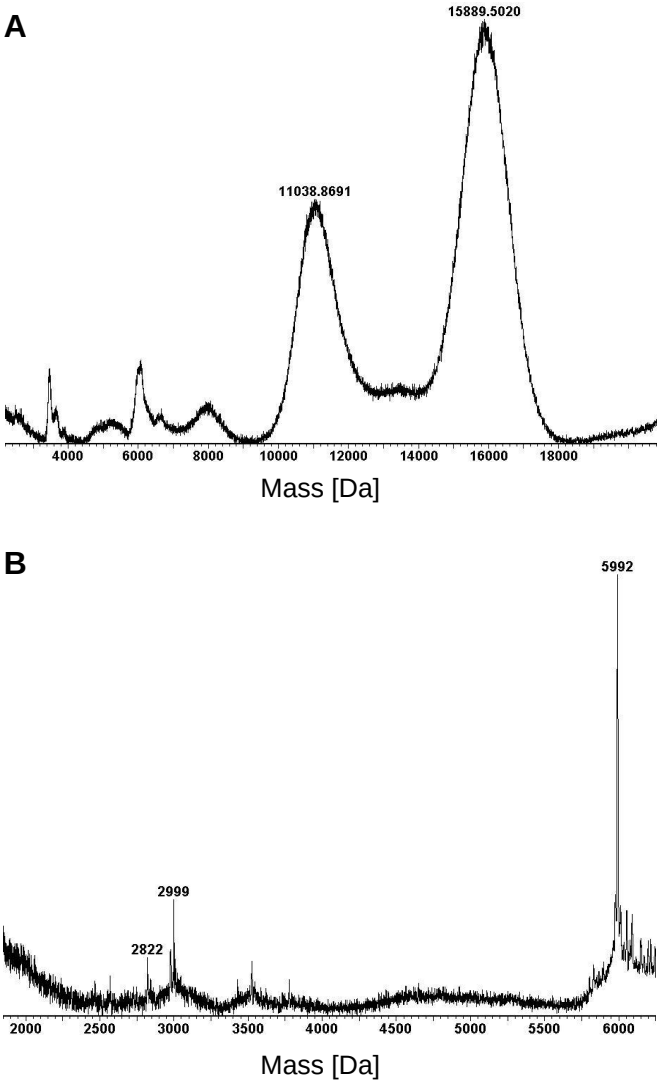


Fig. 2.59: MS analysis of purified insulin analogues. (A) Double PEGylated insulin. Additional mass signals are likely to be derived from loss of PEG or peptide chains during the ionisation process. (B) Bis-product **37**.

In contrast, the purification of maleimide bridged insulin was challenging. The necessary removal of the excess of water-insoluble bridging reagent and diselenide could be achieved by filtration, dialysis or centrifugation. Unfortunately, although the respective filtrates or supernatants contained the desired product, no separation from the side products, notably almost exclusively A chain

products, was accomplished by SEC. Closer examination of the afore discarded material showed that the maleimide bridged insulin analogue is poorly soluble in pure water (as is native insulin) but has acceptable solubility in buffered salt solutions. These properties could be utilised for product purification; the reaction mix was dialysed into water, pelleted and washed with water to remove the soluble A chain products. The residual material was then dissolved in buffer and filtered through a desalting column to remove the insoluble B chain products. This process yielded almost pure double bridged insulin (Fig. 2.59 B) but in a yield of only 2% – probably due to the “leaking” of product into the discarded solutions and the many purification steps.

With the isolated insulin analogues in hand it was then attempted to localise the two maleimide bridges in the products by proteolytic digest combined with MALDI-TOF MS. Thermolysin was found to theoretically generate peptides, which would be suitable to distinguish the disulfide bridges.[410] Indeed treatment of the native insulin with the protease under optimised conditions yielded a characteristic fragmentation pattern (Fig. 2.60). With the help of *in silico* generated digests, allowing for missed cleavages, it was possible to identify numerous peptides (Table 2.15).

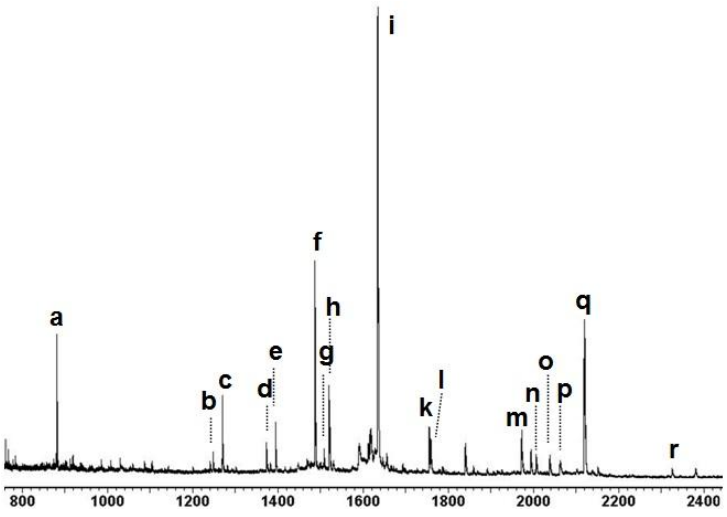


Fig. 2.60: MS analysis of the digest of native insulin with thermolysin. Letters correspond to the index in Table 2.15.

Table 2.15: Identified peptides of native insulin after digest with thermolysin. *Alternatively the fragment IVEQCCTS has a mass of 881.36 Da but upon treatment with monobromomaleimide after reduction, addition of only a single maleimide unit was observed excluding the fragment with two cysteines.

Mass found	Mass calculated	Fragment 1	Fragment 2	Fragment 3	Contained cystine(s)	In-dex
880.93	879.43*	LV**C**GERGF	-	-	-	**a**
1241.35	1241.51	GIVEQ**CC**TSICS	-	-	-	**b**
1269.17	1269.52	GIVEQ**CC**TS	I**C**S	-	A6-A11	**c**
1373.44	1373.57	LENY**C**N	L**C**GSHL	-	A20-B19	**d**
1394.51	1394.58	VEQ**CC**TS	FVNQH	-	A7-B7	**e**
or	1394.58	IVEQ**CC**TS	L**C**GSH	-	A7-B7	**-**
1486.33	1486.65	LENY**C**N	LV**C**GERG	-	A20-B19	**f**
1508.47	1509.66	IVEQ**CC**TS	L**C**GSHL	-	A7-B7	**g**
1520.51	1520.64	LENY**C**N	V**C**GERGF	-	A20-B19	**h**
1633.33	1633.72	LENYCN	LVCGERF	-	A20-B19	**i**
1754.29	1754.73	GIVEQ**CC**TSI**C**S	L**C**GSH	-	A7-B7	**k**
1759.43	1759.72	VEQ**CC**TS	VNQHL**C**GSH	-	A7-B7	**l**
1971.30	1971.87	VEQ**CC**TS	L**C**GSHLVEALY	-	A7-B7	**m**
2006.12	2006.84	VEQ**CC**TS	I**C**SLYQ	L**C**GSH	A6-A11/A7-B7	**n**
2038.63	2037.93	LYQLENY**C**N	L**C**VGERGF	-	A20-B19	**o**
2062.97	2061.95	LENY**C**N	VEALYL-V**C**GERG	-	A20-B19	**p**
or	2062.85	VEQ**CC**TSI**C**S	VNQHL**C**GSH	-	A7-B7	-
2119.24	2119.92	IVEQ**CC**TS	I**C**SLYQ	L**C**GSH	A6-A11/A7-B7	**q**
or	2119.24	VEQ**CC**TS	I**C**SLYQ	L**C**GSHL	A6-A11/A7-B7	-
2325.56	2324.98	IVEQ**CC**TSI**C**SL YQLENY**C**N	-	-	-	**r**

Unfortunately it was not possible to obtain likewise clear results for the digests of the two purified insulin analogues. In both cases a complete digest was achieved in the same incubation time as with the native peptide. This had not been expected for the PEGylated insulin, as this modification has been shown to protect the hormone from proteolytic cleavage.[295] It might be the result of the neutralisation of the protective PEG-shield by the high temperature (70 °C) necessary for the activity of thermolysin. The MS spectra of the digests contained

more noise, some unidentified new peptides and exhibited, instead of the expected upward mass shifts, only the loss of signals (Fig. 2.61).

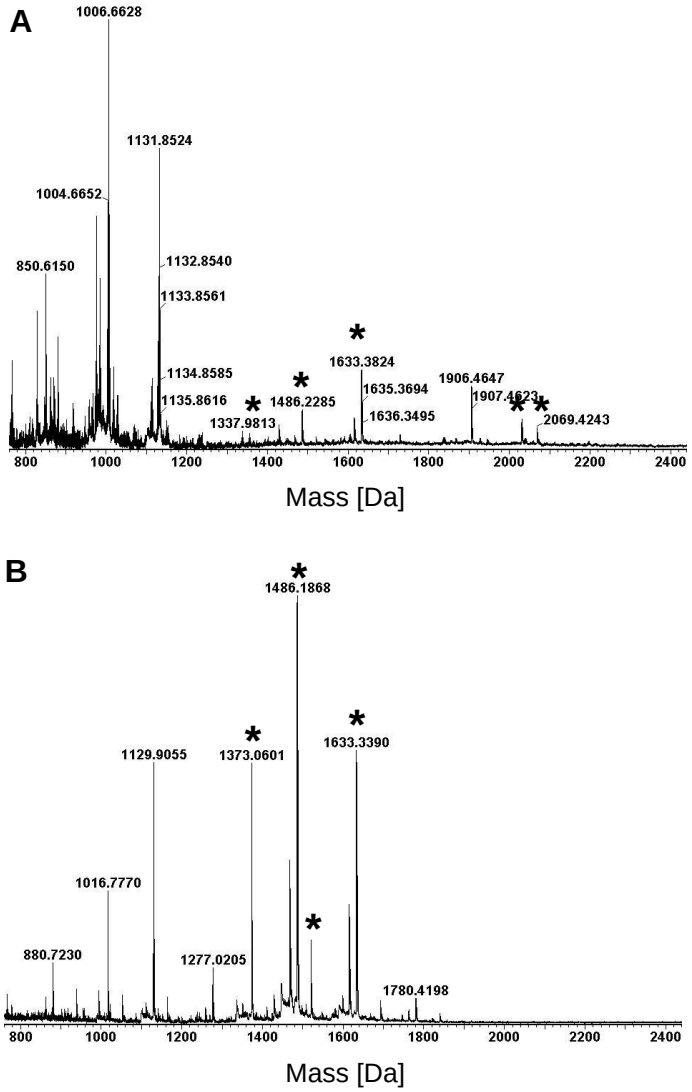


Fig. 2.61: MS analysis of the digest of purified insulin analogues with thermolysin. Peptides containing the A20-B19 disulfide bridge are labelled. (A) Bis-product **37**. (B) Double PEGylated insulin.

A distinguished pattern was found upon comparison with the digest of native insulin, despite the non-ideal results; almost all peptide fragments that contain the A20 – B19 disulfide bond (mainly 1373.44, 1486.33, 1520.51 and 1633.33 Da) were still present, while the MS signals for the polypeptides with the A7 – B7 and A6 – A11 cystines had disappeared. In addition a series of broad higher molecular weight signals were detected (around 6,200, 7,400 and 12,400 Da) in the digest samples of double PEGylated insulin (data not shown) indicating the presence of PEG-conjugated fragments. Taken together this tentatively suggests that the primary sites of maleimide bridging in insulin are the interchain disulfide bond A7 – B7 and the intramolecular disulfide bond A6 – A11 under the assumption that the peptide fragments containing the maleimide bridges

become insoluble or otherwise not accessible to ionisation upon proteolytic cleavage of the hormone. This is supported by the observation that the characteristic yellow colour of the solution of double bridged insulin was quickly lost during the digest with thermolysin and modified hormone did not ionise in LCMS, while the native insulin could be detected readily (data not shown). For clarification, tandem MS induced fragmentation and identification could be used. The modification of the A6 – A11 disulfide bond was a surprise as this structural feature is buried in the hydrophobic core of insulin.[272] This indicates that the bridging of the solvent accessible A7 – B7 cystine has permitted the employed reagents access to this region of the hormone, further implying changes in the overall structure. On the other hand, it has been shown that the modification[411] of the A6 – A11 disulfide bond, or even its complete loss,[307] has a minor impact on the activity of insulin. The A20 – B19 cystine in contrast, which has not been targeted by the maleimide chemistry, is of key importance.[308]

To further examine if persistent changes in the secondary structure of the modified insulins were present, CD spectra in the far UV region of the double maleimide bridged and PEGylated analogues were recorded alongside the native hormone (Fig. 2.62). It should be mentioned that difficulties in determining the exact quantity of PEGylated insulin by standard methods and the limited solubility of the maleimide bridged insulin in the low salt conditions required for CD spectroscopy complicate an exact comparison.

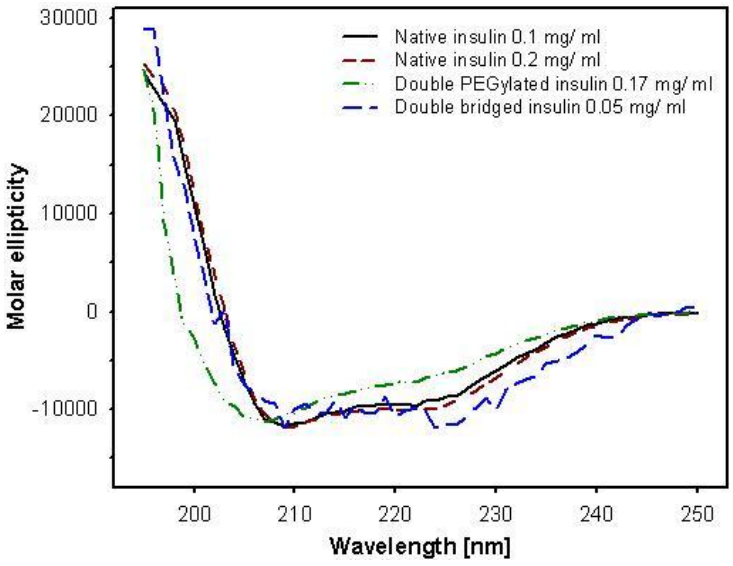


Fig. 2.62: CD spectra in the far UV region of native, double bridged and PEGylated insulin.

All four CD traces show the typical features of a mainly α-helical protein: a local minimum around 222 nm, a global minimum around 208 nm and a sharp increase in signal intensity towards 200 nm.[412] Although noisy, the signal of the double maleimide bridged insulin corresponds well to the unmodified hormone indicating a native fold and successful bridging of the modified disulfides as a mutant without the A6 – A11/ A7 – B7 cystines would have lost most of its helical structure.[302] In contrast, the decreased solubility of this analogue suggests that more hydrophobic surfaces are solvent exposed, which should be reflected in structural changes. Both observations are compatible as insulin is relatively flexible. Its secondary structure elements, which are responsible for the CD signal, are able to perform rigid body movements, possibly to accommodate the maleimide bridges, without significant changes to the overall structure.[413]
The PEGylated hormone on the other hand shows a substantial loss in signal intensity and a small blue-shift of its global minimum. Both features have been observed in analogues, which had lost[302, 308] or scambled[306] the A7 – B7 and/ or A6 – A11 cystines. Still, a loss of signal intensity has also been found in other PEGylated insulins[285] and might originate from the re-packing of amino acid side chains to allow the exit of the attached PEG chain from the protein core or local unfolding which can be induced by large polymers.[265] A thorough amino acid analysis or a high resolution structure of this insulin analogue would allow a more detailed analysis.

In summary, the modification and functionalisation of the insulin disulfide bonds was achieved through the logical evolution of the developed 3,4-substituted maleimide platform. The careful choice of substituents and reducing agent but also of parameters like pH and timing allowed the adaption of the chemistry to the protein target. Although not yet fully optimised, a new generation of insulin analogues was synthesised. Additional experiments on stability, multimerisation behaviour and biological activity would be necessary to determine their potential value as agents for a therapeutic application.

2.8 Modification of an anti-CEA ds-scFv fragment

After characterisation of the maleimide chemistry with somatostatin and the relatively successful modification of insulin, the next step for this project was the

transfer of this approach to antibodies. As full antibodies are complex systems with a multitude of disulfide bonds it was decided to start with a single cystine containing antibody fragment. To this end a collaboration with the lab of Prof Kerry Chester (UCL Cancer Institute) was initiated to access a well characterised disulfide-stabilised single-chain fragment (ds-scFv, see 1.6.3). shMFELL2Cys (for clarity called anti-CEA) is a further development of MFE-23, a scFv derived from a large bacteriophage library prepared from mouse spleen cells[414] and produced in *E. coli*.[415] The murine antibody has been fully humanised by 28 amino acid substitutions.[416] As the original scFv had a strong tendency to form dimers[417] due to its short $(Gly_4Ser)_3$-linker[349] this was elongated to $(Gly_4Ser)_4$. To further stabilise the monomeric form a disulfide bond was introduced[338] at a position (see 4.7.1) previously identified as being ideally suited to tolerate the covalent linkage.[332, 336] Thus, the scFv has been transformed into a highly stable ds-scFv.

The target of the antibody is carcinoembryonic antigen (CEA), which it binds with nanomolar affinity (K_d of MFE-23 = 2.5 nM, K_d of shMFE = 8.5 nM)[338, 418] between the N-terminal two of it's seven Ig domains.[419] CEA is a ~180 kDa highly glycosylated cell surface protein involved in intercellular recognition and attachment and is overexpressed in a number of adenocarcinomas.[420] As the protein is membrane-anchored, it can be released into the blood stream and elevated levels up to 1 nM can be observed in cancer patients.[421] Although the concentration of free CEA is usually too low for diagnostic purposes,[422] monitoring CEA blood levels is the most efficient method of aftercare to control for recurrent disease after surgical resection.[423] This is carried out by competitive immunoradioassay.[424] Therefore a number of antibodies against CEA have been developed[425] and labelled with radioactive compounds,[426] fluorophores[427, 428] and even some immunoconjugates have been reported.[429, 430]

2.8.1 Accessing the anti-CEA disulfide bond

Analogous to the insulin work the first step in the modification process of the anti-CEA ds-scFv was the determination of efficient ways to reduce its artificial disulfide bond. Surprisingly, small amounts of TCEP (up to 20 equiv) had no measurable effect. Potentially the charged reducing agent was not able to reach the cystine buried between the V_H and V_L interface due to unfavourable ionic

interactions,[213] or the immediate environment may not allow for the organisation of enough water molecules around the disulfide bond to support the attack of the phosphine.[214]

Alternatively, reduction of the ds-scFv was attempted using 50 equiv of either TCEP, 2-mercaptoethanol or DTT. Only DTT was efficient in cleaving the cystine of anti-CEA (data not shown) and the amount of the reducing agent and the time to reach complete cleavage were optimised. The most promising conditions (10 and 20 equiv DTT for 1 h at ambient temperature) were repeated under high salt conditions (500 mM NaCl in comparison to 137 mM NaCl of standard PBS) to test if weakening the ionic and Van-der-Waals interactions between the antibody domains[376] would accelerate the reaction (Fig 2.63).

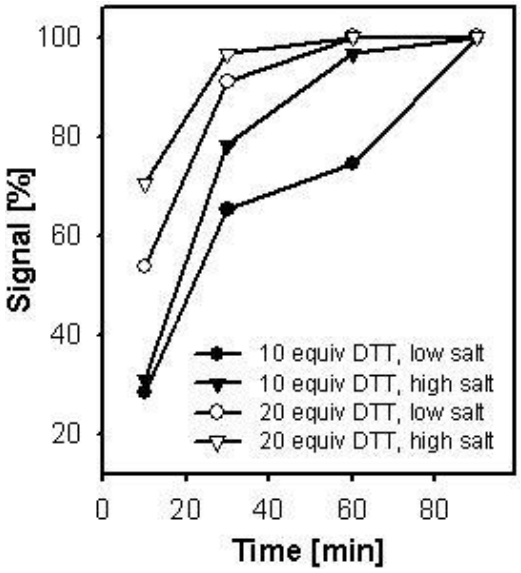


Fig. 2.63: Reduction study of anti-CEA under low salt (137 mM NaCl) and high salt (500 mM NaCl) conditions with DTT. The signal % corresponds to the relative amount of reduced antibody.

Although some increase in the speed of the reduction was found, this observation did not translate into faster bridging reactions later on (see below).

In preparation of an *in situ* protocol anti-CEA was treated with benzeneselenol since TCEP had been unable to cleave the fragment's disulfide bond. As a strong effect of the released leaving group with the target protein had been observed during the modification of insulin, thiophenol was also reacted with anti-CEA (Fig. 2.64).

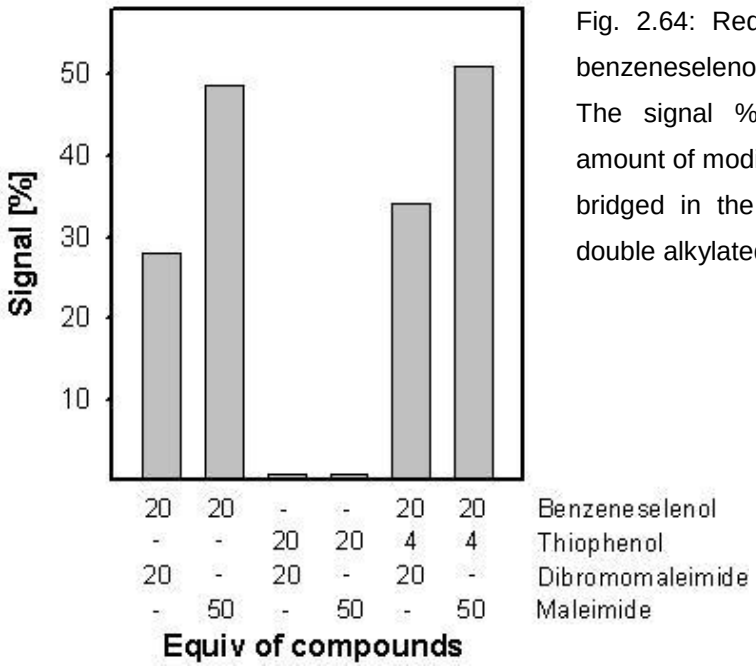
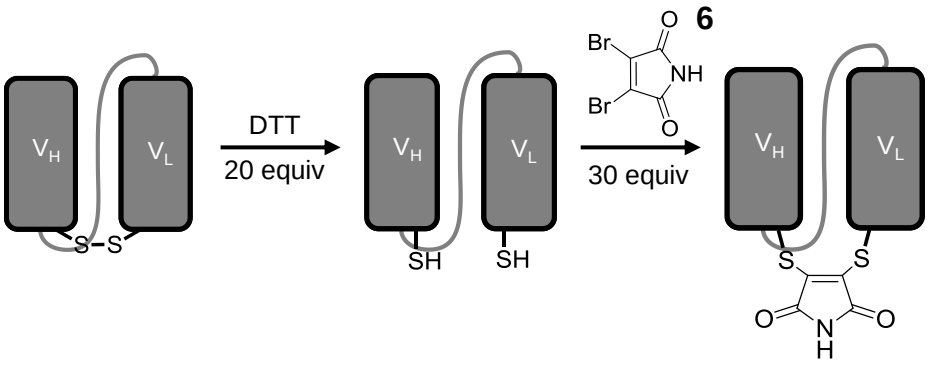


Fig. 2.64: Reduction study of anti-CEA with benzeneselenol, thiophenol and combinations. The signal % corresponds to the relative amount of modified antibody – either maleimide bridged in the case of dibromomaleimide or double alkylated when maleimide was used.

Interestingly benzeneselenol alone was able to cleave the anti-CEA cystine, as observed with insulin, indicating high reactivity of the cysteines involved. Thiophenol in contrast did not reduce the disulfide bond and enhanced the amount of disulfide cleavage in the presence of benzeneselenol only marginally. When maleimide was used to assess the extent of reduction, more modified material was observed than when dibromomaleimide was added. Possible explanations could be again accessibility issues, a faster interaction of the selenol- and thiol-nucleophiles with the halomaleimides in comparison to the unsubstituted compound, thereby reducing its efficient concentration or better ionisation of the alkylated compound resulting in a MS artefact.

2.8.2 Bridging of the anti-CEA disulfide bond

Sequential bridging of anti-CEA was straight forward; addition of an excess of dibromomaleimide (30 equiv) after reduction with DTT yielded the desired product with complete conversion within 5 min (no shorter times were tested) (Scheme 2.14 and Fig. 2.65). The intense and fast yellow colouring of the reaction mixture indicated that the remaining reducing agent was efficiently neutralised by the dibromomaleimide.



Scheme 2.14: Sequential bridging of anti-CEA with dibromomaleimide (**6**).

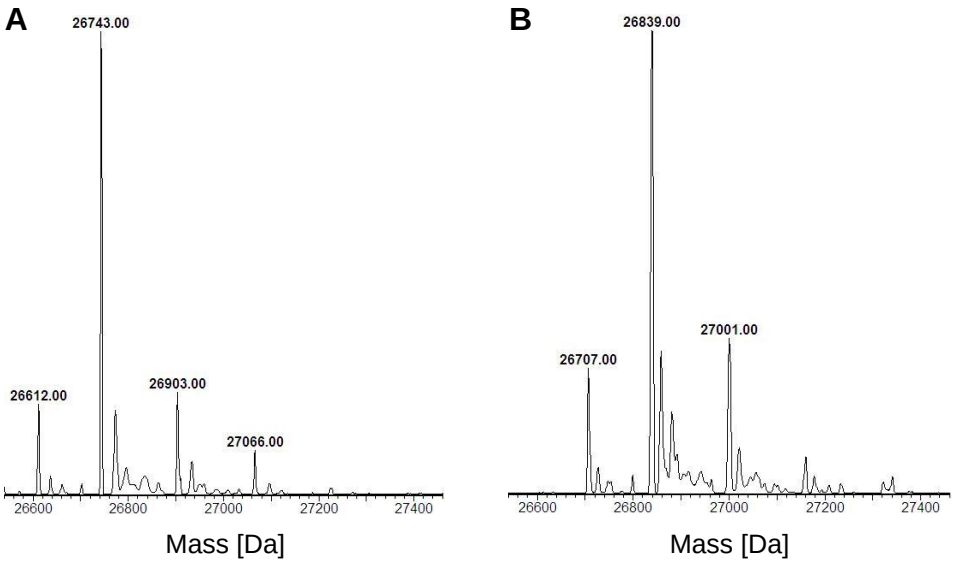


Fig. 2.65: LCMS spectra of the sequential bridging of anti-CEA. (A) Unmodified antibody fragment. (B) Maleimide bridged antibody fragment (requires 26,838 Da).

To establish an *in situ* protocol the ds-scFv was mixed with various amounts of dithiophenolmaleimide and benzeneselenol. Complete bridging was observed with 15 equiv of both compounds (data not shown). With increased quantities of the bridging reagent, solubility related issues were found. Following the reaction by LCMS revealed similar kinetics to the *in situ* modification of somatostatin and complete conversion was reached between 10 and 20 min (Fig. 2.66).

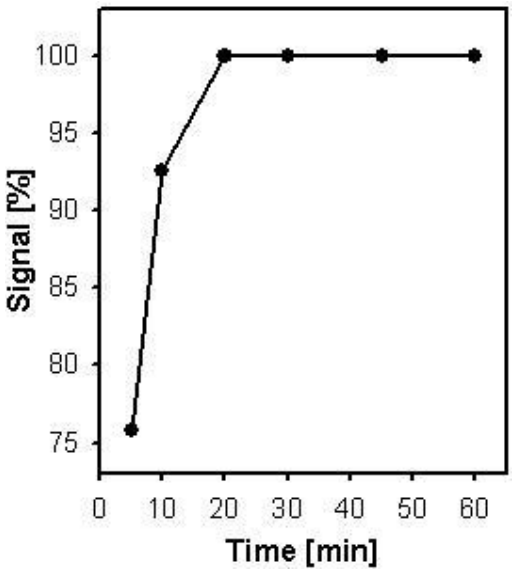


Fig. 2.66: Timed *in situ* bridging of anti-CEA. Conditions: 15 equiv dithiophenolmaleimide/ 15 equiv benzeneselenol, ambient temperature. The signal % corresponds to the relative amount of maleimide bridged antibody fragment.

To further reduce the amount of the potentially precious bridging reagent required, the anti-CEA ds-scFv was also reacted with varying mixtures of reagents where benzeneselenol was used in excess compared to dithiophenolmaleimide. It was possible to prepare fully bridged antibody *in situ* with as little as 2 equiv of the bridging reagent by addition of either 2 portions of 10 equiv of reducing agent (with 15 min reaction time each) or 25 equiv in a single addition-step (Fig. 2.67).

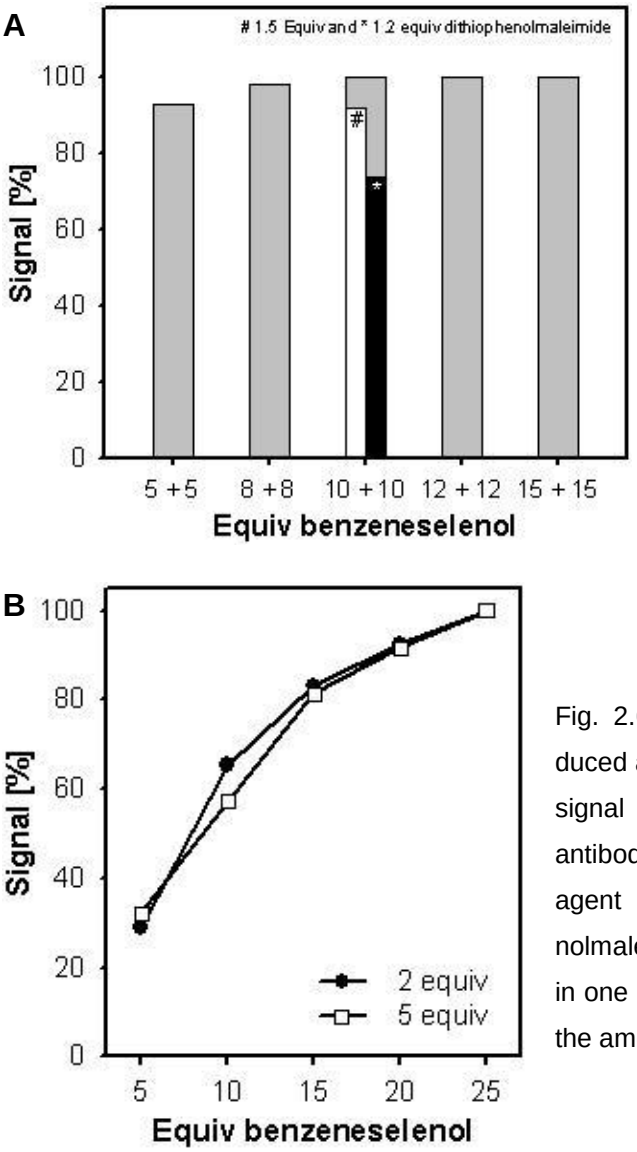
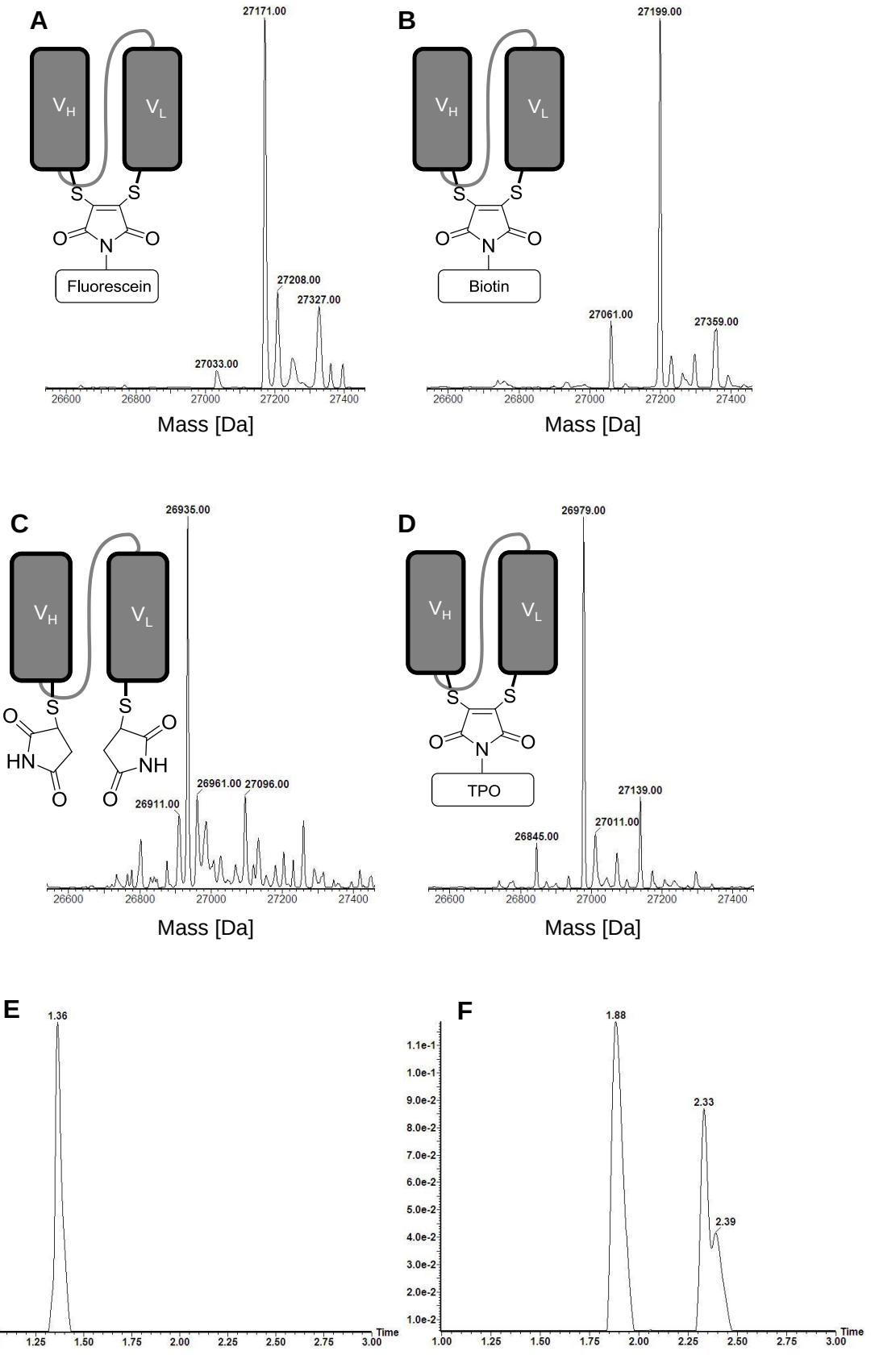


Fig. 2.67: *In situ* bridging of anti-CEA with reduced amounts of dithiophenolmaleimide **30**. The signal % corresponds to the maleimide bridged antibody fragment. (A) Addition of the reducing agent in two portions to 2 equiv of dithiophenolmaleimide. (B) Addition of the reducing agent in one portion. The indicated equiv correspond to the amount of bridging reagent used.

Addition of the same amount of benzeneselenol in two steps was in general more efficient than a single portion of reducing agent, highlighting once more the fast formation of the diselenide. Although reasonable conversion rates were also observed with 1.5 and 1.2 equiv bridging reagent, full modification was not achieved indicating the generation of some unreactive side products. Increasing the amount of dithiophenolmaleimide from 2 to 5 equiv, while keeping the concentration of reducing agent the same, had no impact on the conversion rate (Fig 2.67 B). This adds evidence to the theory that the rate limiting step during the *in situ* modification is not the bridging reaction but the cleavage of the disulfide bond (see 2.5.5).

2.8.3 Functionalisation of anti-CEA

Depending on which version of substituted maleimide had been synthesised, the anti-CEA ds-scFv was functionalised using the established methods. In this respect biotynilation (with *N*-biotin-dibromomaleimide **23**), alkylation (reaction of both cysteines individually with maleimide) and coupling to fluorescein (with *N*-fluorescein-dibromomaleimide **22**) was carried out via the sequential protocol. PEGylation (with *N*-PEG5000-dithiophenolmaleimide **31**) as well as spin labelling (with *N*-TPO-dithiophenolmaleimide **32**) was achieved *in situ*. LCMS and SDS-PAGE in the case of the PEGylated product revealed clean and complete conversion in all cases (Fig. 2.68).


A
B
C
D
E
F
V_H
V_L
Fluorescein
Biotin
TPO
27171.00
27208.00
27327.00
27033.00
27199.00
27061.00
27359.00
26935.00
26961.00
27096.00
26911.00
26979.00
27139.00
27011.00
26845.00
Mass [Da]
1.36
1.88
2.33
2.39
Time

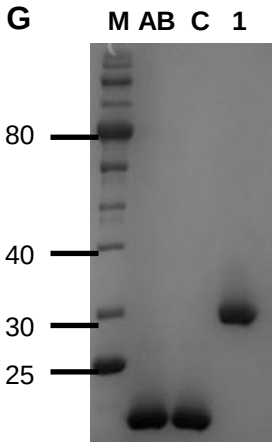


Fig. 2.68: Functionalisation of the anti-CEA antibody fragment. LCMS analysis of (A) anti-CEA-fluorescein (requires 27,172 Da). (B) Anti-CEA-biotin (requires 27,198 Da). (C) Alkylated anti-CEA (requires 26,937 Da). (D) Spin labelled Anti-CEA (requires 26,979 Da). (E) LC elution profile of anti-CEA. (F) LC elution profile of the desalted PEGylation reaction. The main peak (1.88 min) corresponds to the PEGylated antibody fragment and additional peaks (2.33 and 2.39 min) correspond to the *N*-PEG5000-dithiophenolmaleimide. (G) SDS-PAGE analysis of the PEGylation reaction. **M**) Marker. Molecular weight in kDa. **AB**) unmodified anti-CEA. **C**) Processed antibody control. **1**) PEGylation reaction.

A single desalting step or a nickel-resin based immobilised metal ion chromatography (IMAC) in the case of PEGylation afforded the clean and homogeneously modified products without any further purification in yields of 80–90%. Functionalisation via the sequential protocol was practically instantaneous as observed for the bridging with dibromomaleimide. The influence of the largest chemical group, the PEG chain, on the reaction speed of the *in situ* conjugation process was monitored by timed analysis and also found to be identical to that with dithiophenolmaleimide alone (Fig 2.69).

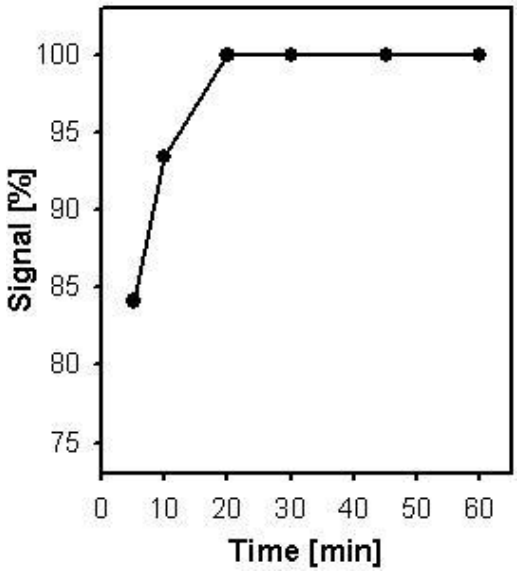


Fig. 2.69: Timed *in situ* PEGylation of anti-CEA. Conditions: 15 equiv *N*-PEG5000-dithiophenolmaleimide/ 15 equiv benzeneselenol, ambient temperature. The signal % corresponds to the PEGylated antibody fragment.

2.8.4 Comparison of sequential and *in situ* modification of anti-CEA

Before the prepared anti-CEA analogues were examined in further detail, the two methods that can in theory be used to synthesise the same derivative

should be briefly compared. Some differences can be concluded from the protocols themselves: Immediately obvious, the amount of substituted maleimide used in the *in* situ sequence is only 15 equiv in contrast to the 30 equiv necessary for the sequential protocol. In addition the *in situ* process holds the possibility to further decrease the concentration of the bridging reagent at the expense of the reducing agent, which usually will be the more inexpensive component. Also, despite the bridging reaction alone being faster in the sequential protocol (around 5 min), the long reduction time involved can be avoided when benzeneselenol is used. This reduces the overall time of the conjugation reaction to around 20 min when carried out *in situ*. On the other hand the neutralisation of the excess of DTT by the halomaleimide in the sequential protocol affords relatively water soluble side products and no precipitates have been found. These are, in contrast, formed by the dibenzeneselenide and over longer time frames by some of the dithiophenolmaleimides, and were observed in the *in situ* protocol. This could be an important issue when one of the processes has to be chosen for a scale up, for example in an industrial size production.

Prior to testing of the biological activity the monomeric state of the anti-CEA analogues had to be verified. This is important as the cleavage of the disulfide bond turns the anti-CEA ds-scFv for a limited time into a scFv with a strong tendency for multimerisation,[417] and the presence of any oligomers can have a significant impact on the binding properties of antibodies and their fragments.[431] Indeed, when analysed by non-denaturing SDS-PAGE a small amount of a higher molecular weight species was found in the sequential bridged sample but not in *in situ* bridged ones. FPLC analysis of the same material confirmed this observation (Fig. 2.70). Corresponding signals were also found in sequential functionalised anti-CEA analogues (e.g. Fig. 2.74) but not in *in situ* conjugated material (e.g. Fig. 2.68 G).

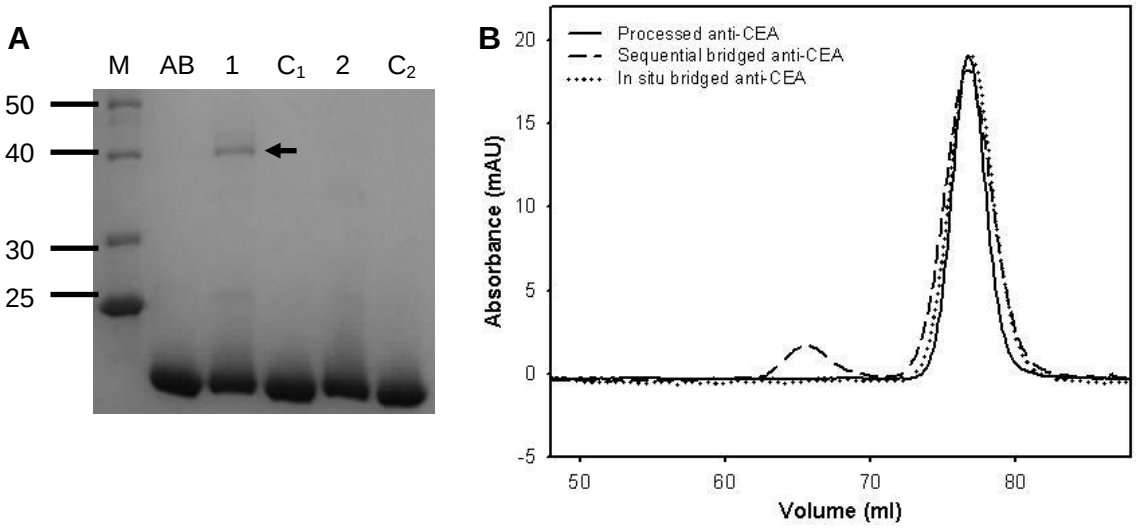


Fig. 2.70: Analysis of oligomer formation during the preparation of maleimide bridged anti-CEA. (A) Non-denaturing SDS-PAGE. 1) Sequential bridged anti-CEA. 2) *In situ* bridged anti-CEA. C_1) and C_2) are the corresponding processed controls. (B) FPLC traces of processed, sequential and *in situ* bridged anti-CEA.

During the production process of the anti-CEA ds-scFv in *E. coli* dimers are also formed. These are connected by intermolecular disulfide bonds (personal communication B. Tolner and data not shown). With the employed maleimides being able to bridge two free cysteines it is possible that the oligomers observed after modification were formed also through intermolecular maleimide bridges. Unfortunately, when testing for this, DTT was necessary to reduce any potential interfragment disulfide bonds but did also cleave any maleimide bridges (see 2.2.5). Thus the dimer signal was completely lost upon treatment of the bridged material with the reducing agent both in high salt and low salt conditions which were used to control the reactivity of the involved thiol groups (data not shown).[432] The weak MS traces that could be obtained form the dimeric form of the anti-CEA after the reaction revealed the presence of two maleimide units per dimer (data not shown). This suggested that if intermolecular linkages are formed all four cysteines are modified. Alternatively the correctly bridged cystines have been formed but the two antibody fragments are now interlocked similar to two links in a chain.

In conclusion these results clearly emphasise the usefulness of the *in situ* approach in avoiding the usually unwanted and often problematic formation of dimers when working with scFv fragments,[417] achieved through the rapid nature

of the reaction. Altogether the one-step modification process is clearly superior to the sequential protocol in this context.

2.8.5 Biological activity of anti-CEA analogues

Testing the influence of the inserted maleimide bridge and the accompanying functionalisation on the biological activity of the anti-CEA ds-scFv was carried out by enzyme-linked immunosorbent assay (ELISA) against full length human CEA. The experiment was performed with all prepared analogues of the antibody fragment (Fig. 2.71).

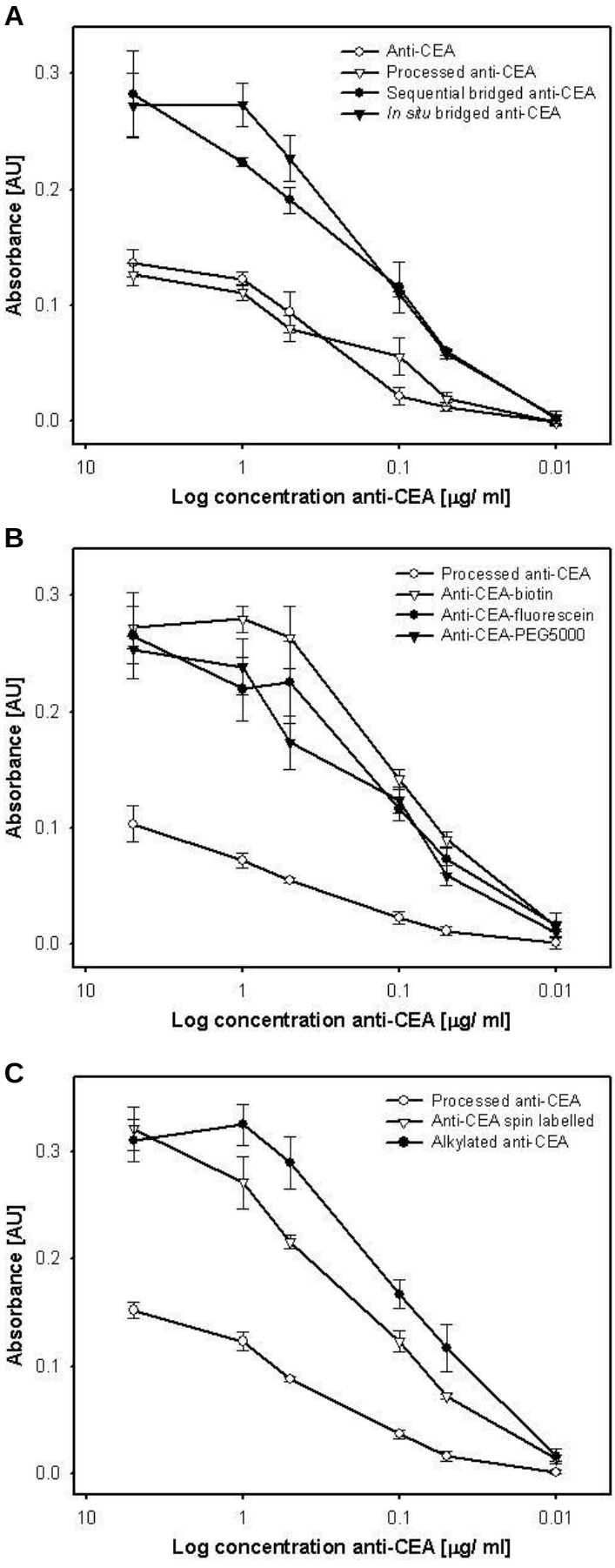


Fig. 2.71: ELISA of anti-CEA analogues and controls against full length human CEA. (A) Maleimide bridged anti-CEA. (B) Functionalised anti-CEA. (C) Functionalised and alkylated anti-CEA.

All modified versions of the antibody fragment showed consistently not only retention of the binding activity, but in fact an approximate 3x increase. This was found even in the case of the PEGylated analogue, despite frequent literature reports on the loss of activity upon polymer-conjugation to antibody fragments.[157] The effect could not have been caused by the presence of oligomers as the sequential and *in situ* bridged samples had similar activity. Interestingly the K_d of MFE-23 increased around 3x upon insertion of the disulfide bond[338] indicating that the additional covalent connection between the Ig domains had a negative impact on antigen recognition. This was supported by the observation that the double alkylated anti-CEA fragment, which basically has lost the cystine, exhibited a similar increase in binding activity as the bridged analogues. Potentially geometric strain had been introduced into the molecule through the disulfide bond, hindering the induced fit of the structure upon antigen binding. Evidence suggests that this is partially relived by the elongation of the cystine by the maleimide bridge.

A Biacore assay, performed by Dr Kim Vigor (UCL Cancer Institute), using a selection of anti-CEA analogues confirmed the overall increase in activity with the following K_ds: 20.8 ± 2.9 nM for the unmodified fragment, 6.4 ± 0.3 nM for the *in situ* bridged fragment and 8.7 ± 0.3 nM for the *in situ* PEGylated anti-CEA.

2.8.6 Functionality of anti-CEA analogues

Chemically modified scFvs and other stabilised antibody fragment formats are employed as recognition and quantification elements in a multitude of research and diagnostic applications. A few of these scenarios were used to test the functionality of the synthesised anti-CEA analogues.

Fluorescently labelled antibodies targeting CEA have been prepared for ADEPT to support surgery,[425] to analyse microarrays[427] and coupled to nanoparticles in the process of the development of new bioimaging techniques.[428] As the antibody is usually present only in very small amounts a strong fluorescence of the conjugate is important. The fluorescence intensity of the anti-CEA-fluorescein analogue was thus measured. It was found to be high even at nanomolar con-

centrations despite the presence of a single fluorophore unit per antibody (Fig. 2.72). Currently employed conjugation methods target lysine residues and thus often couple more than one imaging reagent to each individual antibody.

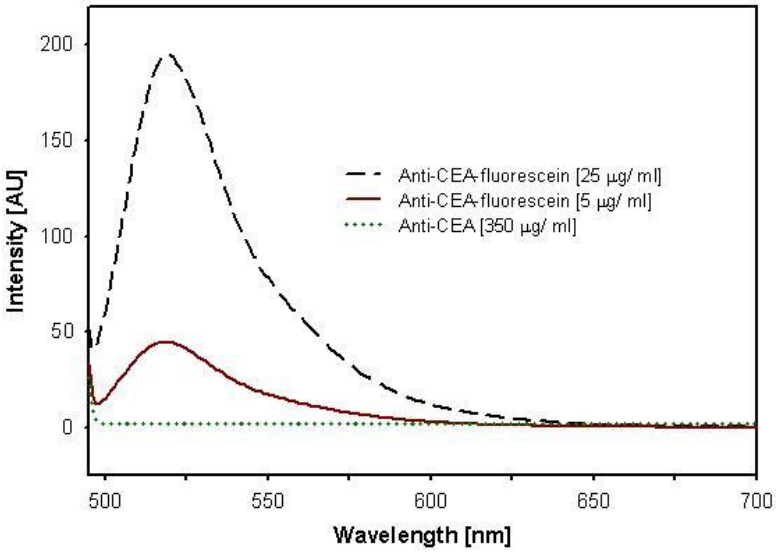


Fig. 2.72: Fluorescence of anti-CEA-fluorescein. Excitation wavelength = 488 nm.

To test the combination of antigen-binding activity and fluorescence, dilution series of the CEA-expressing cell line CAPAN-1[433] and the CEA-negative cell line A375[434] were prepared alongside suitable controls and treated with the *N*-fluorescein-maleimide bridged ds-scFv. Data acquisition with a fluorescence-ELISA reader after removal of non-binding antibody revealed specific binding activity and a good correlation of the signal intensity to the theoretical number of cells per sample (Fig. 2.73).

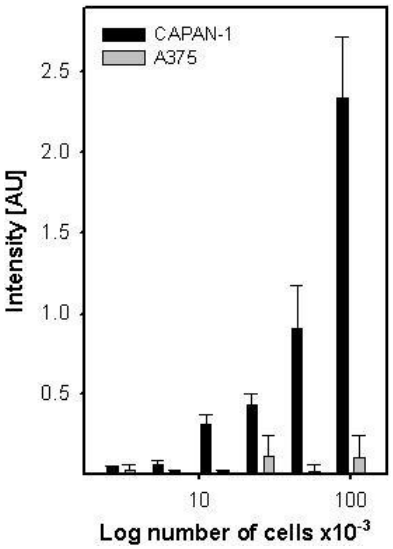
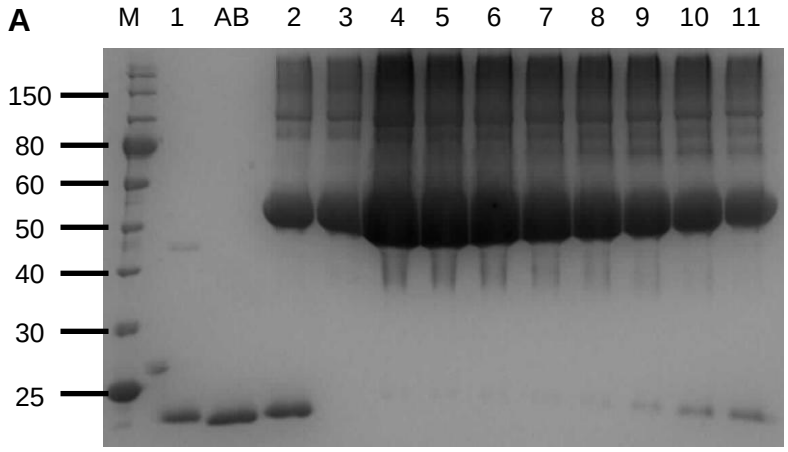


Fig. 2.73: Fluorescence read-out after the treatment of dilution series of CEA-expressing (CAPAN-1) and non-expressing (A375) cell lines. Excitation wavelength = 488 nm.

As few as 8,000 cells could be reliably detected in this simple experimental layout. The signal to cell number correlation, enabled by the homogeneous product of the labelling-reaction, suggests the potential for an easy quantification method of antigen expressing cells in bulk samples.

Similar to fluorescence labelling, coupling of antibody fragments to biotin followed by addition of a (strept-)avidin labelled enzyme is a very common technique, particularly for the use with the enzyme horseradish peroxidase (HRP).[435] This direct coupling of the primary antibody to the signalling entity as opposed to using a labelled secondary antibody is desirable in a number of applications, including competition assays and same-species immunohistochemistry.[375] The nature and length of the linker plays an important role due to the steric bulk of the enzyme, which can interfere with the antigen-interaction. A loss of binding activity of the antibody is thus often observed even when site-specific cysteine labelling is used as the method for chemical coupling.[371, 436]

To understand the performance of the developed maleimide chemistry against this background, the anti-CEA-biotin antibody fragment was mixed with increasing amounts of a commercially available horseradish peroxidase-streptavidin conjugate (HRP/STREP), and the putative anti-CEA-biotin-streptavidin-horseradish peroxidase (in short anti-CEA-HRP) complexes were purified via immobilised metal ion affinity chromatography (Fig. 2.74).



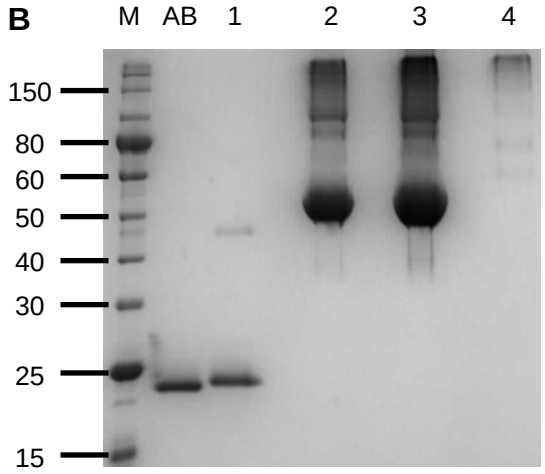


Fig. 2.74: Preparation of the anti-CEA-HRP conjugate. (A) Synthesis. 1) Anti-CEA-biotin. 2) Mix of unmodified ds-scFv and the HRP/STREP conjugate. 3) HRP/STREP conjugate. 4) Mix of 15 µl of biotin-sscFv (20 µM) with 15 µl, 5) 12 µl, 6) 10 µl, 7) 8 µl, 8) 6 µl, 9) 4 µl, 10) 2 µl and 11) 1 µl of HRP/STREP solution (1.25 mg/ ml). (B) Purification. 1) Anti-CEA-biotin. 2) HRP/STREP conjugate. 3) Mix of anti-CEA-biotin with a 3x excess (by mass) of HRP/STRP. 4) Purified anti-CEA-HRP.

SDS-PAGE analysis of the HRP/STREP conjugate showed that the commercial available material was highly heterogeneous with a large amount of unconjugated HRP (band at around 50 kDa) present. Varying the ratios of anti-CEA-biotin to the HRP/STREP conjugate revealed that all of the antibody fragment was consumed through binding of the biotin moiety to the available sites of the streptavidin, when a 3x excess by mass of the enzyme conjugate was added. The resultant anti-CEA-HRP complexes could be isolated as high molecular weight species from the coupling mixtures by IMAC.

As the molecular composition of these species could not be further identified the material was characterised by its activity. To this end, a dilution series of an anti-CEA-HRP solution with a defined OD_{280} were subjected to the substrate conversion reaction, alongside the HRP-coupled secondary antibody used in the established ELISA and the HRP/STREP conjugate (data not shown). A good signal intensity was obtained with a 1 : 500 dilution of an OD_{280} = 0.4 solution of the anti-CEA-HRP, which was subsequently used as a reference.

The applicability of both the anti-CEA-biotin and anti-CEA-HRP conjugates was tested in modified versions of the activity ELISA (Fig. 2.75).

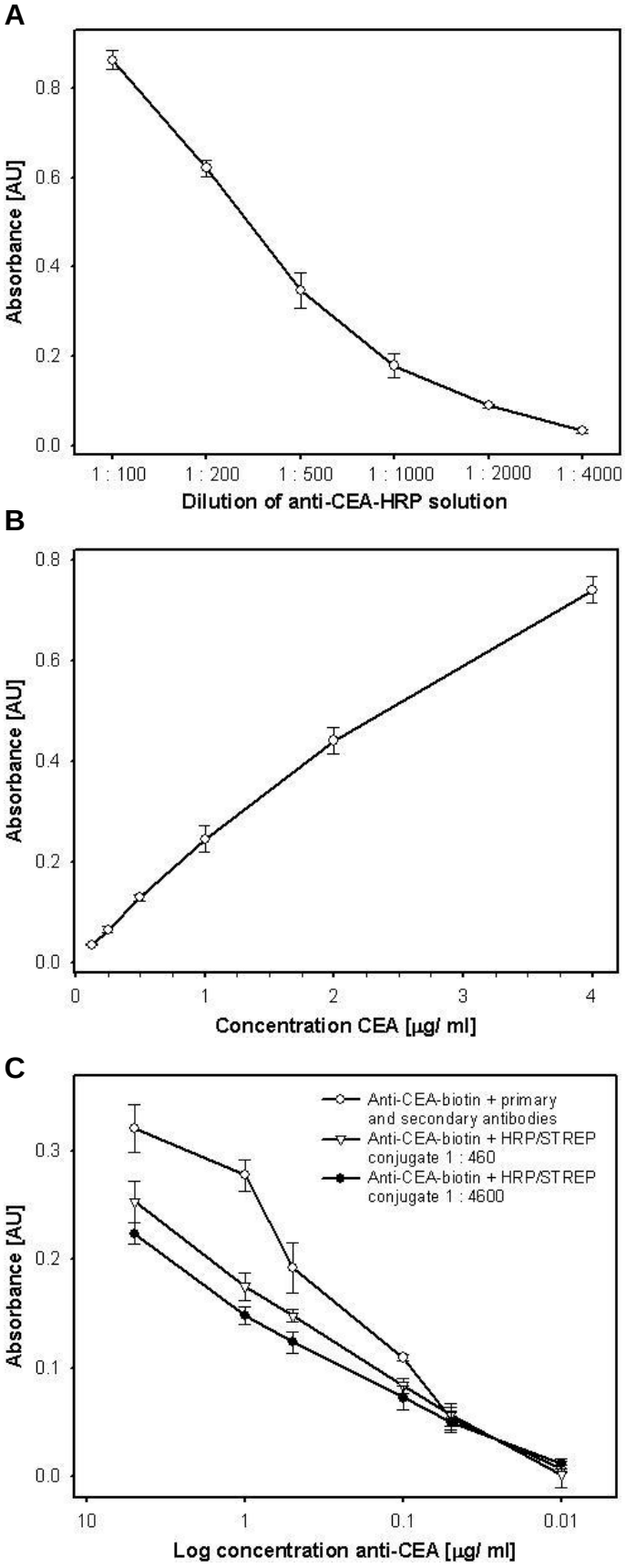


Fig. 2.75: Activity of anti-CEA-HRP conjugates. (A) One-step ELISA with various dilutions of the anti-CEA-HRP conjugate. (B) One-step ELISA against varying amounts of antigen. (C) Two-step ELISA with on-plate formation of the reporter complex. No background signal was observed with the 1 : 4600 dilution.

First ELISA plates coated in a constant amount of antigen were treated with different dilutions of the standardised anti-CEA-HRP solution, which confirmed previous observations. Then the amount of antigen was varied in combination with the 1 : 500 dilution of the anti-CEA-HRP solution and good signal linearity over a range of CEA concentrations was found. These two experiments were carried out as one-step ELISAs where antigen detection is facilitated by the addition of only a single agent. This reduces the amount of expended materials, the total assay time and washing steps, as well as possible sources of error. In order to demonstrate the versatility of the system and to reduce the amount of consumed HRP/STREP conjugate, a two-step ELISA was also performed. In this case, the anti-CEA-biotin analogue was incubated with CEA coated 96-well plates, following which the HRP/STREP conjugate was added in different dilutions. Initially, more concentrated mixtures in PBS resulted in a very strong background signal, which could be eliminated by increasing the dilution in blocking buffer. This showed that the binding of streptavidin to the antibody-coupled biotin is robust under different conditions, in addition to reducing the amount of consumed HRP/STREP conjugate to 2.1 µl per 96-well plate. This ELISA format also allows the reporter system to be changed readily, as the varying connection is now the available streptavidin-enzyme, and not the expensive antibody-enzyme link.

Although not an imaging reagent, PEGylation is a common modification of antibody fragments, often used to increase their short circulation half-lifes.[169] To compare the introduced disulfide-based conjugation, the ds-scFv was modified with 5 kDa PEG chains via the traditional method of lysine targeting. PEGylation was carried out with a NHS-ester activated polyethylene glycol polymer, using varying amounts of the compound and different reaction times inspired by published procedures.[162, 166] The aims here were to either produce mainly single-PEGylated antibody fragments or to reach full conversion with the reaction as achieved with the substituted maleimides (Fig. 2.76).

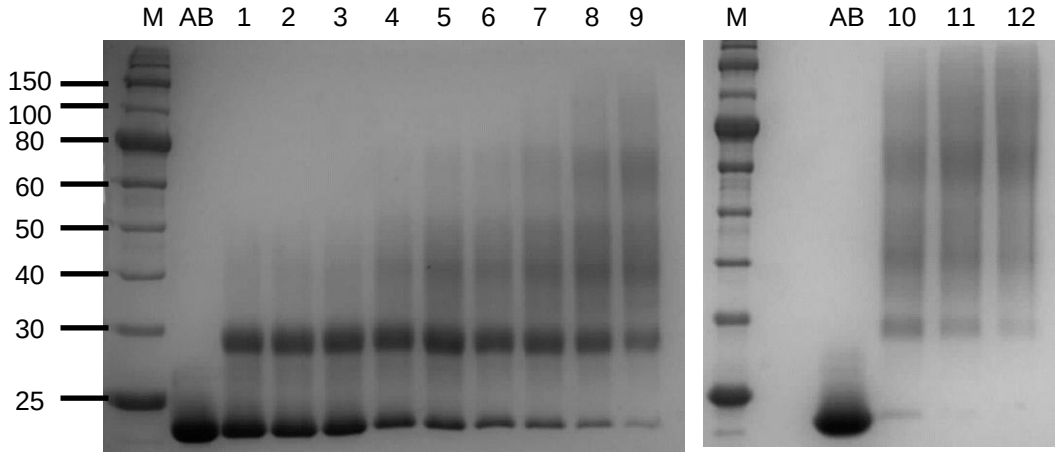


Fig. 2.76: Lysine based PEGylation of anti-CEA. 1) 2 equiv NHS-PEG5000/ 30 min. 2) 2 equiv/ 2 h. 3) 2 equiv/ 4 h. 4) 5 equiv/ 30 min. 5) 5 equiv/ 2 h. 6) 5 equiv/ 4 h. 7) 10 equiv/ 30 min. 8) 10 equiv/ 2 h. 9) 10 equiv/ 4 h. 10) 12 equiv/ 4 h. 11) 15 equiv/ 4 h. 12) 18 equiv/ 4 h.

SDS-PAGE analysis revealed an expected reaction pattern; the conjugation was relatively fast and small amounts of reagent produced mainly mono PEGylated antibody species but in poor yield. The conversion rate was increased with more equivalents of the NHS-PEGylation reagent, however the overall heterogeneity of the product also increased, appearing as a “ladder” of multiple PEGylated ds-scFvs on the gels. Full conversion was achieved with 18 equiv of the NHS-PEG reagent yielding a mixture of antibody fragments with an estimated 1 to 6 polymers attached (anti-CEA itself contains 15 lysine residues). The fully modified product was purified via IMAC as well as the mono-PEG antibody fragment via SEC (in yields of 28% and 14% respectively). The activity of these analogues was compared by ELISA to the unmodified antibody and its mono-PEG version prepared using the developed disulfide-bridging methodology (Fig. 2.77).

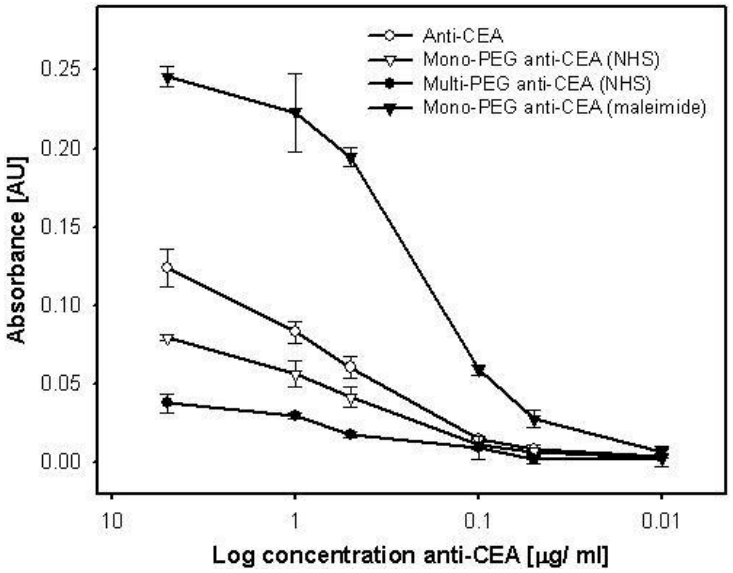


Fig. 2.77: ELISA of variously PEGylated anti-CEA analogues against full length CEA.

Both randomly modified antibody fragments were clearly inferior to the site-specific PEGylated species. The mono-PEG fragment produced with NHS-chemistry had lost around 40% of its activity, which might be a result of direct blocking by conjugation to lysines in the CDR loops or indirect blocking through the polymer being attached close to the binding site. The sample produced by the complete conversion conditions exhibited almost no activity. Again likely to be due to the same but exaggerated effects. It is also possible that the antibody fragments with higher polymer-loading are completely inactive and the residual binding observed here is entirely based on the presence of a small amount of the mono, double and perhaps triple PEGylated species. Overall these results are a perfect example to highlight the importance of site-specificity of bioconjugation reactions.

Spin labelled antibodies and antibody fragments are of specific interest for the development of new biosensors. The tumbling motion of these conjugates should in theory change significantly upon binding of the antigen. This effect would be visible in the EPR spectra of a spin label attached to an antibody (or indeed an antigen). As the differential signal would depend on the population of the antigen-antibody complex, the concentration of antigen in a sample could be determined by addition of a known amount of spin labelled antibody in excess. Any attempts to realise this concept in the literature have been thus far unsuccessful.[437, 438] The commonly used conjugation chemistry allows the spin label too much segmental mobility[54] and uncouples the motions of protein and label.

Ultimately the rotation of a protein can only be determined if the label has two points of attachment.[231] Bridging an antibody disulfide bond with a spin label would be ideal to create a very rigid connection between the two molecules, and although such compounds have been prepared, this was not by targeting native disulfide bonds.[232, 233] To investigate this possibility the anti-CEA fragment was reacted *in situ* with *N*-TPO-dithiophenolmaleimide and after purification EPR spectra in or without the presence of NA1, a fragment of CEA comprising its two most N-terminal Ig domains, were recorded (Fig. 2.78). This experiment was performed with Mr Vishal Sanchania (UCL Institute for Structural and Molecular Biology, ISMB).

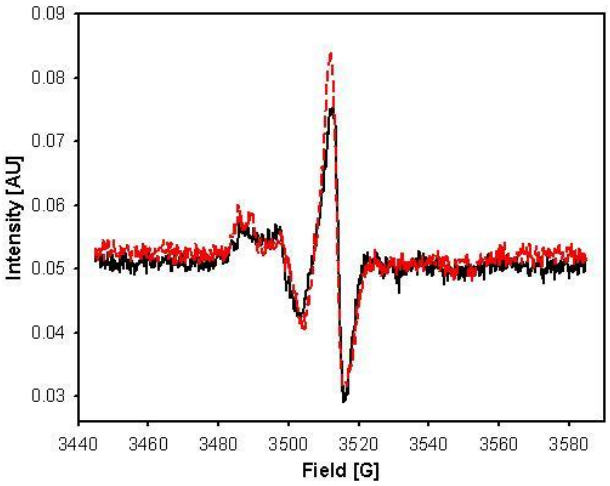


Fig. 2.78: EPR spectra of spin labelled anti-CEA only (black trace, 10 µM) and in the presence of antigen NA1 (red trace, 128.3 µM).

The spectra of the labelled antibody fragment were, taking into account the low concentration of the protein, as clear as observed for spin labelled somatostatin (see 2.4.2). Notably the 3rd line, indicative for the motion of nitroxide radicals,[439] was almost not visible. More important substantial changes in the line shape were observed upon addition of the antigen, here in saturating amounts, in comparison to when BSA was added as a control (data not shown). Furthermore, similar changes were found when antibody and antigen were mixed in human plasma as well as in full human blood (data not shown). This is of special interest as not only is there no system to date using optical detection that works in opaque solutions such as blood, but also because spin labels are usually highly instable in this medium due to reduction by ascorbate.[440] Taken together spin labels based on substituted maleimides might be the key to turn the theory outlined above into reality and thus enable the development of EPR-based immunosensors.

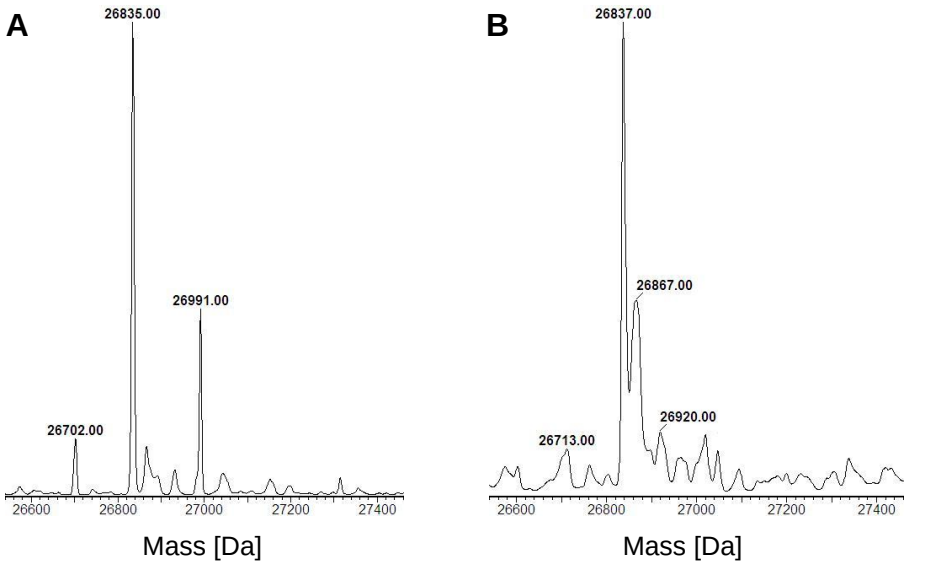
It should be mentioned that Mr Vishal Sanchania (UCL ISMB) has developed these findings further with great success culminating in the filing of a patent related to disulfide bond-based spin labelling.[441]

2.8.7 Stability of the maleimide bridge in anti-CEA

An important aspect in the development of new bioconjugation strategies is the stability of the introduced chemical bonds under the various conditions of potential applications, including simple storage. Thus a set of experiments were carried out with modified anti-CEA antibody fragments to explore this parameter for maleimide-based disulfide bond bridging.

As a first very basic test, the bridged and PEGylated ds-scFv were synthesised and stored in PBS at 4 °C for a week mimicking storage conditions. Then the same material was prepared again and its activity tested alongside the aged samples by ELISA (data not shown). The activity observed was virtually identical, suggesting good resistance to aggregation, degradation and also full solubility of the anti-CEA analogues.

The next step was to examine the stability of the maleimide bridge in a setting closer to potential *in vivo* applications. To this end the maleimide bridged ds-scFv was incubated alongside suitable controls in human plasma at 37 °C for 7 d and aliquots analysed by SDS-PAGE and LCMS at various time points, after purification via IMAC (Fig. 2.79).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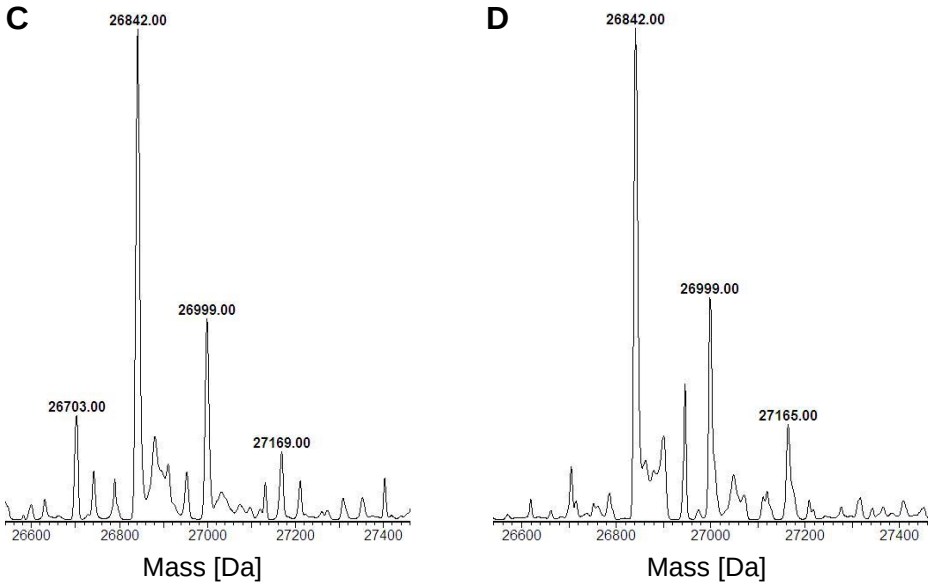
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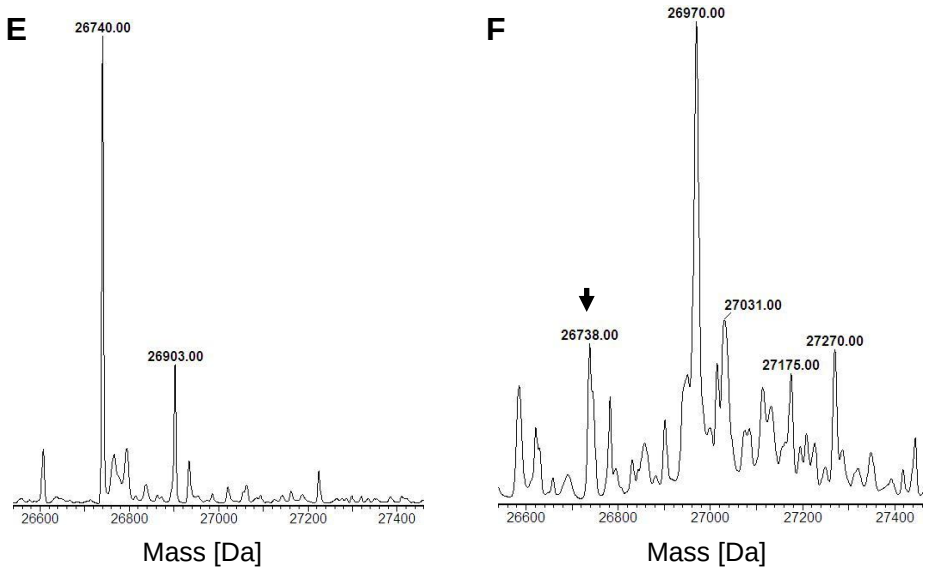
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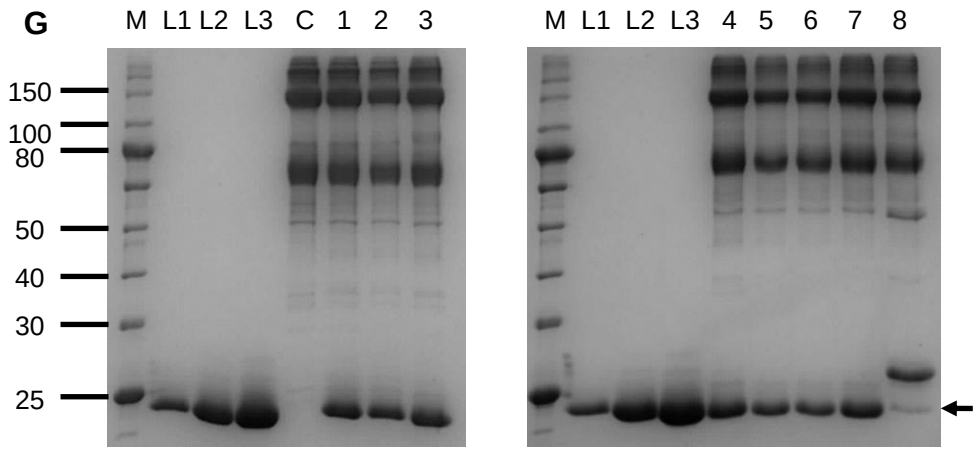


Fig. 2.79: Stability of the maleimide bridge in human plasma. (A) Maleimide bridged anti-CEA isolated after 1 h, (B) 24 h, (C) 3 d and (D) 7 d. The increase in molecular weight is potentially based on the inability of the deconvolution software to separate non-hydrolysed from hydrolysed species. (E) Unmodified anti-CEA after 7 d. (F) Alkylated anti-CEA after 7 d. The increase in molecular weight corresponds to both succinimide units being hydrolysed (requires 26,973 Da). (G) SDS-PAGE analysis. L1) Loading control of 1 µg, L2) 3 µg and L3) 5 µg of unmodified anti-CEA. C) IMAC purification of human plasma without antibody present. 1) Maleimide bridged anti-CEA after 1 h, 2) 4 h, 3) 24 h, 4) 3 d, 5) 5 d and 6) 7 d. 7) Unmodified anti-CEA after 7 d. 8) Alkylated anti-CEA after 7 d.

Relatively constant amounts of the antibody fragment could be isolated from the plasma samples as compared by loading controls on SDS-PAGE, with some losses occurring after 5 d. Similar observations were made in the case of the unmodified and alkylated controls. Interestingly, this last control contained some alkylated material that had lost both succinimides (black arrows) and even re-formed the disulfide bond, as indicated by the lower running band on the gel (proteins with intact disulfide bonds move faster during PAGE due to a more compact shape).[199] LCMS analysis of the isolated sample material revealed that this was not the case when a maleimide had been inserted into the cystine, as the modified antibody fragments were intact even after 7 d, except for some potential hydrolysis occurring. This demonstrates that the maleimide unit is more stable than the succinimide, thereby highlighting a crucial advantage of the substituted maleimides. Even more important, this shows that the bioconjugates prepared by cystine-bridging are stable towards the free thiol content of human blood in the form of small molecular weight compounds such as GSH (around 18 µM) and free cysteines on the surface of plasma proteins (up to 500 µM).[82, 83]

To test if cleavage of the maleimide bridge was still possible, as observed with somatostatin (see 2.2.5), the maleimide bridged anti-CEA analogue was treated with a 100x excess of 2-mercaptoethanol, DTT or GSH (Fig. 2.80).

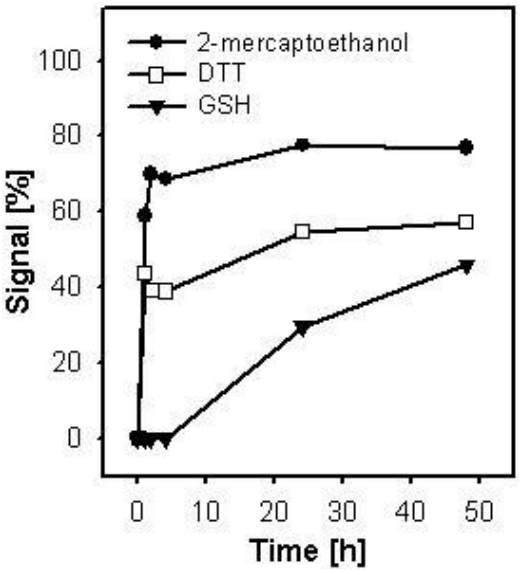


Fig. 2.80: Stability of maleimide bridged anti-CEA against various reducing agents. The signal corresponds to the un-bridged antibody fragment.

Indeed the regeneration of the unmodified antibody fragment could be observed but was relatively slow and did not reach completion in the experimental time window. Potentially the maleimide inserted into the cystine is protected by the steric bulk of the surrounding protein structure, which restricts the access of the reducing agent. The data suggests that such conjugates would be stable even towards the high intracellular concentrations of GSH for a few hours, enabling *in vivo* imaging and similar in-cell applications.

Although the performed plasma experiment had shown that the substituted maleimide-based modification is stable, the exact quantity and biological functionality of this material after incubation in human plasma was not clear. To address this question a similar test was set up where the aliquots removed over time were diluted in PBS, and their relative antigen-binding capacity analyzed by ELISA (Fig. 2.81). The assay was performed with unmodified, maleimide bridged, PEGylated and alkylated antibody analogues.

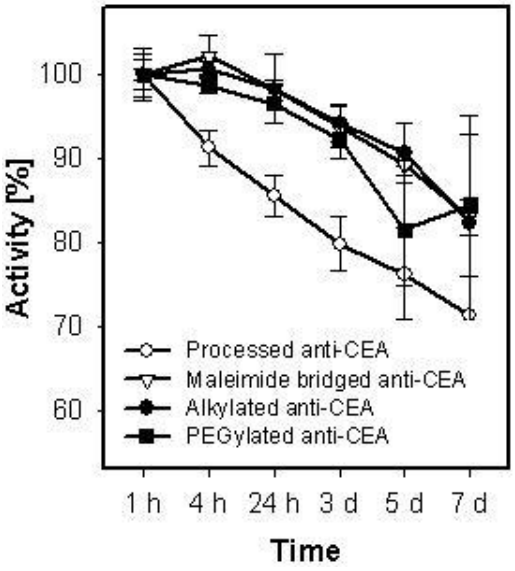
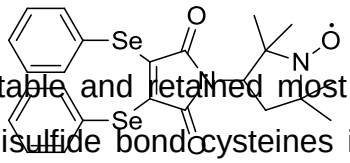


Fig. 2.81: Relative activity of various anti-CEA analogues after incubation in human plasma.



In general the ds-scFv itself was very stable and retained most of its activity after 7 d at 37 °C. Modification of the disulfide bond cysteines increased the stability but this was independent of their re-connection as the alkylated analogue exhibited the same properties. This implies a role of the cystine in the pathways that lead to a loss of binding capacity for example, potential participation in the formation of aggregates or mixed disulfides with plasma proteins. PEGylation of the anti-CEA fragment appeared to have no influence on its resilience. However, the main factors where the polymer contributes to stability, protection from proteolytic degradation and reduced renal clearance, had not been tested for in the assay. Overall the findings of the initial plasma test were mirrored relatively well with more than 95% activity preserved over the first 3 days, followed by a more pronounced loss of antigen-binding over the next 4 days. Taken together this suggests that the maleimide bridge is stable in human plasma, that the conjugates, which are intact, are active, that these are the majority of the material present and that the slow but gradual loss of protein and its activity is not correlated to the presence of the maleimide.

Overall the modification of the anti-CEA ds-scFv antibody fragment was straight forward and simple to optimise. Process properties including; quantitative conversion, fast and site-specific reaction, easy purification and protocol versatility, full biological activity and functionality of the homogeneous products, and prevention of oligomerisation, promise great potential of the developed platform in

the field of antibody-conjugation. In the light of this success, modification of full antibodies rather than their fragments was thus the next target.

2.9 Modification of anti-FLAG antibodies

As first models to transfer the methods of maleimide-based modification to full length antibodies the M1 and M2 clones of the anti-FLAG tag mouse antibody were chosen. These antibodies are robust, have a defined binding activity (to the FLAG tag, an artificial octapeptide)[341] and are commercially available. They have different isotypes which is IgG1 in the case of M2 with 2 disulfides in the hinge region, and IgG2b in the case of M1 that comprises four disulfide bridges in the hinge region.[311] The light chains have a molecular weight of approximately 23 kDa and the heavy chains 52 kDa adding up to 150 kDa of the intact complex.

2.9.1 Bridging of the M2 anti-FLAG antibody

To determine whether disassembly of the cystine connected complex can be observed, various amounts of TCEP were added to the M2 clone and the mixture incubated on ice over night. Analysis was performed by SDS-PAGE as the SDS disrupts the strong non-covalent interactions between the 4 peptide chains, enabling their separation after reduction of the cystines by electrophoresis. Cleavage of more than one disulfide bridge (the reduction of a single cystine in the hinge region would not result in isolated antibody chains) was observed only when the glycerol was removed from the buffer by dialysis prior to experimentation (see 4.2.2) and 20 equiv or more reducing agent were used (Fig. 2.82 lanes 1 and 2). The addition of equal amounts of dithiophenolmaleimide before the reducing agent completely prevented the structural loss of the antibody in the case of 20 equiv of the phosphine and to a large extent when 50 equiv of TCEP were used (Fig. 2.82 lanes 3 and 4).

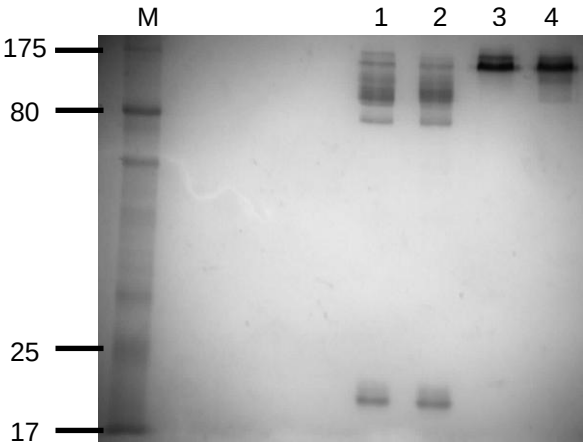


Fig. 2.82: Dithiophenolmaleimide **30** protects the M2 anti-FLAG antibody from reductive disassembly. 1) 20 equiv TCEP. 2) 50 equiv TCEP. 3) 20 equiv TCEP and 20 equiv **30**. 4) 50 equiv TCEP and 50 equiv **30**.

These results suggest that the antibody disulfide bridges are relatively stable when compared to the disulfide bonds in somatostatin and insulin, as greater amounts of TCEP are necessary to open them. The observation that mixing the antibody with dithiophenolmaleimide and the phosphine led to the retention of the full antibody structure can be a result of successful bridging but also due to the inactivation of the reducing agent by cross-reaction with the maleimide.

2.9.2 Functionalisation of the M2 anti-FLAG antibody

In order to obtain evidence for the successful modification of the antibodies' disulfide bonds *N*-PEG5000- **31** and *N*-TPO-dithiophenolmaleimide **32** were used with both *in situ* modification protocols (TCEP and benzeneselenol) to prepare PEGylated and spin labelled anti-FLAG antibodies. Unfortunately no signal was obtained by EPR spectroscopy with the potentially modified material. This was likely to be due to the extremely low concentration of protein that was subjected to analysis. The protein was supplied in 6.3 µM which decreased further by repeated dialysis (removal of glycerol, removal of excess spin label) and a weakness of the EPR method is its low sensitivity.

In contrast PEGylation of the target protein was observed by MALDI-TOF MS (Fig. 2.83) and SDS-PAGE (Fig. 2.84).

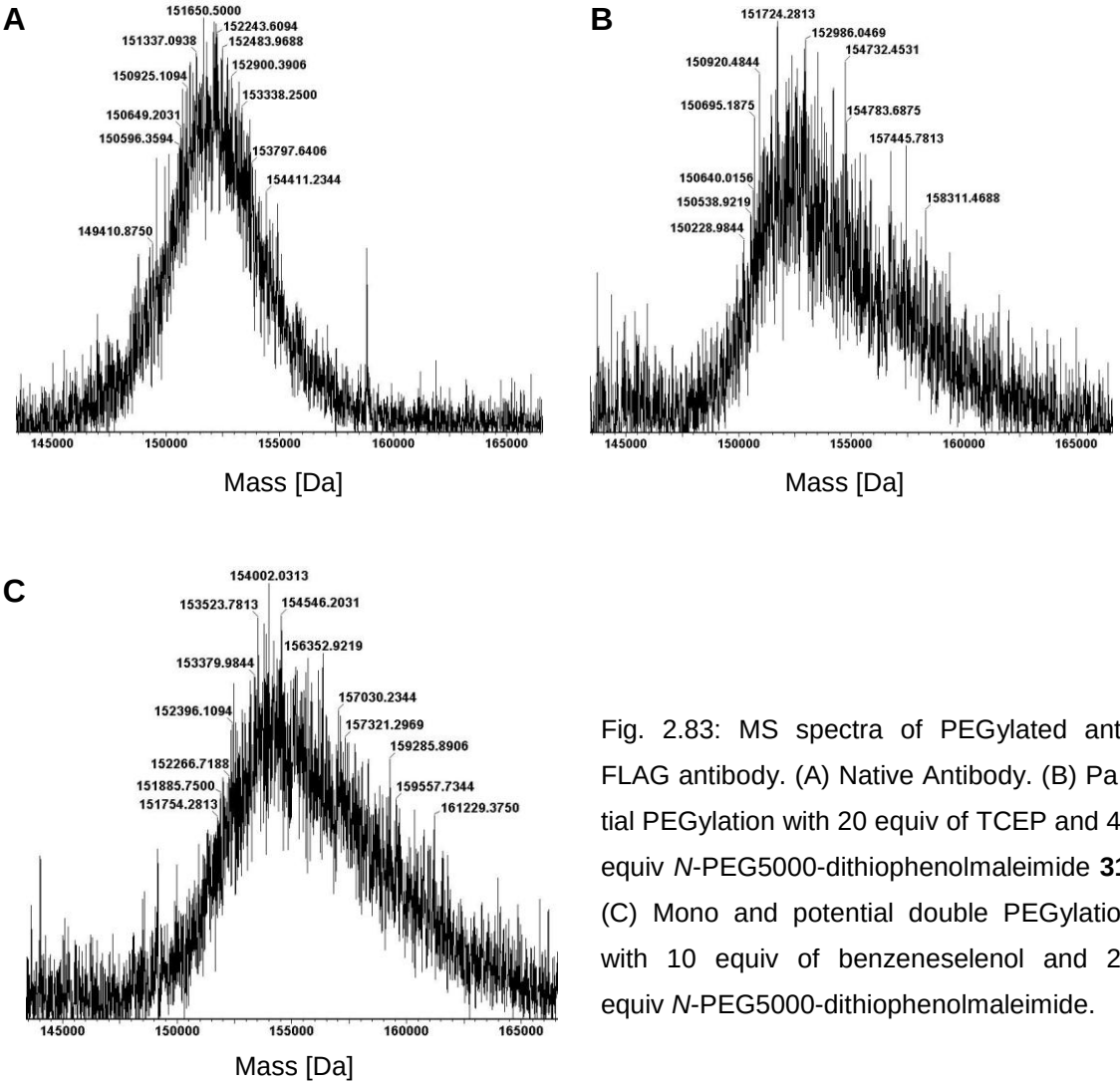


Fig. 2.83: MS spectra of PEGylated anti-FLAG antibody. (A) Native Antibody. (B) Partial PEGylation with 20 equiv of TCEP and 40 equiv *N*-PEG5000-dithiophenolmaleimide **31**. (C) Mono and potential double PEGylation with 10 equiv of benzeneselenol and 20 equiv *N*-PEG5000-dithiophenolmaleimide.

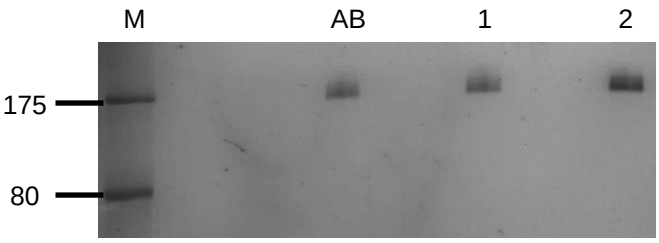


Fig. 2.84: PEGylation of the anti-FLAG antibody. 1) 20 equiv of TCEP and 40 equiv *N*-PEG5000-dithiophenolmaleimide **31**. 2) 10 equiv of benzeneselenol and 20 equiv *N*-PEG5000-dithiophenolmaleimide.

Due to the small mass of the PEG chain compared to the antibody only a slight shift in the positions of the protein in the SDS-PAGE was observed but the MS data supported that modification had occurred. An additional shoulder peak was visible to the right of the mass signal of the native antibody when TCEP was used as a reducing agent. This peak had an increased mass by approximately 5 kDa suggesting the presence of mono PEGylated antibody in the reaction mixture. If selenol was used instead of the phosphine, movement of the base

peak to a position approximately 4.5 kDa higher was observed with an additional shoulder of around 8–10 kDa higher indicating that efficient mono and double PEGylation had occurred.

Although some modifications were observed the data was very noisy and no EPR signal could be obtained. In order to omit the dialysis steps necessary to remove the glycerol from the solution and thus prepare quantitatively more modified material for analysis, both anti-FLAG clones were purchased in solutions containing sodium azide (0.02%) as a preservative instead of glycerol.

2.9.3 *In situ* bridging of the M2 anti-FLAG antibody

To establish if TCEP would be sufficiently active in the presence of azides a reduction study was performed with both antibodies (Fig. 2.85).

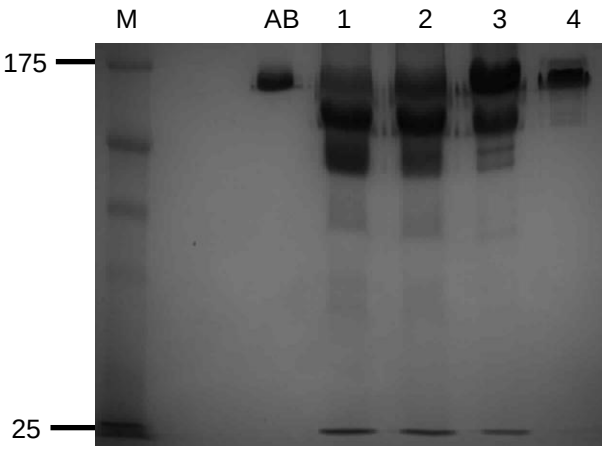


Fig. 2.85: Reduction of M2 with TCEP in azide solution. 1) 480 equiv TCEP. 2) 240 equiv TCEP. 3) 20 equiv TCEP. 4) 20 equiv TCEP in the glycerol containing buffer.

This experiment clearly shows that the glycerol has not only led to low process yields, but has also hampered the reduction of the antibody disulfide bonds. In contrast the presence of the azide was relatively well tolerated and separation of the antibody chains was observed without prior buffer exchange. A similar picture was obtained with the M1 clone (data not shown). In both cases 100 equiv of TCEP cleaved most of the cystines.

To confirm also the compatibility of dithiophenolmaleimides **30**, **31** and **32** with the new preservative, *in situ* bridging of the M2 antibody was performed (Fig. 2.86).

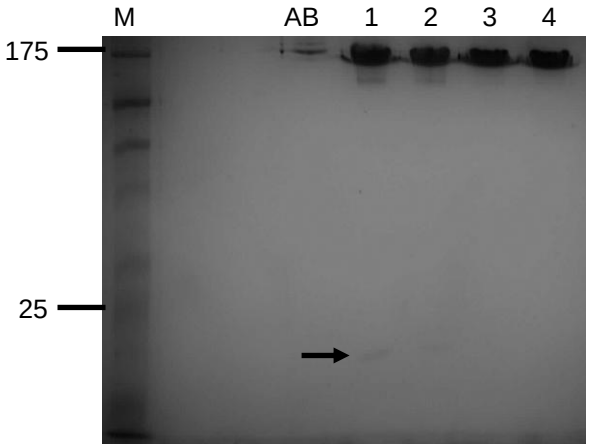


Fig. 2.86: *In situ* bridging of M2 with TCEP in azide solution. The arrow indicates the position of free L chains. Equimolar amounts of TCEP and dithiophenolmaleimide **30** were used. 1) 120 equiv. 2) 60 equiv. 3) 20 equiv. 4) 10 equiv.

The reaction of equimolar amounts of reducing and bridging reagent, which this time was carried out at ambient temperature for only 1 h, looked very promising by SDS-PAGE analysis. Only very faint signals for the light chains (black arrow) from the complex were observed, which corresponded to the appearance of bands in the region of HHL antibody fragments. No disassembly of full antibodies was observed when small amounts of TCEP were used.

2.9.4 *In situ* spin labelling of anti-FLAG antibodies

Again to obtain physical proof that a modification reaction had taken place an attempt was made to spin label the M1 and M2 antibodies. To confirm the stability of the spin label during the reaction *N*-TPO-dithiophenolmaleimide **32** was mixed in the antibody buffer with an excess of sodium azide, which represented the concentration of the preservative in the solution (~500x) and incubated with and without TCEP (100x excess over the label) at ambient temperature. The constantly recorded EPR spectra showed no loss of signal intensity over a 2 h period – the maximum time the label would be exposed to these conditions (data not shown).

Both antibodies were then reacted with 100 equiv of TCEP and spin label **32**. The excess of label, as well as the azide solution, was carefully removed by repeated desalting and the concentrated samples analysed by EPR (Fig. 2.87).

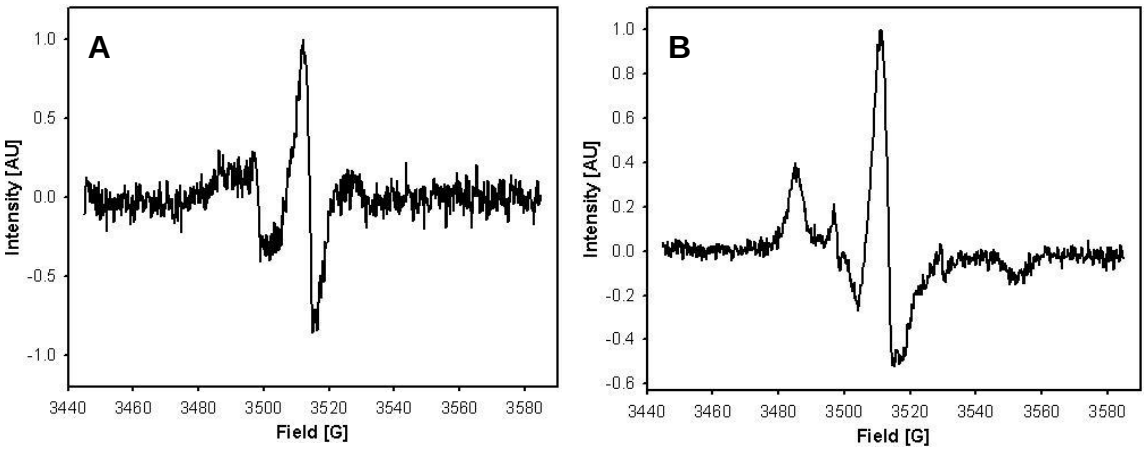


Fig. 2.87: EPR spectra of spin labelled anti-FLAG antibody. (A) M1 clone. (B) M2 clone.

Both samples were free of unbound label and showed signals representative of highly immobilised spin labels suggesting conjugation to a molecule of high mass: the antibodies. An interesting result was obtained when the antibody concentration was compared to the number of spins in the samples. For the M1 clone 2.04 spins (and thus labels) per antibody were found on average, and for the M2 antibody 1.01 spins per antibody. As the main difference between the two clones is their isotype, which differ in the number of the disulfide bonds in the hinge region, this might be a clue that the disulfide bridging methodology offers a certain amount of selectivity or certain antibody products can be prepared when the correct isotype is used. Clearly further research on this matter would be necessary.

It should be mentioned that *in situ* PEGylation of the antibodies was also attempted in the azide solution, however the small sample size did not allow for efficient removal of the excess of *N*-PEG5000-dithiophenolmaleimide, which prevented analysis by MALDI-TOF MS.

2.9.5 Binding activity of spin labelled M2 anti-FLAG antibody

Full antibodies have been spin labelled before, although not via their disulfide bonds but on the oligosaccharides attached to the C_H2 domain.[437, 438] Using this material the authors were not able to visualise the binding event by EPR. To test for the possibility that the disulfide bridged spin labelled M2 antibody would be able to detect the presence of antigen, it was incubated with an excess of the FLAG peptide and the mixture analysed by the same technique. No signifi-

cant changes in the EPR spectra were observed except for the slow appearance of a highly mobile species with similar characteristics to the free spin label (see Fig. 2.15 A). A potential explanation might be derived from the fact that the antigen had been synthesised with an additional C-terminal cysteine to allow for labelling. Thus the FLAG-Cys peptide potentially acted as a reducing agent cleaving the maleimide bridged disulfide bonds thereby releasing, or accepting, the spin label. Given the stability of the maleimide bridge in the anti-CEA ds-scFv (see 2.8.7) this was unexpected. However, the disulfide bonds in the anti-FLAG antibody might be more accessible, those at least would be the most likely to have been modified, or the cysteine in the FLAG peptide is very reactive due to its charged environment (sequence: DYKDDDDKC).[90] As a control the thiol group in the peptide was blocked by reaction with maleimide and the experiment repeated with this material. The increase of spin label mobility was not observed anymore but a possible binding event was also not indicated by the recorded spectra (data not shown).

To test if the activity of the antibody had been effected by the modification reaction, an ELISA of the labelled M2 against ras like GTPase bearing a N-terminal FLAG tag was performed by Mr Vishal Sanchania (UCL ISMB) (Fig. 2.88).

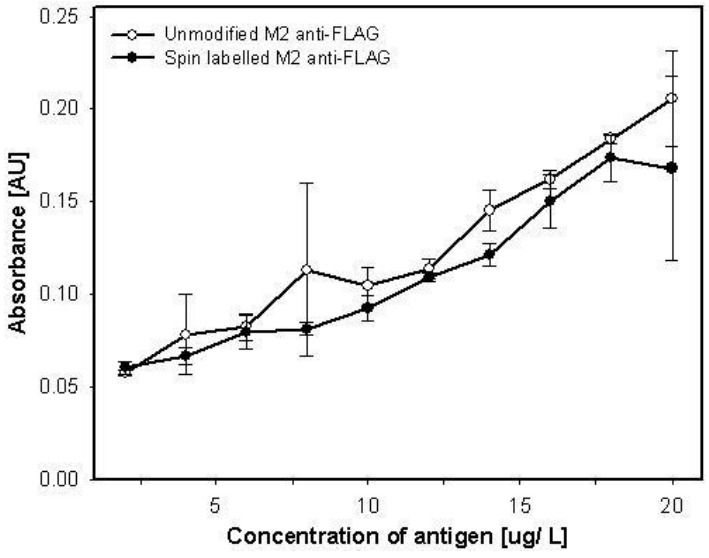


Fig. 2.88: ELISA of spin labelled M2 anti-FLAG antibody. Antigen: Ras like GTPase with a N-terminal FLAG tag.

The assay clearly showed that the antibody had retained more than 90% of its binding activity towards the FLAG-tag. This suggests that the insertion of functionalised maleimides into the antibody disulfide bonds had not perturbed its

structure, and is tolerated well in terms of biological activity. The negative result of the EPR measurement thus most likely originated from the insufficient change of the tumbling motion of the labelled antibody upon antigen binding. This is not surprising taking the mass difference into account (the molecular weight of the peptide is 1,116 Da).

Despite the promising results obtained with the anti-FLAG antibodies the system had a number of disadvantages that hampered the intended process development. For instance, the material is very costly enforcing small sample sizes, the presence of preservatives, problems with purification (no binding to protein A or G), as well as the fact that mouse antibodies have almost disappeared from clinical pipelines,[363] and the structural differences compared to human antibodies.[316] It was thus decided to work on a more relevant model, which was found in the anti-MSP1 antibody kindly supplied by Dr David Matthews (MRCT).

2.10 Modification of an anti-MSP1 antibody

The anti-MSP1 antibody was the most efficient immunoglobulin from a library of antibodies developed in 1994 against the merozite surface protein 1 (MSP1).[442] This is a 19 kDa C-terminal truncation of a larger protein complex on the surface of *Plasmodium falciparum* merozites, which is revealed by proteolysis after release of the parasite intermediate from infected erythrocytes. Antibodies against MSP1 have been found in malaria patients and it is hoped that the clinical development of such agents would lead to drugs that would stop the replication cycle of the parasite in a human host. The anti-MSP1 antibody was not developed further due to its expensive production and tendency for aggregation (personal communication D. Matthews). It is a chimeric antibody with a human framework and mouse variable domains and has a molecular weight of approximately 150 kDa.

2.10.1 Cleavage of the disulfide bonds of the anti-MSP1 antibody

To establish conditions for the modification of the anti-MSP1 antibody and as a follow up to the work on the anti-FLAG antibodies, a reduction study with TCEP was performed first (Fig. 2.89).

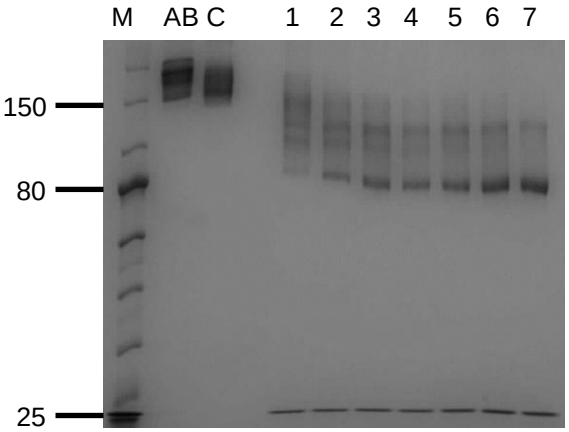


Fig. 2.89: Reduction of the anti-MSP1 antibody with TCEP. 1) 10 equiv. Bands top to bottom: HHL, HH, HL, H and L chains. 2) 15 equiv. 3) 20 equiv. 4) 25 equiv. 5) 30 equiv. 6) 40 equiv. 7) 80 equiv.

The disassembly of the antibody into the different combinations of the 4 polypeptide chains under various reducing conditions was easily observed by SDS-PAGE. As published,[229] small amounts of TCEP are sufficient to cleave some antibody disulfide bonds. In fact by using as little as 1 equiv of the phosphine reagent, faint bands of the free light chains were visible (data not shown). Treatment with 25 equiv of TCEP left only some of the hinge region cystines intact, and 100 equiv of the reducing agent were sufficient to break almost all interchain disulfide bonds of the anti-MSP1 antibody (see Fig. 2.91). It should be noted that the antibody samples were not thermally denatured prior to loading onto the SDS-PAGE. This explains the unusually high position of the H chain signal (expected mass is 50 kDa); the Ig domains are still folded and give the polypeptide together with the oligosaccharides, a very elongated shape that manifests in a reduced movement through the polyacrylamide gel.

2.10.2 *In situ* bridging of the anti-MSP1 antibody

Using the results of the reducing agent investigation on anti-MSP1, *in situ* bridging reactions with a wide range of reagents mixtures were tested (Fig. 2.90).

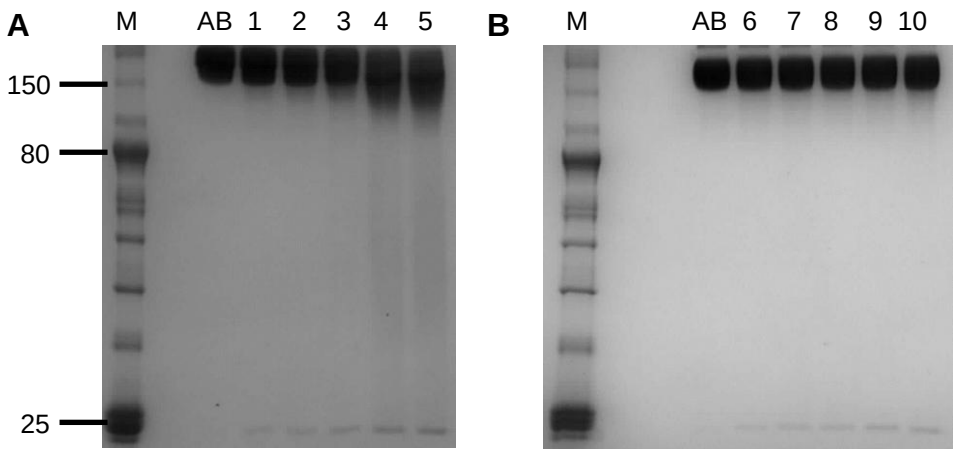


Fig. 2.90: *In situ* bridging of the anti-MSP1 antibody. (A) Equimolar mixtures of dithiophenolmaleimide **30** and benzeneselenol. 1) 2 equiv. 2) 5 equiv. 3) 10 equiv. 4) 50 equiv. 5) 100 equiv. (B) 5x excess of dithiophenolmaleimide. 6) 1 equiv TCEP. 7) 2 equiv TCEP. 8) 5 equiv TCEP. 9) 10 equiv TCEP. 10) 20 equiv TCEP.

In comparison to what had been found in the case of the mouse antibodies, the anti-MSP1 antibody lost some of its light chains during the modification reaction. Although the bands were visible only when gels were overloaded, the signals were present even with a 5x excess of the bridging reagent over the reducing agent added. It is possible that under these conditions the simultaneous reaction of the two revealed cysteines with two maleimide reagents is faster that the re-bridging reaction. However, this is unlikely especially in light of the results obtained with insulin, where the bridging of two cysteines localised on different peptides was efficient, although the structure was kept together only by the non-covalent interactions of the proximate environment.

Structural differences between mouse and human (or chimeric) antibodies have been revealed by crystallisation of intact full length proteins of that class from both species.[310, 315, 316] Notable is the observation that human antibodies adopt much less the usually depicted Y shape, and the angles between the Fab and the Fc fragments can vary widely within the same molecule. This results in two structurally different Fabs, and can be reflected in some cases by a difference in the stability of the interaction of the H and L chain of one of the “arms” of the antibody. Together with the possibly varying accessibility of the disulfide bonds connecting the H and L chains which may result, this might have been the cause of the observed small loss of light chains during the *in situ* bridging reaction.

2.10.3 *In situ* PEGylation of the anti-MSP1 antibody

Again in parallel to preceding work a physical proof that the protein disulfide bonds have been modified during the reaction was required. Thus the anti-MSP1 antibody was PEGylated *in situ* utilising both TCEP and benzeneselenol (Fig. 2.91). Whilst an effect had been observed with smaller amounts of reagents, 100 equiv of the bridging and reducing compounds were used for better comparability with previous data.

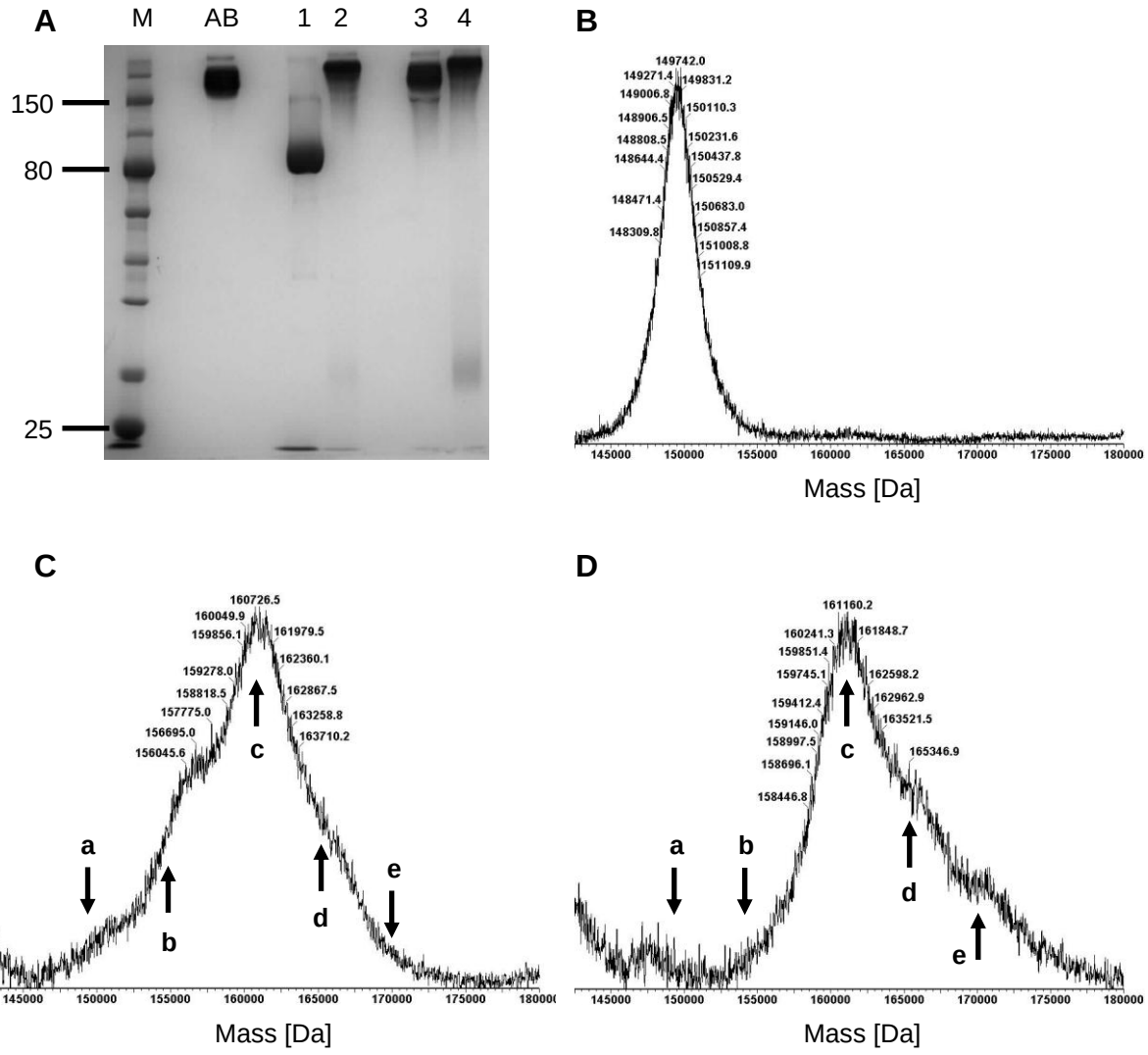


Fig. 2.91: *In situ* PEGylation of the anti-MSP1 antibody. (A) SDS-PAGE analysis. 1) 100 equiv TCEP. 2) 100 equiv TCEP and 100 equiv *N*-PEG5000-dithiophenolmalemide **31**. 3) 100 equiv benzeneselenol. 4) 100 equiv benzeneselenol and 100 equiv PEGylation reagent **31**. (B) MS of the unmodified antibody. (C) MS of the product from lane 2. Arrows indicate the proposed number of attached PEG chains. **a** = 0, **b** = 1, **c** = 2, **d** = 3, **e** = 4. (D) MS of the product from lane 4.

The sample analysis provided important insights into the reaction: Benzeneselenol alone was not efficient in breaking the target disulfide bonds permanently, save for a faint signal of released light chains. Similar weak signals of unmodified and PEGylated L chains were observed in both bridging samples. The presence of small amounts of un-PEGylated L chains despite the excess of the maleimide compound, underlines that mono-peptidylcysteine maleimide adducts are cleaved easier by reducing agents than maleimides inserted into a disulfide bridge.[396] If the occurrence of the PEGylated L chains is caused by inefficient bridging, or individual modification of both cysteines with a maleimide, can not be concluded from the available data.

In both PEGylation samples a clear upwards shift of the intact antibody band is visible, and more prominent when benzeneselenol is used. This was confirmed by analysis of the material by MALDI-TOF MS. Only a small amount of unmodified antibody was observed when TCEP was used as a reducing agent. The double PEGylation product appears as the most intense signal followed by some mono- and possible triple-PEG conjugates. Together with the observed release of light chains during the reduction studies, this suggests that TCEP-based modification protocols might afford mainly antibodies conjugated via their switch region disulfide bonds. In contrast benzeneselenol was found to be more active, in spite of the seemingly inefficient cystine reduction when employed without bridging reagent. Although the main product was still the double PEGylated antibody, other strong signals for triple and even quadruple PEGylated species are seen. The selenol reagent seems to be either fairly dependent on the presence of thiols in order to fully reduce disulfide bonds, or opens the disulfide bonds only reversible. No indication of a preference of benzeneselenol for a certain type of antibody disulfide bond could be drawn from this data. Overall, the *in situ* PEGylation of the anti-MSP1 antibody proceeded efficiently with marginal losses of intact antibody complexes and good conversion rates.

A few experiments were then carried out with the aim to further improve the reaction. However, an increase in time or temperature did not result in a different product pattern (data not shown).

2.10.4 *In situ* spin labelling of the anti-MSP1 antibody

After the successful PEGylation of the anti-MSP1 antibody, an attempt was made to spin label the protein via the same protocol (Fig. 2.92)

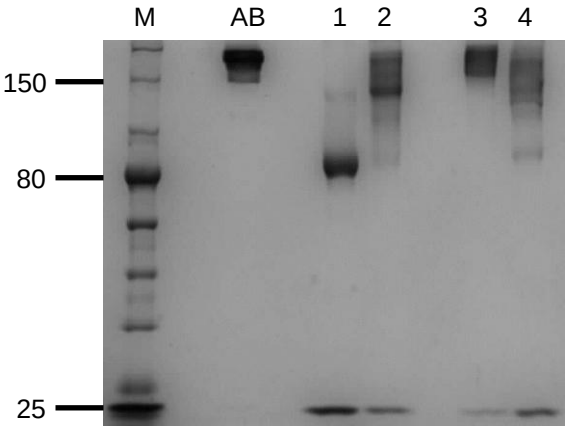


Fig. 2.92: *In situ* spin labelling of the anti-MSP1 antibody. 1) 100 equiv TCEP. 2) 100 equiv TCEP and 100 equiv *N*-TPO-dithiophenolmaleimide **32**. 3) 100 equiv benzeneselenol. 4) 100 equiv benzeneselenol and 100 equiv *N*-TPO-dithiophenolmaleimide **32**.

The poor reducing activity of benzeneselenol was reproducible, but the bridging samples appeared very different by SDS-PAGE. The re-bridging of reduced disulfide bonds had not happened efficiently and prominent bands for all possible antibody fragments, especially the light chain, dominate the gel. Despite this, the presence of multiply spin labelled antibody species was confirmed by EPR spectroscopy (data not shown). Two explanations are possible; either the reactivity of the spin label *N*-TPO-dithiophenolmaleimide was lower than that of other maleimide compounds employed thus far, or the solubility of the label in combination with the fragility of the target antibody was a problem.

To solve this problem a number of experiments were carried out. First the antibody concentration was measured regularly before and after the reactions to ensure that the protein's concentration was maintained. This indeed confirmed that the anti-MSP1 antibody had a tendency to aggregate over time.

Next the previous experiment was repeated but this time with only 10 equiv of both reducing agents (data not shown). The results showed that a reduced amount of TCEP and selenol furnished more of the intact antibody product. To further optimise the process the anti-MSP1 antibody was treated with the amount of TCEP with which most of the switch region disulfide bonds had been reduced (25 equiv, see Fig. 2.89), in the presence of different amounts of the spin label (Fig. 2.93). Benzeneselenol was omitted from further optimisation, as

the presence of this compound induced too much aggregation of the antibody (data not shown).

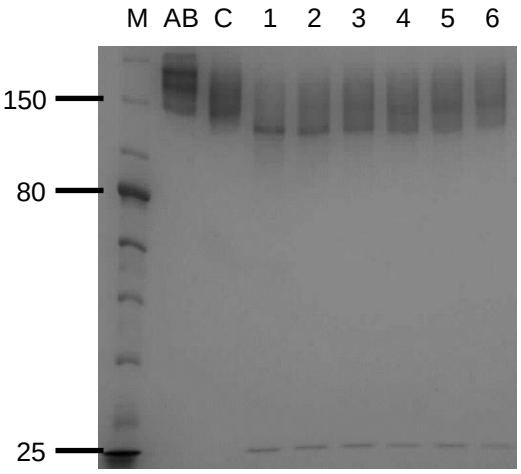


Fig. 2.93: Optimisation of the *in situ* spin labelling of the anti-MSP1 antibody with *N*-TPO-dithiophenolmaleimide **32**. 25 equiv of TCEP were used in all samples. 1) 5 equiv. 2) 10 equiv. 3) 15 equiv. 4) 20 equiv. 5) 25 equiv. 6) 50 equiv.

The results revealed that a combination of 25 equiv of both bridging and reducing agent produces a conjugation product that resembles the control reaction well. The presence of more of the maleimide compound did not improve this any further. It was also tested if the optimised reagent mix could be added stepwise, in the same fashion that was established for insulin. To this end either TCEP or both compounds were added in 1, 2 or 3 portions over a time course of 1 h to the antibody. However, SDS-PAGE analysis showed no further improvements (data not shown).

2.10.5 Preparation and activity of anti-MSP1 analogues

For an industrial scale process for the production of antibody conjugates, it is desirable to use as little as possible of the chemical compounds required per batch. As the optimised protocol for the *in situ* spin labelling of the anti-MSP1 antibody consumed only one quarter of the maleimide as well as the TCEP, transfer of this protocol to the PEGylation process was attempted. Only 15 equiv of *N*-PEG5000-dithiophenolmaleimide were necessary to efficiently bridge the reduced disulfide bond in the presence of 25 equiv of TCEP (see below).

PEGylated and spin labelled antibody samples were then carefully prepared for further analysis and activity testing (Fig. 2.94).

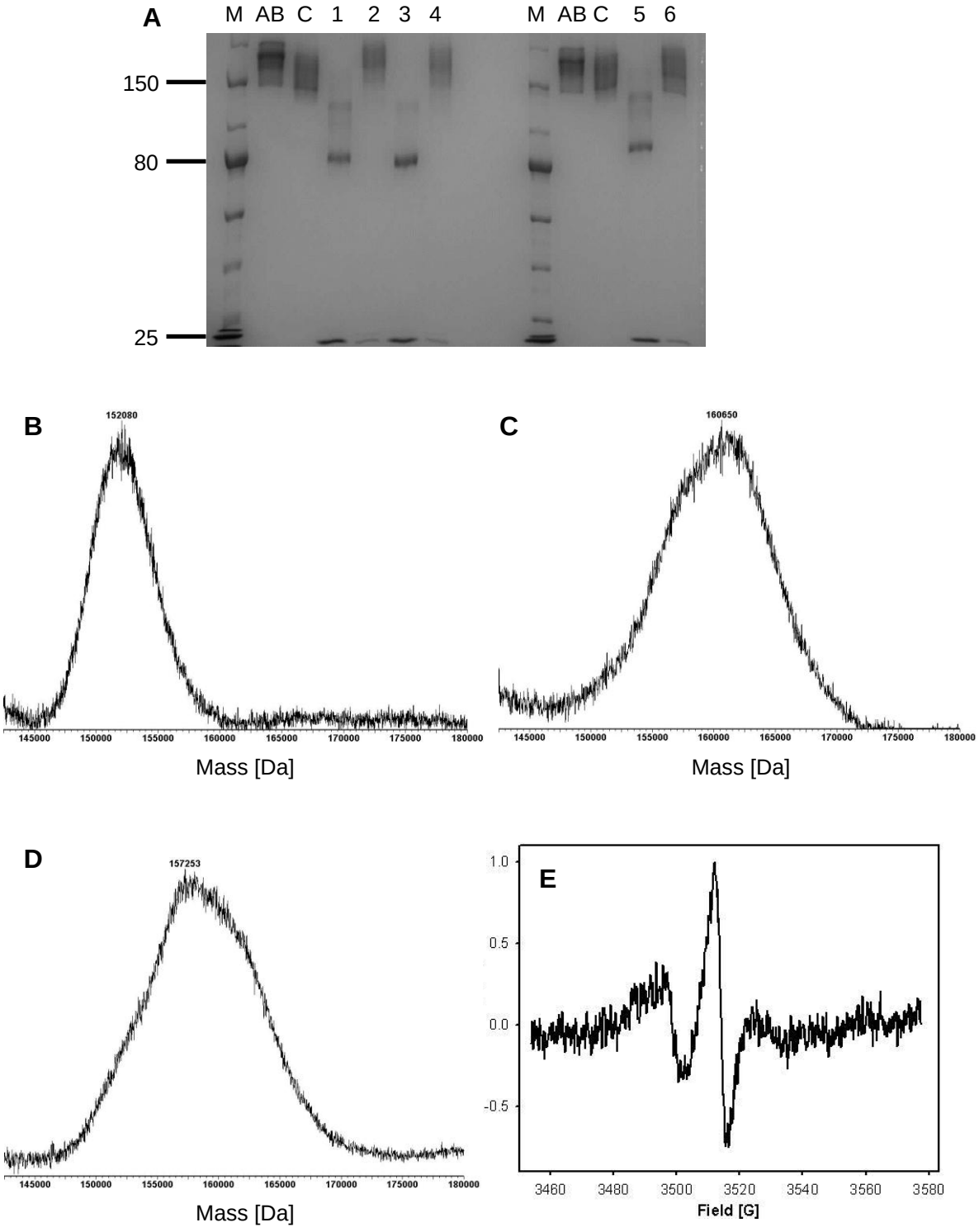


Fig. 2.94: Preparation of anti-MSP1 analogues. (A) SDS-PAGE analysis. 1) 25 equiv TCEP. 2) 25 equiv TCEP and 15 equiv *N*-PEG5000-dithiophenolmaleimide **31**. 3) 100 equiv TCEP. 4) 100 equiv TCEP and 100 equiv *N*-PEG5000-dithiophenolmaleimide **31**. 5) 25 equiv TCEP. 6) 25 equiv TCEP and 25 equiv *N*-TPO-dithiophenolmaleimide **32**. (B) MS of the unmodified antibody. (C) MS of the product from lane 2). (D) MS of the product from lane 4). (E) EPR spectra of the spin labelled antibody.

All conjugation reactions were reproducible despite the declining quality of the antibody sample, as observed by SDS-PAGE. The MS analysis of the PEGylated anti-MSP1 analogues was surprising; the products were very similar to previous attempts despite the amounts of the reactants being so different. The main signals indicated predominantly mono and double PEGylation, with small amounts of the unmodified antibody and possibly some triple PEGylation products. This result on its own would indicate that the limiting factor in the reaction is the time required for full (4x) PEGylation, but such an experiment had been performed (see 2.10.3) and excluded this possibility. An alternative explanation is the structural inaccessibility of certain disulfide bonds to the bulky PEGylation reagent. In a zipper-like fashion, some cystines might only be accessible if others have been reduced. If the bridging reaction of these first “gating” disulfide bonds happens too fast, the others cannot be modified anymore. However, if a second reaction can take place in the meantime, products with a higher conjugation number would appear which would also not be subject to further modification. This is clearly dependent on the structure, as well as the dynamics of the individual antibody and a deeper structural insight would be necessary to verify such a theory. The EPR spectrum of the spin labelled material clearly indicates the immobilisation of the label onto the antibody and the absence of free label. A spin count showed the presence of an average of 1.84 labels per protein, which fits relatively well with the PEGylation results.

The binding activity of all anti-MSP1 analogues were then tested alongside the unmodified antibody in an ELISA against recombinant MSP1 antigen (Fig. 2.95)

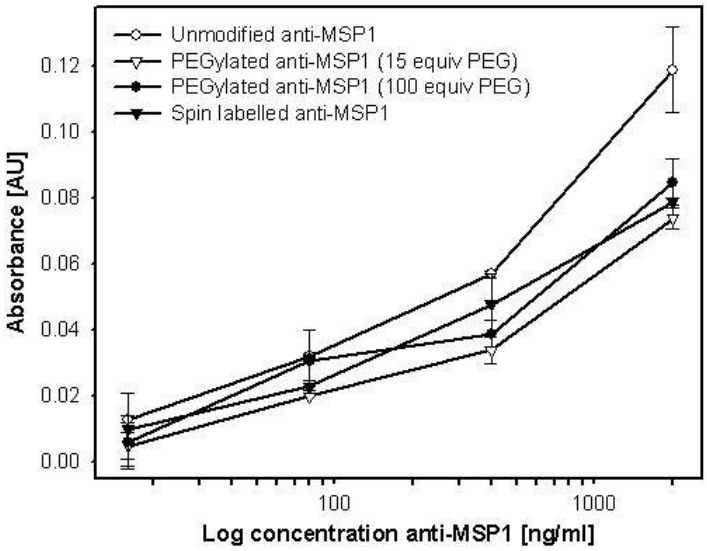


Fig. 2.95: ELISA of anti-MSP1 analogues. Antigen: Recombinant MSP1 (1-19).

Due to the limited supply of antigen, only a few dilutions could be tested. Still the experiment clearly showed that in contrast to the mouse antibodies, anti-MSP1 had lost some of its binding activity (~ 40%) when conjugated to functional groups via its disulfide bonds. Still the conjugation of proteins to PEG or antibodies to drugs potentiates their half life[154] or biological activity[443] often several times, so that a limited loss of antigen-binding can be tolerated in certain cases. In addition it is known, and became even more apparent during this study that the anti-MSP1 antibody would be a difficult target due to its fragility and tendency to aggregate. Against this background the almost complete modification of a full length antibody via its disulfide bonds with retention of a significant amount of its activity can be seen as a success.

Both the anti-FLAGs as well as the anti-MSP1 antibody have been model compounds to test the general feasibility of antibody conjugation based on disubstituted maleimides. To evaluate the methodology in the context of products relevant for therapeutic applications, it was necessary to work with material that was optimised and prepared for this use – a clinically approved antibody.

2.11 Modification of rituximab

Rituximab was chosen as an ideal candidate. This antibody was readily approved for clinical use in 1997, and is today seen as a hallmark in antibody-based cancer therapy.[444] Marketed as “MabThera” or “Rituxan”, rituximab is used as a first-line treatment for large B cell lymphoma and rheumatoid arthritis.[445] Initially the antibody was used alone, but now commonly in combination with a regime of chemotherapeutics.[446] Rituximab is a chimeric mouse (murine V_H and V_L domains)/ human IgG1 antibody produced in mammalian cells, and binds to a discontinuous epitope of the orphan cell-surface receptor CD20, a B cell marker, with a K_d of 5.2 nM.[447] Although the exact *in vivo* mechanism has not been elucidated, it is known that the antibody is able to induce cell-killing upon binding by activation of apoptosis pathways, CDC and ADCC.[448] Despite the fact that a number of studies have shown that CD20 does not internalise after antibody-binding,[449] ADCs of rituximab have been prepared and successfully demonstrated improvements in the therapeutic index, with receptor recycling being proposed as a potential entry mechanismn.[450, 451]

Against this background rituximab seemed an ideal and clinically relevant system to test the novel bioconjugation chemistry developed in this project.

2.11.1 Targeting of rituximab disulfide bonds

After transfer of the antibody from its clinical formulation into PBS, rituximab was treated in parallel to the anti-MSP1 experiments with various amounts of TCEP. The effect of the reducing agent on the protein complex was visualised by SDS-PAGE (Fig. 2.96).

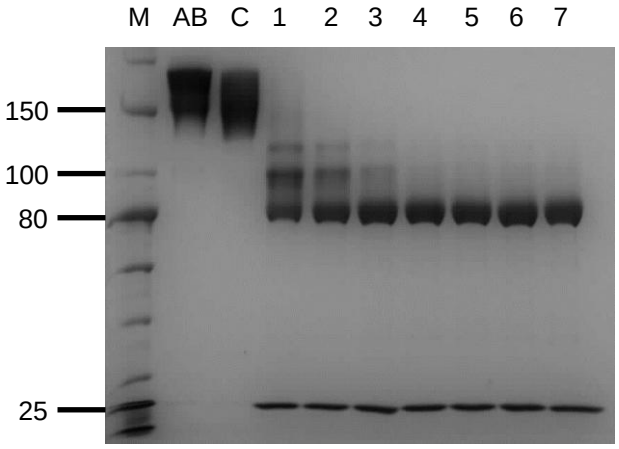


Fig. 2.96: Reduction of rituximab with TCEP. 1) 5 equiv. Bands top to bottom: HHL (very faint), HH, HL, H and L chains. 2) 10 equiv. 3) 20 equiv. 4) 40 equiv. 5) 60 equiv. 6) 80 equiv. 7) 100 equiv.

Overall the disulfide bridges of this anti-CD20 antibody seemed slightly less stable towards the reducing agent in comparison to the other full antibodies tested. 10 equiv of TCEP were sufficient to fragment every molecule of full rituximab, and 40 equiv were enough to disassemble the protein complex completely into the H and L chains. These two values were used as reference points for the next step, a TCEP-based *in situ* modification of rituximab with dithiophenolmaleimide (Fig. 2.97).

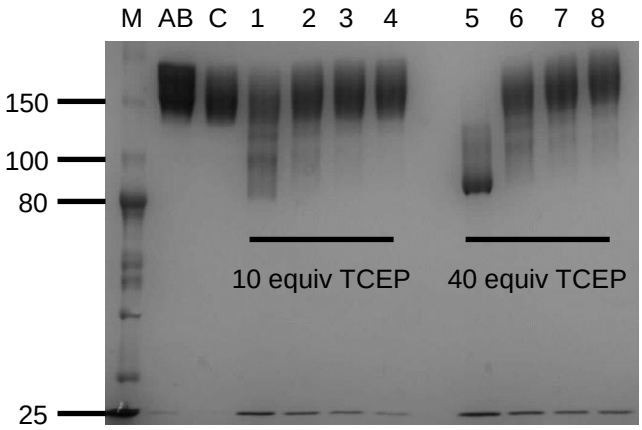


Fig. 2.97: *In situ* bridging of rituximab with TCEP and dithiophenolmaleimide. 1) 3 equiv bridging reagent. 2) 5 equiv. 3) 10 equiv. 4) 20 equiv. 5) 5 equiv. 6) 20 equiv. 7) 40 equiv. 8) 80 equiv.

However, similar results to the anti-MSP1 antibody, were obtained. Weak L chain signals and fragmentation patterns were observed, when twice the amount of bridging reagent than reducing agent were used. This was somewhat disappointing. Clinical grade antibodies are usually selected for their stability amongst other properties, and this, potentially based on a more coherent domain complex, had promised a higher tolerance to the re-bridging reaction.

To understand the efficiency of the reaction the experiment was repeated with *N*-PEG5000-dithiophenolmaleimide as the bridging reagent. SDS-PAGE revealed a similar picture but MALDI-TOF MS showed another surprising issue (Fig. 2.98).

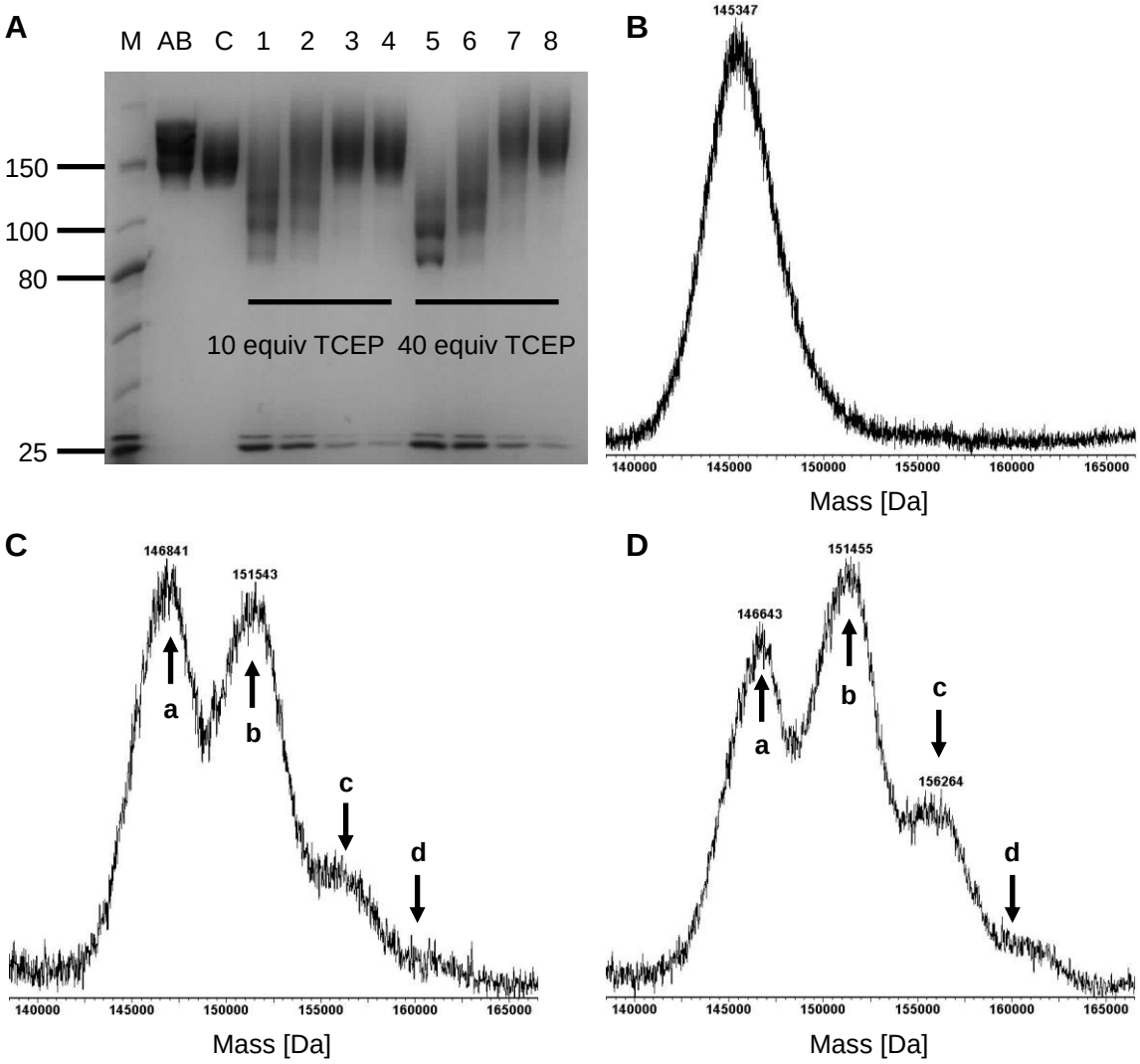


Fig. 2.98: *In situ* PEGylation of rituximab with TCEP. (A) SDS-PAGE analysis. 1) 3 equiv *N*-PEG5000-dithiophenolmaleimide **31**. 2) 5 equiv. 3) 10 equiv. 4) 20 equiv. 5) 5 equiv. 6) 20 equiv. 7) 40 equiv. 8) 80 equiv. (B) MS of the unmodified antibody. (C) MS of the product from lane 4). Arrows indicate the proposed number of attached PEG chains. **a** = 0, **b** = 1, **c** = 2, **d** = 3. (B) MS of the product from lane 8).

The mass corresponded well to the theoretical molecular weight of rituximab (144,544 Da without glycosylation),[447] which clearly shows that the PEGylation even of the most efficiently bridged samples was incomplete. The product mix contained antibody molecules with 1, 2 and potentially 3 PEG chains, but most importantly a strong signal of the unmodified material was present. This was the case even when 40 equiv of the reducing agent were used. Here possible explanations might be found in the properties of the rituximab disulfide bonds. The cysteines may be more reactive and thus re-oxidise faster. Alternatively the disulfide bonds might be less accessible for the PEGylation reagent in this antibody, slowing down the bridging reaction. This would allow for more time for side reactions to occur, consuming TCEP and maleimide. Also, despite the excess of functionalised maleimide present in the samples, the SDS-PAGE showed signals for non PEGylated free L chains. This potentially indicates cleavage of PEGylation reagent, which has reacted only with a single cysteine and thus not formed a bridge, by TCEP. Such a reaction has been observed with substituted maleimides before.[396]

Whatever the reason, the TCEP-based *in situ* protocols were not efficient enough to completely modify the disulfide bonds of rituximab. To overcome this problem the alternative, benzeneselenol, was tested.

2.11.2 Benzeneselenol-based *in situ* modification of rituximab

Since a reduction study comparable to the one performed with TCEP is not possible with the selenol reagent, various mixtures of rituximab with different ratios of benzeneselenol and *N*-PEG5000-dithiophenolmaleimide **31** were prepared and analysed by SDS-PAGE (Fig. 2.99).

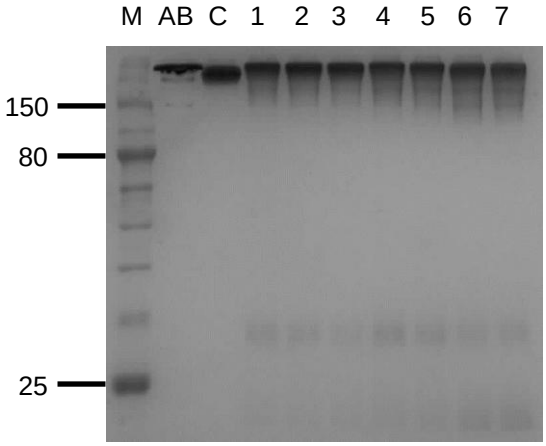


Fig. 2.99: *In situ* PEGylation of rituximab with benzeneselenol. Ratios of benzeneselenol : *N*-PEG5000-dithiophenolmaleimide **31** are: 1) 40 : 80, 2) 30 : 60, 3) 20 : 40, 4) 40 : 40, 5) 40 : 20, 6) 40 : 10 and 7) 30 : 10.

The experiment displayed a better efficiency than the TCEP based protocol. Only a small amount of L chains, mainly PEGylated, were lost. The formation of HHL, HH and HL fragments was accordingly low. Both observations were less pronounced in samples where the amount of benzeneselenol was decreased or the maleimide reagent increased. To confirm that the modification had actually taken place the 3 best samples were prepared again with appropriate controls and analysed by PAGE and MS (Fig. 2.100).

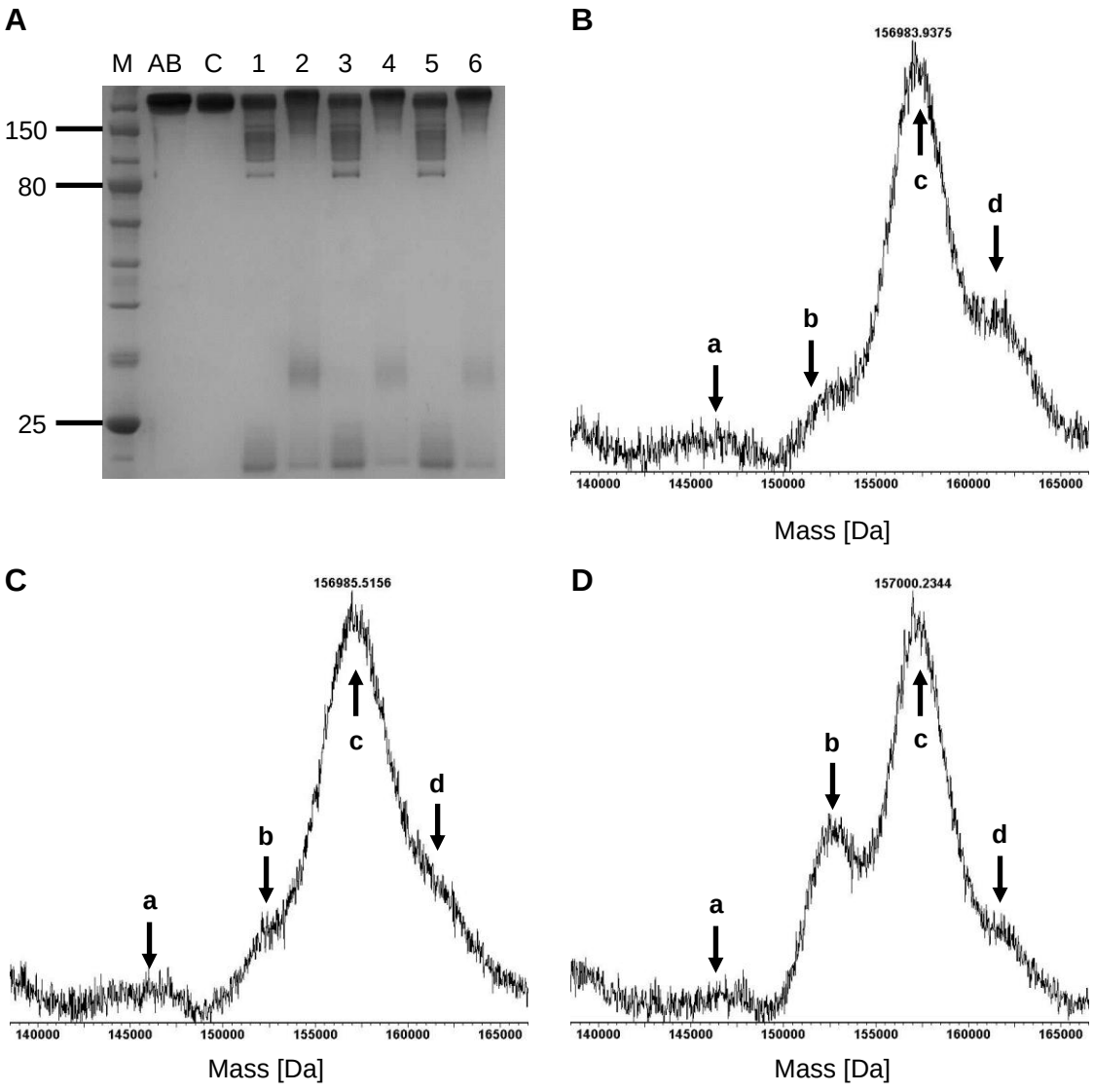


Fig. 2.100: Optimised *in situ* PEGylation of rituximab with benzeneselenol. (A) SDS-PAGE analysis. 1) 40 equiv benzeneselenol. 2) Benzeneselenol : *N*-PEG5000-dithiophenolmaleimide **31** = 40 : 10. 3) 30 equiv benzeneselenol. 4) Benzeneselenol : PEGylation reagent **31** = 30 : 60. 5) 20 equiv benzeneselenol. 6) Benzeneselenol : PEGylation reagent **31** = 20 : 40. (B) MS of the product from lane 2). Arrows indicate the proposed number of attached PEG chains. **a** = 0, **b** = 1, **c** = 2, **d** = 3. (C) MS of the product from lane 4). (D) MS of the product from lane 6).

The previous results were reproducible by SDS-PAGE. Although benzeneselenol alone cleaved only a fraction of the antibody disulfide bonds in the controls, the mass spectra of all 3 samples indicate clearly modification with nearly full conversion taking place. The main product is a double PEGylated antibody with small amounts of rituximab with 1 or 3 PEG chains attached. The ratio of these can be shifted towards the lower modification number when less benzeneselenol is used. The overall pattern of conjugation might indicate a certain preference of the reduction system used here for one type of disulfide bonds. These are likely to be those in the switch region cystines, as mainly two modifications are observed and L chains but no H chains are lost.

2.11.3 Sequential modification of rituximab

Although further improvements are still possible, the selenol-mediated *in situ* modification of rituximab seemed to furnish predominantly double-conjugated antibody. While with the advent of the "Thiomab" technology, where two free cysteines are engineered into full antibodies,[177] this view is shifting, a drug load of around 4 is still considered ideal or even necessary in the context of ADCs.[230, 378] To access higher modification numbers than seemingly possible with the *in situ* protocols, the idea of sequential modification, with reduction followed by bridging, was tested. Somatostatin and the anti-CEA ds-scFv fragment were successfully conjugated via this process, and the antibody complex should in theory also tolerate the loss of its interchain disulfide bonds for a limited time.[377]

To this end rituximab was reduced with 40 equiv of TCEP for 1 h after which various amounts of dithiophenolmaleimide were added. Analysis by SDS-PAGE revealed that even with 80 equiv of the bridging reagent none of the antibody molecules had retained its full structure (data not shown), although the formation of some HH and HHL complexes was found. As a potential cause, the presence of a high amount of organic solvent (20% DMF had been added before the reduction step) was identified. Essen and Skerra have reported that this can disrupt the structure of non-disulfide bonded antibody constructs,[331] and others have observed similar effects.[202] To test this hypothesis the experiment was repeated without DMF in the protein solution and with *N*-PEG5000-

dithiophenol-maleimide as the bridging reagent to avoid the introduction of organic solvent completely (Fig. 2.101).

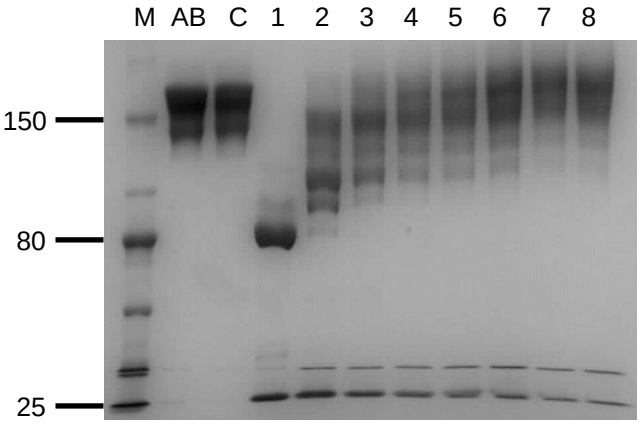


Fig. 2.101: Organic solvent free sequential PEGylation of rituximab. The antibody was reduced with 40 equiv of TCEP for 1 h. 1) Reduced antibody. 2) 5 equiv *N*-PEG5000-dithiophenolmaleimide **31**. 3) 10 equiv. 4) 20 equiv. 5) 30 equiv. 6) 40 equiv. 7) 60 equiv. 8) 80 equiv.

The results supported the hypothesis, and this time complete and presumably PEGylated antibody was observed. Despite this fact, the reaction was as inefficient as found with the TCEP-based *in situ* modification in terms of producing a clean product. This suggests that the problem is not the cleavage of the cystines but the bridging reaction in the presence of a large excess of the phosphine. In the search for alternative methods to reduce the antibody, rituximab was treated with various amounts of 2-mercaptoethanol, DTT, mixtures of small amounts of TCEP and DTT, as well as TCEP immobilized onto resin beads. Only DTT and a mixture of DTT with TCEP yielded adequately reduced antibody (data not shown). Optimised versions of all established reduction protocols were then used to generate sequential methods based on the lowest amounts of PEGylation reagent required. It should be noted that the dibromomaleimide version **25** was used when DTT was present to neutralise the excess of bridging reagent (Fig. 2.102).

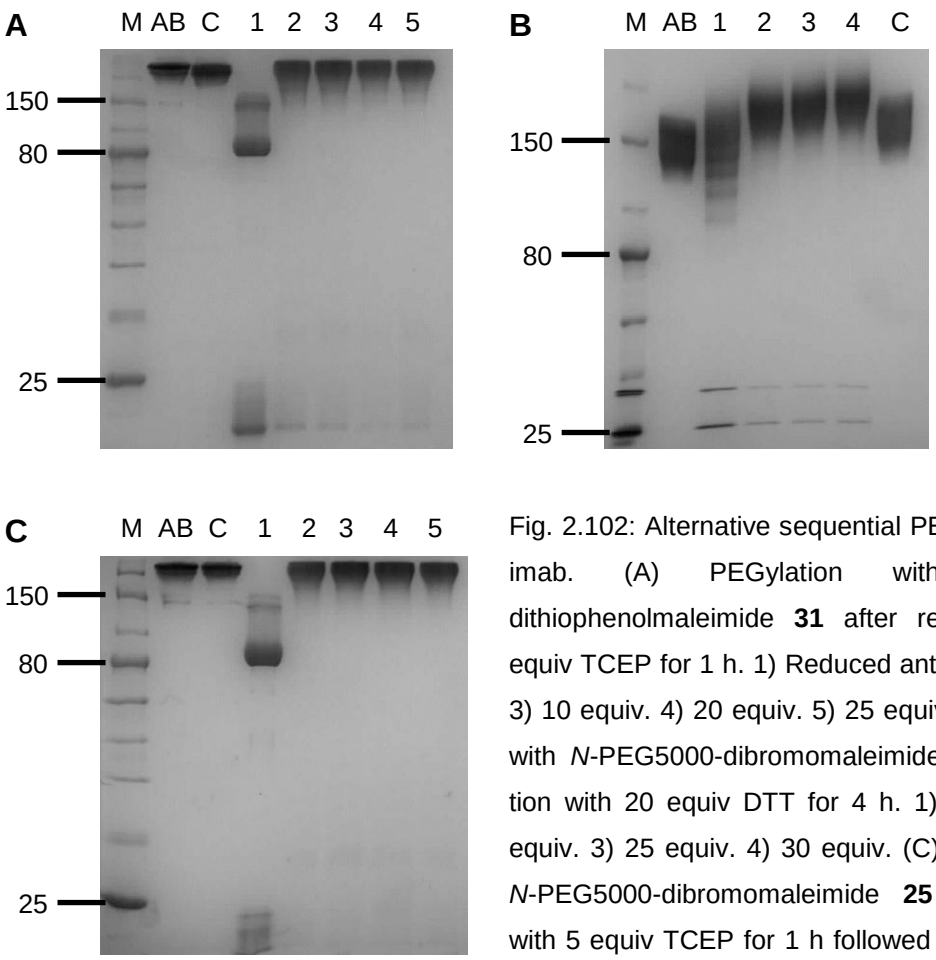


Fig. 2.102: Alternative sequential PEGylation of rituximab. (A) PEGylation with *N*-PEG5000-dithiophenolmaleimide **31** after reduction with 10 equiv TCEP for 1 h. 1) Reduced antibody. 2) 5 equiv. 3) 10 equiv. 4) 20 equiv. 5) 25 equiv. (B) PEGylation with *N*-PEG5000-dibromomaleimide **25** after reduction with 20 equiv DTT for 4 h. 1) 15 equiv. 2) 20 equiv. 3) 25 equiv. 4) 30 equiv. (C) PEGylation with *N*-PEG5000-dibromomaleimide **25** after reduction with 5 equiv TCEP for 1 h followed by 10 equiv DTT for 3 h. 1) Reduced antibody. 2) 15 equiv. 3) 20 equiv. 4) 25 equiv. 4) 30 equiv.

All 3 methods furnished the bridged antibody with small amounts of fragmentation. 20–25 equiv of the PEGylation reagent were sufficient to obtain mainly intact rituximab in the presence of the non-reacted reducing agent.

As before to confirm the conjugation of the polymer chains to the antibody, the best protocols were repeated and analysed by MALDI-TOF MS in addition to SDS-PAGE (Fig. 2.103).

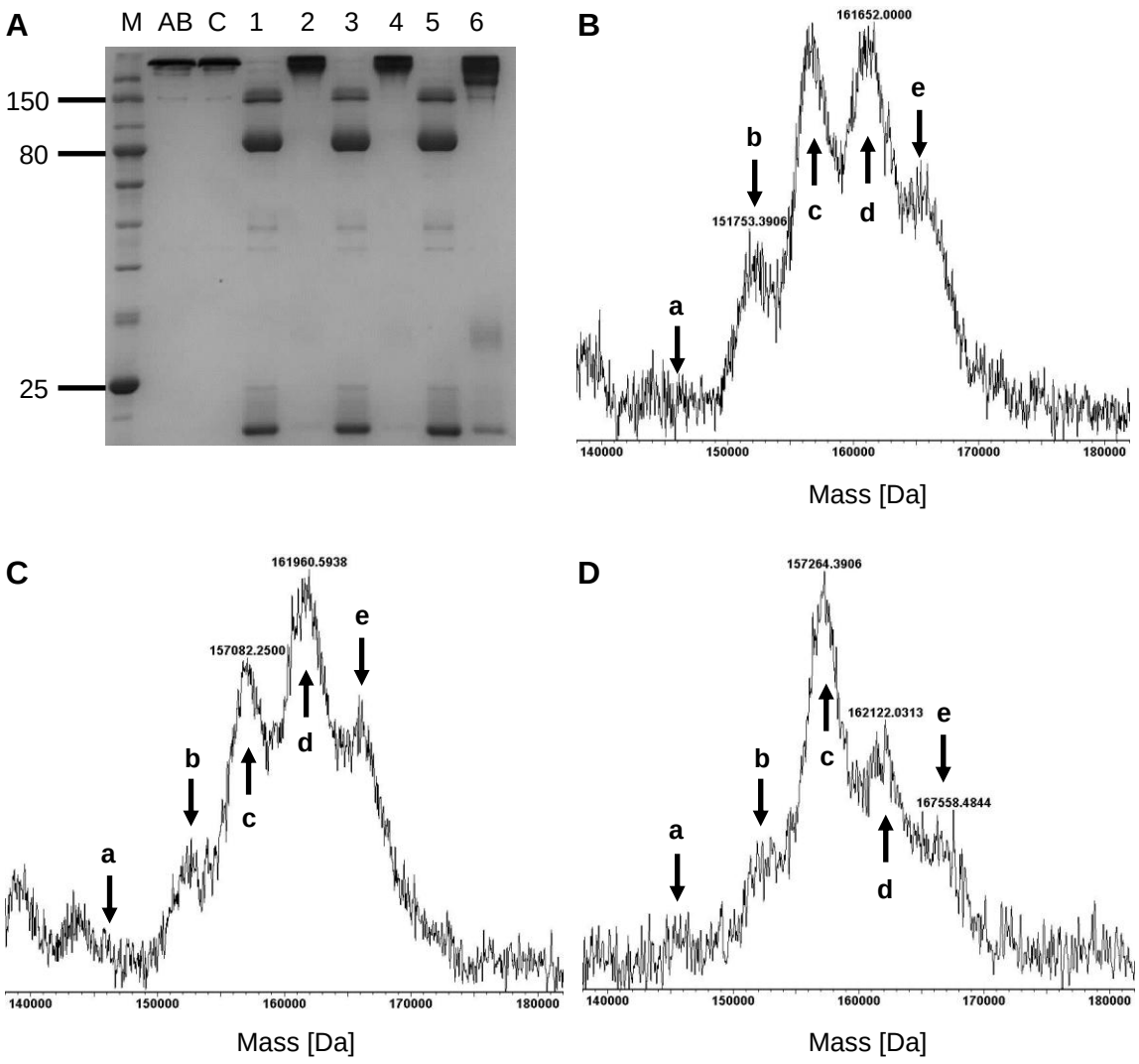


Fig. 2.103: Optimised sequential PEGylation of rituximab. (A) SDS-PAGE analysis. 1) Reduced antibody (5 equiv TCEP 1 h, then 10 equiv DTT 3 h). 2) Reduced antibody from lane 1) PEGylated with 20 equiv *N*-PEG5000-dibromomaleimide **25**. 3) Reduced antibody (20 equiv DTT 4 h). 4) Reduced antibody from lane 3) PEGylated with 25 equiv *N*-PEG5000-dibromomaleimide **25**. 5) Reduced antibody (10 equiv TCEP 1 h). 6) Reduced antibody from lane 5) PEGylated with 20 equiv *N*-PEG5000-dithiophenolmaleimide **31**. (B) MS of the product from lane 2). Arrows indicate the proposed number of attached PEG chains. **a** = 0, **b** = 1, **c** = 2, **d** = 3, **e** = 4. (C) MS of the product from lane 4). (D) MS of the product from lane 6).

In all samples PEGylation of the antibody had taken place and overall conversion rates seemed to be close to complete. Depending on the reducing agent used a mix of variously modified products was obtained. Protocols that utilised DTT yielded mainly double and triple PEGylated rituximab, with substantial amounts of the single and quadruple modified products. It should be emphasised again that MALDI-TOF MS can not reliably be used for quantification, es-

pecially here due to the difficult ionisation of PEGylated proteins, and any statements refer to a comparison of the samples with each other. This might also be a factor in explaining why the main product by MS is not the fully modified antibody as would be expected. Another is certainly the presence of some half-antibody (the HL fragment) before the reaction, visible on the SDS-PAGE. Interestingly with TCEP as a reducing agent mainly double PEGylation was achieved with some higher modifications also present. Here side reactions between the maleimide compound and the phosphine could again be responsible for this pattern. However, a potential preference of this reagent combination for a certain antibody region, as observed with benzeneselenol, is conceivable.

2.11.4 Stepwise modification of rituximab

Although the sequential protocol allows the quick and seemingly complete PEGylation of rituximab, while re-forming most of the disulfide bonds, the products were relatively heterogeneous. To solve this problem and to investigate the decreased reaction efficiency in the presence of TCEP, a stepwise method was designed. Here, inspired by the published methods for antibody modification via their disulfide bonds after complete reduction,[228] the reducing agent was removed before addition of the bridging reagent. To test this, rituximab was treated with 80 equiv of TCEP, purified on a PD G-25 desalting column and immediately reacted with various amounts of *N*-PEG5000-dithiophenolmaleimide (Fig. 2.104).

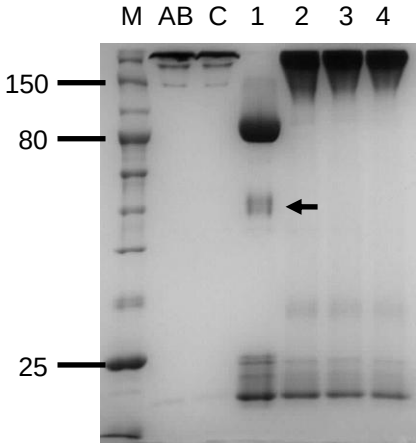


Fig. 2.104: Initial stepwise PEGylation of rituximab. The antibody was reduced with 80 equiv TCEP for 1 h. 1) Reduced antibody after purification. To this sample were added 2) 5 equiv, 3) 8 equiv and 4) 10 equiv *N*-PEG5000-dithiophenolmaleimide **31**.

Despite the addition of an excess of PEGylation reagent over the disulfide bonds, a relatively strong signal for free L chains was found. Also an additional

signal was observed (black arrow) in the sample of the reduced antibody after the desalting step. Estimated from its mass, this could only arise from disulfide bond formation between two free L chains, which is possible but has been described as slow.[377] Both observations pointed towards the disruption of the antibody complex in the purification step, or inefficient maintenance of the reduced state. To find ideal conditions for the preparation of clean but reduced antibody an auto-oxidation study was carried out. Rituximab was treated with TCEP and desalted as before, but simultaneously buffer exchanged into a solution that would suppress oxidation of the protein cystines and support formation of the domain complex. This buffer had a medium pH (6.8) and contained EDTA (1 mM) to stop re-oxidation,[379, 452, 453] as well as a low salt concentration (50 mM phosphate) to strengthen the non-covalent ionic interactions.[376] The sample was kept under an argon atmosphere in the dark for 40 h and aliquots were carefully removed over this time and alkylated with maleimide to preserve their state of oxidation. The formation of disulfide bonds was quantified by densitometry after SDS-PAGE (Fig. 2.105).

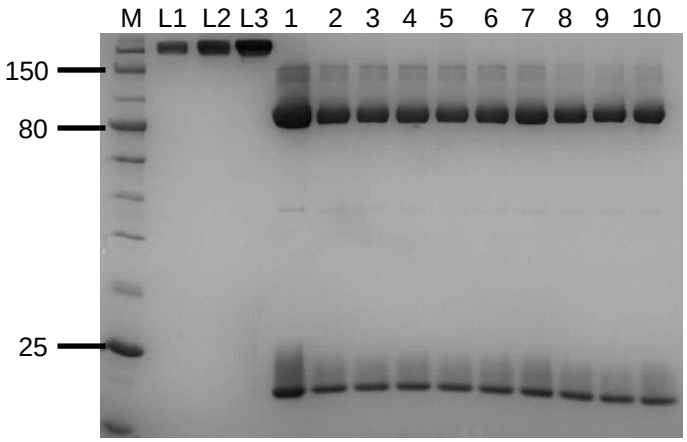


Fig. 2.105: Re-oxidation study with rituximab. L1) Loading control of 1 µg, L2) 2 µg and L3) 4 µg of intact antibody. 1) Freshly reduced antibody. 2) Reduced antibody alkylated after 5 min, 3) 20 min, 4) 40 min, 5) 1 h, 6) 2 h, 7) 4 h, 8) 20 h, 9) 30 h and 10) 40 h.

A fully reduced antibody can reform largely with intact disulfide bonds within hours by simple air oxidation even after physical separation of the different peptide chains.[37, 377] In contrast the conditions used here were clearly sufficient to keep rituximab in its non-cystine bonded form. Densitometric analysis of the gel showed that less than 4% of the total interchain disulfide bonds were intact even after 40 h.

Based on this, a second experiment to modify rituximab via a stepwise protocol was performed. After reduction, the antibody was desalted and buffer swapped into the anti-oxidising solution. It was kept under argon in the dark for 24 h to allow for potential re-association of the protein chains, and then treated with *N*-PEG5000-dithiophenolmaleimide. In addition, dithiophenolmaleimide was added to a separate set of samples in enough DMF to yield a final concentration of 20% organic solvent in the protein solution. All reactions were analysed by SDS-PAGE and later the best performing mixtures also subjected to MALDI-TOF MS (Fig. 2.106).

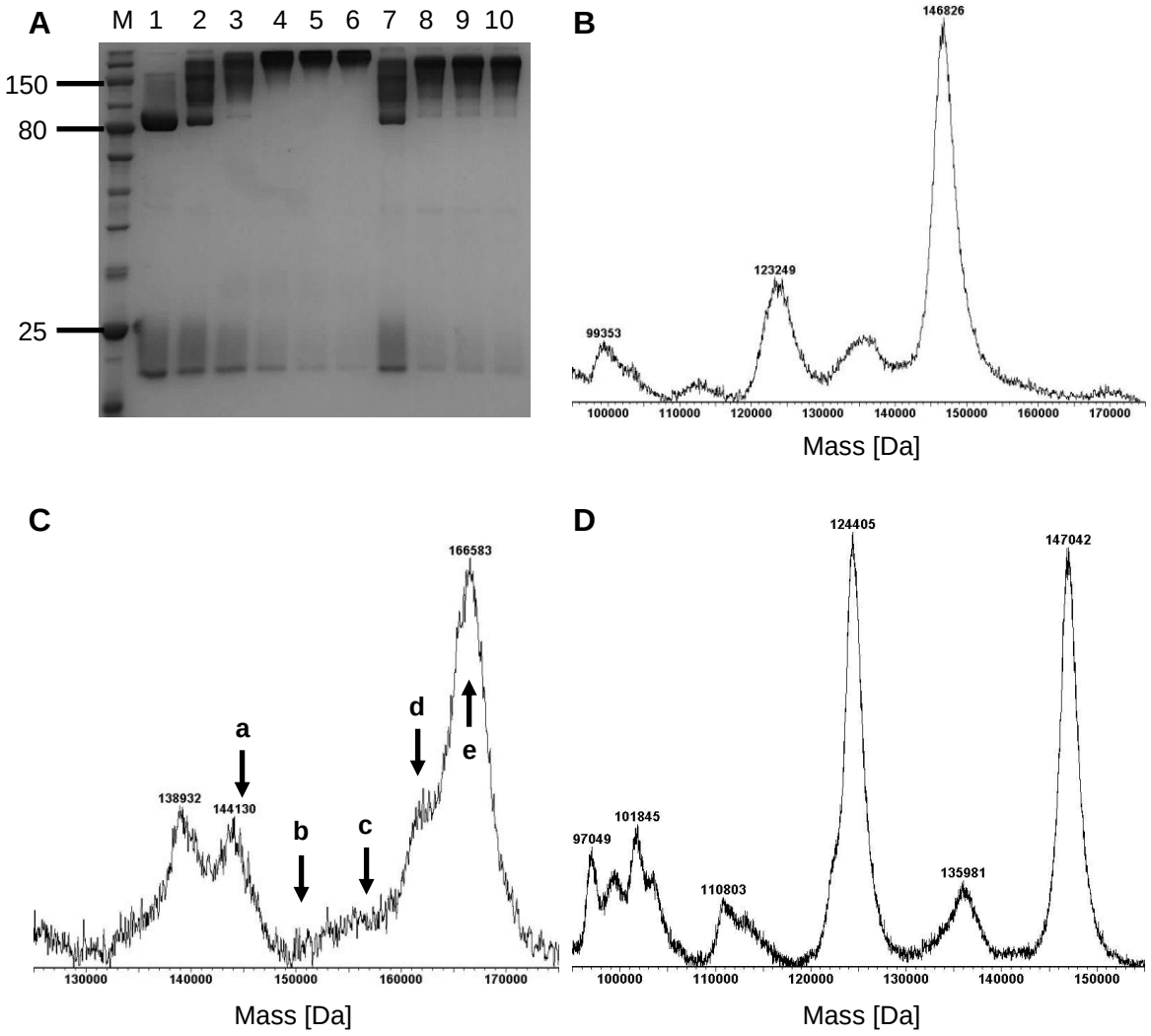


Fig. 2.106: Stepwise modification of rituximab. (A) SDS-PAGE analysis. 1) Reduced antibody (80 equiv TCEP, 1 h). 2) Control with 20% DMF added to the reduced antibody. 3) 4 equiv of *N*-PEG5000-dithiophenolmaleimide **31**. 4) 8 equiv. 5) 12 equiv. 6) 16 equiv. 7) 4 equiv of dithiophenolmaleimide **30** in 20% DMF. 8) 8 equiv. 9) 12 equiv. 10) 16 equiv. (B) MS of unmodified rituximab. (C) MS of the product from lane 6). Arrows indicate the proposed number of attached PEG chains. **a** = 0, **b** = 1, **c** = 2, **d** = 3, **e** = 4. (D) MS of the product from lane 10).

The samples from the organic solvent free PEGylation reaction, where 12 or more equiv of the maleimide were used, looked almost perfect by SDS-PAGE, and MS analysis showed a similar picture with the main product being the 4 times modified antibody. A certain amount of fully and incompletely PEGylated HHL fragments are also visible (at the position of the unmodified antibody). However, the same fragment is found in the spectrum of the unmodified antibody, and so is at least partially an artefact of the MS technique (see 2.3.2). In contrast, fragmentation is clearly more pronounced when organic solvent was added to the reaction for simple bridging. The main product here is the HHL fragment. The resolution of MALDI-TOF MS is not good enough to define if bridging had taken place, but an upwards mass shift (380 Da would be expected in the case of 4 maleimide bridges) which was consistently observed in subsequent reactions, served at least as a positive indicator. The incomplete preservation of the antibody structure was probably again caused by the presence of DMF. In fact, in the control reaction where the solvent alone was added to the reduced antibody (Fig. 2.106 A, lane 2), the appearance of HL, HH and HHL fragments suggests that complex and potentially even disulfide bond formation is actually induced by DMF. This effect is difficult to rationalise and has not been mentioned in the literature where similar reaction sequences have been carried out.[228] Nevertheless, as many potential candidate compounds for attachment to an antibody will require the presence of organic solvent, it was decided to try to find optimised conditions for the stepwise bridging of rituximab.

To this end the procedure was repeated with different organic solvents in varying amounts, dibromomaleimide or dithiophenolmaleimide in different quantities, and analysis carried out after different reaction times (data not shown, see 4.10.12). The best conditions were found to be 16 equiv of the dithiomaleimide reagent added in 10% DMSO (total final concentration) to the antibody and purification after 30 min (Fig. 2.107). For comparison bridging was also carried out using the established *in situ* protocol with benzeneselenol and dithiophenolmaleimide (in a 30 : 60 ratio), and the DTT-based sequential method (30 equiv dibromomaleimide were added in DMF to a final concentration of 20% after reduction with 20 equiv DTT for 4 h).

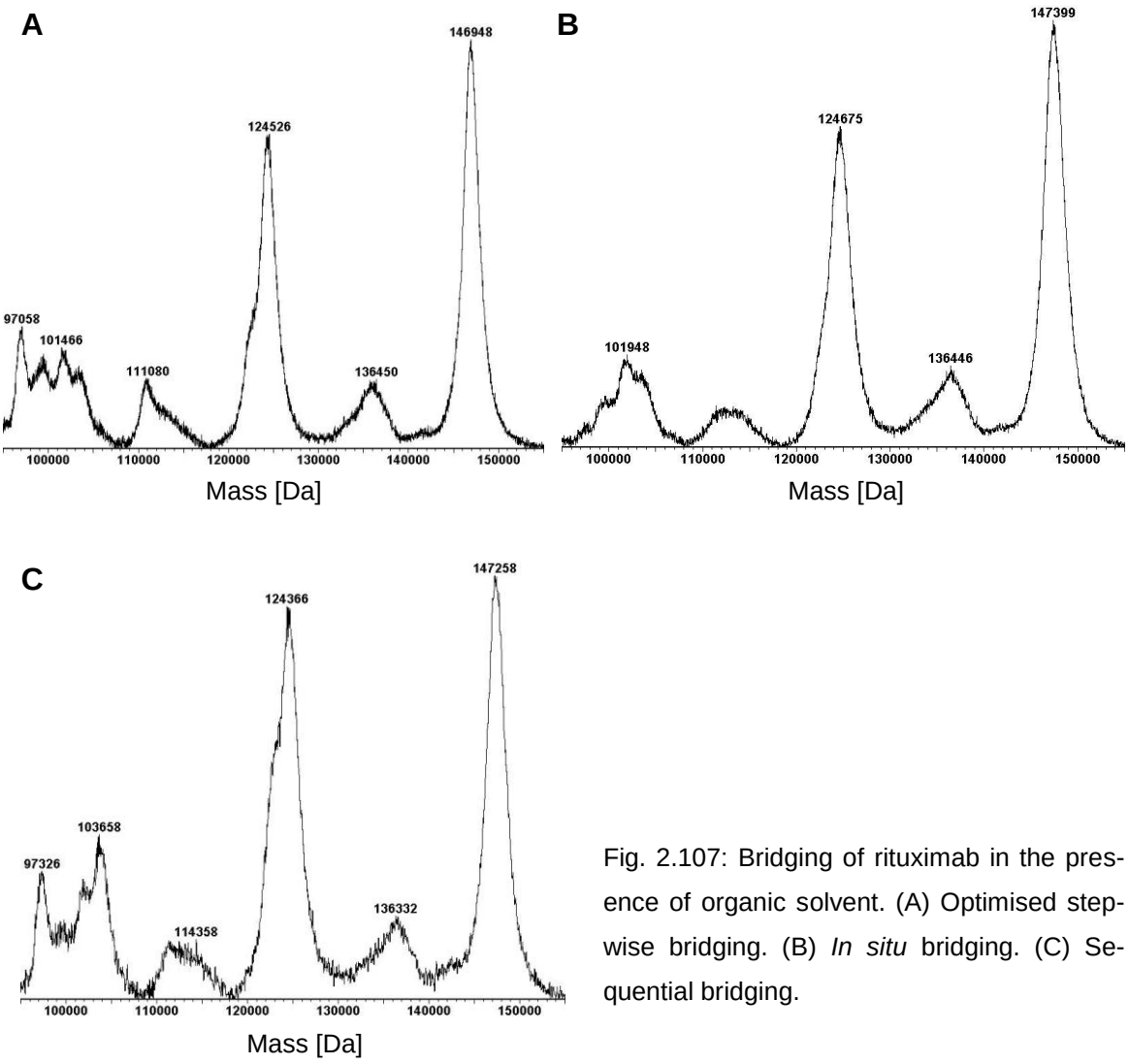


Fig. 2.107: Bridging of rituximab in the presence of organic solvent. (A) Optimised stepwise bridging. (B) *In situ* bridging. (C) Sequential bridging.

The main signal under all three conditions corresponded to the full antibody, but the MS spectra still suggested the presence of a substantial amount of fragments. As in contrast the stepwise PEGylation performed much better, it seems clear that the efficiency of the reaction is context dependent. For this reason no further refinement of the process was attempted. Good water solubility of the bridging reagent is definitely an important parameter. Overall the stepwise protocol afforded the most homogeneously modified products of all the developed methods, as well as the highest modification number.

2.11.5 Site-selectivity of the *in situ* and sequential modification of rituximab

In comparison to the stepwise protocol the *in situ* and sequential methods yielded mixtures of variously modified antibody products, with very distinctive patterns when benzeneselenol or TCEP were used as reducing agents. This sug-

gested the potential existence of a preference of reducing agents for the cleavage of certain disulfide bonds. This would thus result in a desirable site-specificity of the modification. Such a phenomenon has been described in the literature but the overall picture is far from uniform. Early work showed good specificity of 2-mercaptoethanol and 2-mercaptoethylamine for the hinge region cystines,[454, 455] which is supported by the observation that these cysteines are the most reactive.[452] DTT in contrast, was found to have a preference for the switch region disulfides by Sun and colleagues[229] as well as Liu and co-workers.[313] However, other reports claim specificity of the dithiol for the hinge region.[381, 450, 456] Another group again published only recently that they had observed no targeting of specific antibody disulfide bonds with both TCEP and DTT.[379] As the most striking difference in all of these studies is the identity of the antibody, it is quite plausible that each of these molecules behaves differently, based on sequence and isotype.[313]

To understand rituximab in this regard, a MS based approach was initially chosen. The anti-CD20 antibody was PEGylated using the established *in situ* and sequential conditions and digested with papain, which would cleave rituximab into the Fab and the Fc fragments.[328] These were separated on protein A columns, and subjected individually to MALDI-TOF MS. The following example has been generated with a 40 : 10 mixture of benzeneselenol and *N*-PEG5000-dithiophenolmaleimide **31** (Fig. 2.108).

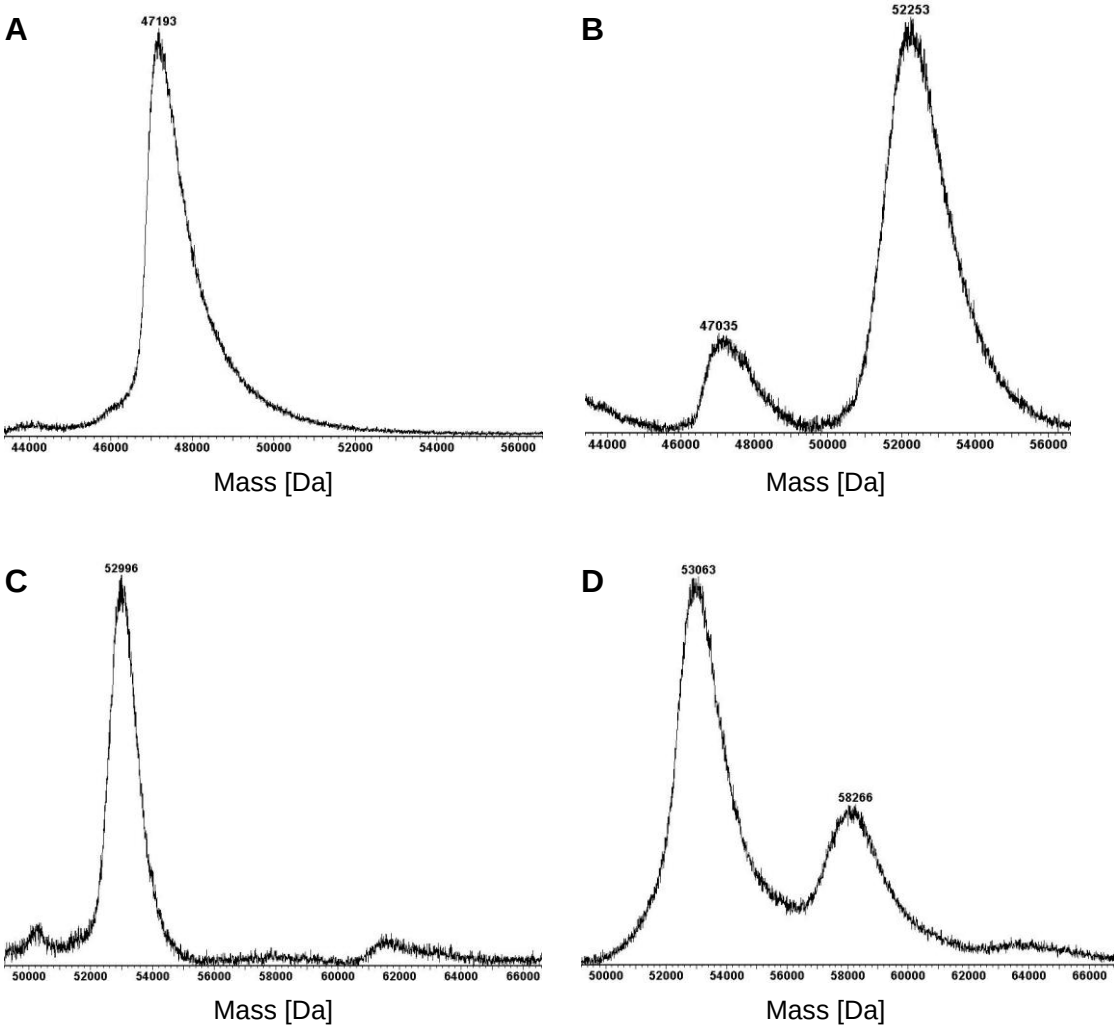


Fig. 2.108: MS analysis of rituximab fragments after *in situ* PEGylation. (A) Fab fragment of the unmodified antibody. (B) Fab fragment after PEGylation. (C) Fc fragment of the unmodified antibody. (D) Fc fragment after PEGylation.

The initial data looked encouraging as the MS spectra contained a strong signal of the PEGylated Fab fragment and a clearly weaker signal for mono, and almost no double PEGylated Fc fragment. Together this suggests a preference of the benzeneselenol-based method for the switch region disulfide bonds. Unfortunately a control of all prepared samples by SDS-PAGE revealed that the proteolytic cleavage was not quantitative (Fig. 2.109).

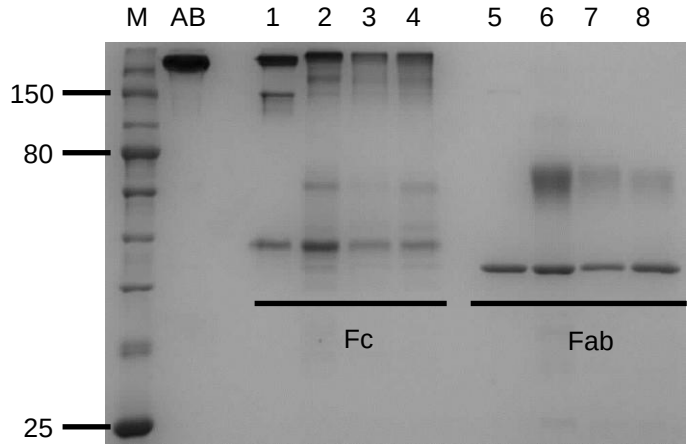


Fig. 2.109: SDS-PAGE analysis of papain digests of rituximab and PEGylated analogues. 1+5) Unmodified antibody. 2+6) *In situ* PEGylation (40 : 10 ratio of benzeneselenol : N-PEG5000-dithiophenolmaleimide **31**). 3+7) TCEP-based sequential PEGylation (10 equiv TCEP for 1 h, then 20 equiv **31**). 4+8) DTT-based sequential PEGylation (20 equiv DTT for 4 h, then 25 equiv *N*-PEG5000-dibromomaleimide **25**).

With an incomplete digest the data could not be used for quantification. Still some interesting features were found; the strongest Fab-PEGylation signal was obtained with benzeneselenol as the reducing agent. The overall yield of fragments from the digests dropped from 25% in the case of the *in situ* modification, to 14% with TCEP, and 7% with DTT as the reducing agents. As PEGylation is used to protect proteins from proteolytic degradation this could be an indicator for increased hinge region modification due to the proximity of these disulfide bonds to the papain cleavage site.

This of course also revealed a general flaw of the approach; the combination of PEGylation and proteolytic digest. To avoid this problem rituximab was simply bridged with 3,4-substituted maleimides using the developed methods, digested, deglycosylated to generate a homogenous sample, and the fragments separated and analysed by LCMS (data not shown). Although the fragment yields were increased to the around 35% observed with unmodified antibody, quantification was again impossible. Still the LCMS data followed the observed trends; stronger signals of bridged Fab fragments generated by the *in situ* method, more modifications in the hinge region containing Fc fragment with TCEP, and DTT seemingly targeting both types of cystines equally.

To obtain further confirmation for these observations a fragment-modification approach was adopted. This time the Fc and Fab were prepared by digest from

the unmodified antibody and then subjected to the developed chemistry. Although this introduces a deviation from the real system, crystal structures of intact antibodies show that the presence of the Fab fragments has a limited impact on the solvent accessibility of the Fc disulfide bonds and vice versa, with distances of 20 Å and more between the Ig domains.[310] In addition, the antibody structure is quite flexible in the hinge region,[316] and the Fab sequence containing the cysteines interacts little with the surrounding heavy chain domains.[457]
To this end the purified rituximab fragments were treated with the *in situ* and sequential procedures using the PEGylation reagent to visualise the modification by SDS-PAGE (Fig. 2.110).

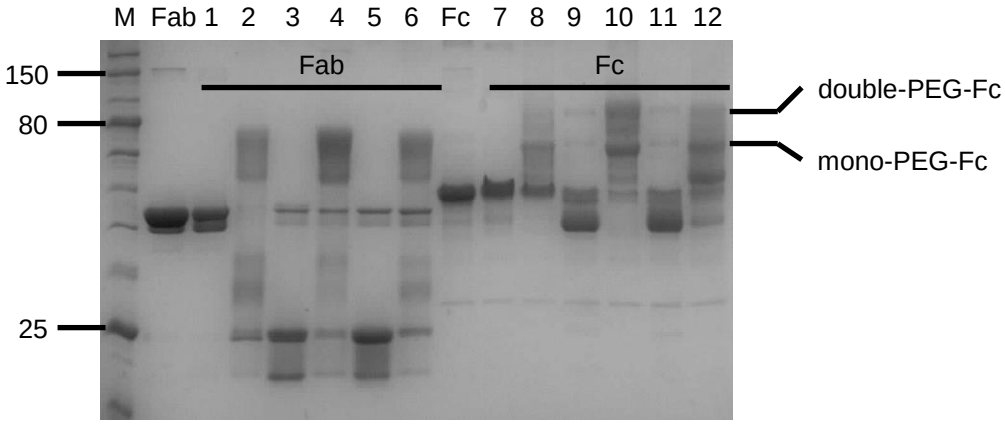


Fig. 2.110: PEGylation of rituximab fragments. 1+7) Reduction control with 40 equiv benzeneselenol. 2+8) *In situ* PEGylation with a 40 : 10 ratio of benzeneselenol : *N*-PEG5000-dtihiophenolmaleimide **31**. 3+9) Reduction control with 10 equiv TCEP (1 h). 4+10) Sequential PEGylation with 20 equiv of PEGylation reagent **31** after reduction with 10 equiv TCEP (1 h). 5+11) Reduction control with 20 equiv DTT (4 h). 6+12) Sequential PEGylation with 25 equiv of *N*-PEG5000-dithiophenolmaleimide after reduction with 20 equiv DTT (4 h).

Here the overall picture became more distinct. Full PEGylation of the Fab fragment was achieved only with the *in situ* method, while the same protocol yielded only small amounts of modified Fc. Conversely, the TCEP based conjugation generated a lot of mono and double PEGylated Fc, but Fab modification was incomplete. DTT again showed no preference for a particular fragment, and it was thus concluded that at least for rituximab no site-specificity could be achieved using this reducing agent. It should be noted that in this experiment all reagents were present in twice the amount per disulfide bond than before, as each fragment solution contained only half the cystines of the full antibody.

In order to obtain a more detailed picture, it was decided to add the reducing agent in limiting amounts, when both of the fragments are present. Therefore they would then “compete” for the reducing agent. To find the ideal conditions, the Fab and Fc of rituximab were treated separately with different concentrations of TCEP for varying times, and completeness of reduction was analysed by SDS-PAGE (data not shown). 10 Equiv of the phosphine (a 1 : 5 ratio of cystine to reducing agent) were sufficient to break all Fc disulfide bonds in 2 h. In contrast a 4 h incubation time was required with 5 equiv TCEP (in the same ratio) to cleave the Fab cystine almost completely. These findings were used in the outlined experiment; Fab and Fc fragments were mixed in a 2 : 1 ratio as would be the case in a full antibody, treated with 2–15 equiv TCEP for 1 h, and *N*-PEG5000-dithiophenolmaleimide added (Fig. 2.111).

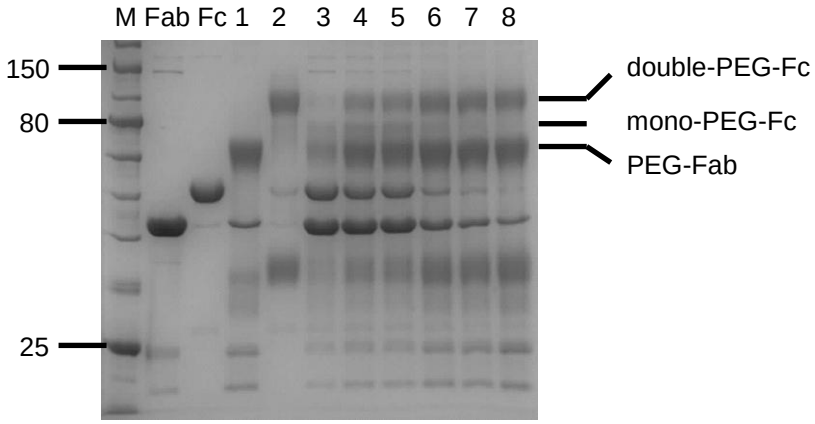


Fig. 2.111: Sequential PEGylation of a mix of rituximab Fab and Fc fragments. Samples were treated with TCEP for 1 h, followed by addition of 20 equiv *N*-PEG5000-dithiophenolmaleimide **31** and thermally denatured before loading. 1) Fab fragment treated with 10 equiv TCEP and PEGylation reagent **31**. 2) Fc fragment treated with 10 equiv TCEP and PEGylation reagent **31**. 3) 2 : 1 mix of Fab and Fc treated with 2 equiv, 4) 4 equiv, 5) 6 equiv, 6) 8 equiv, 7) 10 equiv and 8) 15 equiv TCEP before addition of the PEGylation reagent **31**.

The results strongly suggest that the sequential PEGylation reaction based on TCEP has a clear, but not exclusive, preference for the hinge region disulfide bonds. Both fragments were modified relatively equally when small amounts of the phosphine were added. However, using 8 equiv and more the signal intensity of the unmodified Fc fragment disappears more rapidly than of the Fab fragment. The possibility that this was simply a statistical effect can be excluded by the observation that the band of the mono PEGylated Fc product was very faint

in low TCEP samples. This became even weaker with increasing amounts of reducing agent. This together with the presence of still unmodified Fc further implies, that reduction of one of the hinge region cystines is required for access to the next one. An effect which has been found to be the case in other antibodies.[216, 313] Indeed, observing the effect in this experiment supports the idea that the fragment-modification approach simulated the full antibody correctly. Opposing to this was the appearance of many more free chains than observed with the full intact antibody (see Fig. 2.102 A). Their presence can be attributed to an overall loss of stability of the complex in the antibody fragments. Alternatively, the thermal denaturation of the samples before loading onto the gel to improve the resolution between the relatively similar antibody fragments may be the cause.

The experiment was repeated with the *in situ* protocol. Since benzeneselenol does not allow for a reduction study to be carried out, a relatively wide margin of reagent mixtures were used (Fig. 2.112).

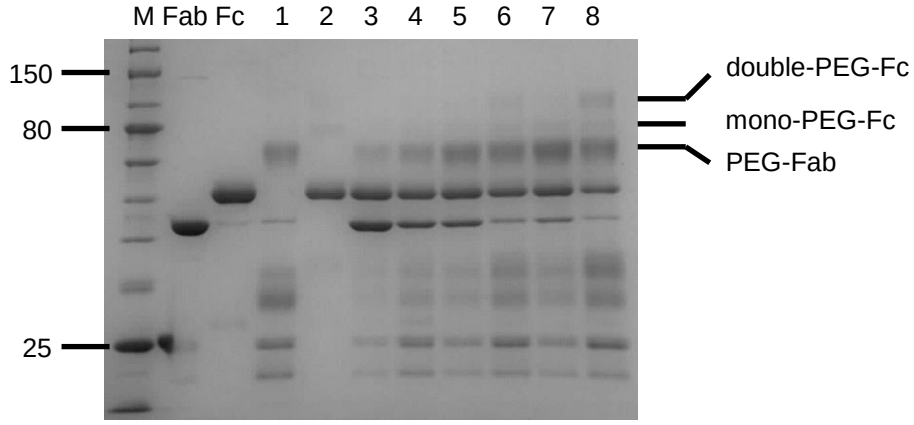


Fig. 2.112: *In situ* PEGylation of a mix of rituximab Fab and Fc fragments. Samples were treated with following ratios of benzeneselenol : *N*-PEG5000-dithiophenolmaleimide **31** and thermally denatured before loading. 1) Fab fragment treated with 30 : 5. 2) Fc fragment treated with 30 : 5. 3) 2 : 1 mix of Fab and Fc treated with 30 : 2, 4) 60 : 2, 5) 30 : 5, 6) 60 : 5, 7) 30 : 10 and 8) 60 : 10.

Already the controls with single fragments revealed the inability of the benzeneselenol-based method to target the Fc disulfide bonds efficiently. In agreement with this observation, the mixed samples contained predominantly PEGylated Fab and only very faint signals for the modified Fc. Both the amount of reducing agent and maleimide were important to achieve specificity, in addition to good

conversion rates. As observed previously, larger amounts of released single chains were found. This suggests, in the light of the site-specificity of the *in situ* method, that the presence of intact hinge region disulfide bonds seems to support the maintenance of the protein complex during the process of modification.

In summary, despite the impossibility to perform all of the experiments designed to elucidate the potential of site-specificity of the introduced chemical methodology on the native system, conclusions can be made. All experiments revealed a common picture; TCEP-based sequential protocols offer some specificity for the hinge region, the *in situ* methods are targeting the switch region cystines with good specificity and any method based on DTT comes without any discernable preference for a certain type of disulfide bond. These findings were well reflected in the data collected so far with the full antibody. However, in the light of previously published studies they should for the moment be perceived as confined to rituximab.

2.11.6 Activity and stability of rituximab analogues

After establishing a number of efficient protocols to modify the target antibody and the identity of their products, the activity of these analogues was tested by flow cytometry. To broaden the approach, functionalised versions of rituximab were also prepared; fluorescent antibody via the sequential DTT-based protocol, and bridged and spin labelled antibody via the *in situ* method (see legend Fig. 2.107). The intensity of the fluorescein-rituximab emission was strong even at low pM concentrations (data not shown), indicating the presence of numerous fluorescein molecules per antibody. The spin labelled analogue was found to have an average of 2.42 labels per protein. This was a bit higher compared to what would have been expected from the PEGylation data. However, issues with the MALDI-TOF MS quantification or a different labelling efficiency of the spin label due to a less bulky substituent are valid hypothesis for this difference.
Flow cytometry was carried out in parallel to a published procedure with rituximab.[445] Raji cells, a Burkitt's lymphoma B cell line, were treated alongside positive, negative and processed controls with the rituximab analogues. Fluorescently labelled anti-human IgG $F(ab)_2$ fragment was then added and subsequently fixed. 5,000 Cells were counted per sample and the fluorescence com-

pared after gating for live cells (overall survival rate was 60–70%) (Fig. 2.113). The assay was performed with Ms Carolina Fernandes (MRCT).

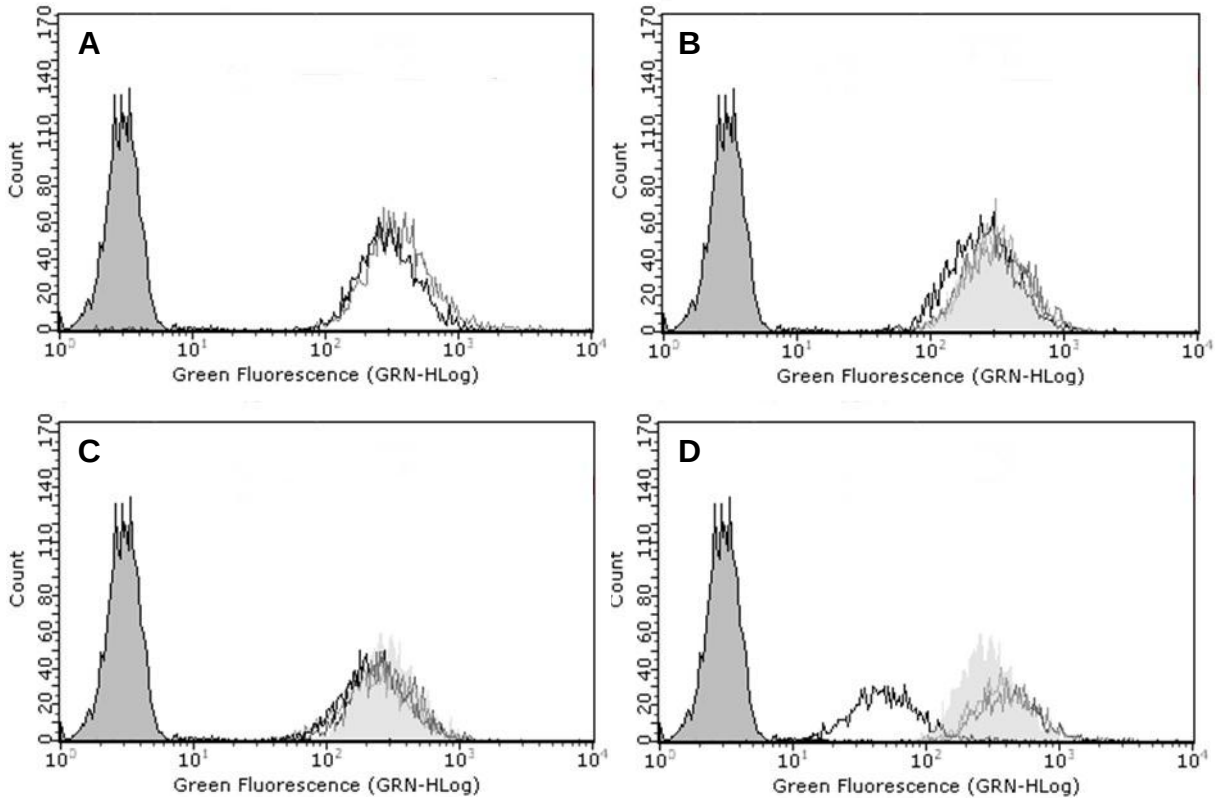


Fig. 2.113: Flow cytometric analysis of rituximab analogues. The solid grey filled signal is the negative control. (A) Positive controls with unmodified (black line) and processed rituximab (grey line). (B) *In situ* PEGylated rituximab with benzeneselenol : PEGylation reagent ratios of 40 : 10 (light grey line), 20 : 40 (grey line) and 30 : 60 (black line). The light grey filled signal is the processed sample. (C) Sequential PEGylated rituximab with TCEP-based (light grey line), TCEP+DTT-based (grey line) and DTT-based (black line) samples. (D) Functionalised rituximab with bridged (light grey line), spin labelled (grey line) and fluorescent (black line) sample.

Overall the fluorescence profiles of almost all samples overlaid very well with the signal of the unmodified antibody, indicating retention of activity. The only exception is the fluorescently labelled rituximab. However, this is based on the presence of a different fluorophore on the secondary antibody, which could not be used with this analogue (here the direct fluorescence of the sample was used for detection). Unfortunately the stepwise protocol had not been developed at the time of the experiment. But as the sequential PEGylated samples contain some of the fully modified antibody, and were seemingly fully active, a similar result for the stepwise modified analogues is likely. For comparison the

same assay was performed with rituximab labelled via its lysine residues,[451] and reduced disulfide bonds.[450] These revealed a loss of up to 40% and 30% of activity respectively. This indicates that both site-specific labelling as well as the preservation of the disulfide bonds are favourable features of the herein described chemistry.

To follow up on the importance of the integrity of the cystine network, the thermostability of selected rituximab analogues was examined. To simulate the different states of a disulfide bond a few additional samples were prepared. The antibody was sequentially bridged (20 equiv DTT for 4 h, then 25 equiv dibromomaleimide) and modified in the same way with *N*-phenyl-dibromomaleimide (kindly provided by Dr. Mark Smith, UCL Chemistry), followed by incubation for 16 h at 37 °C to hydrolyse the maleimide bridge. Furthermore, rituximab was alkylated using a partial reduction approach as published by Sun *et al.* (method B).[229] Here the antibody was treated with a small amount of TCEP (2.75 equiv) followed by addition of 4.4 equiv of plain maleimide, analogous to the established sequential modification method. Analysis of these analogues by SDS-PAGE revealed the expected substantial fragmentation of the non-rebridged sample (Fig. 2.114).

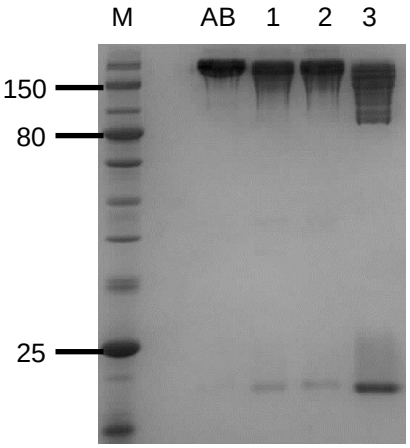


Fig. 2.114: Rituximab analogues prepared for the thermostability assay. 1) DTT-based sequential bridged antibody. 2) Antibody sequential (DTT-based) bridged with *N*-phenyl-dibromomaleimide and incubated at 37 °C for 16 h. 3) Alkylated antibody prepared via partial reduction.

For the thermostability assay the samples were heated stepwise in the presence of the hydrophobic dye Sypro orange. Upon heat induced unfolding of the protein, this compound associates with the revealed lipophilic sequences by which its fluorescence is activated. This temperature dependent increase of fluorescence represents the read-out and was fitted to obtain an average melting

temperature (Fig. 2.115). The experiment was carried out with the help of Ms Carolina Fernandes (MRCT).

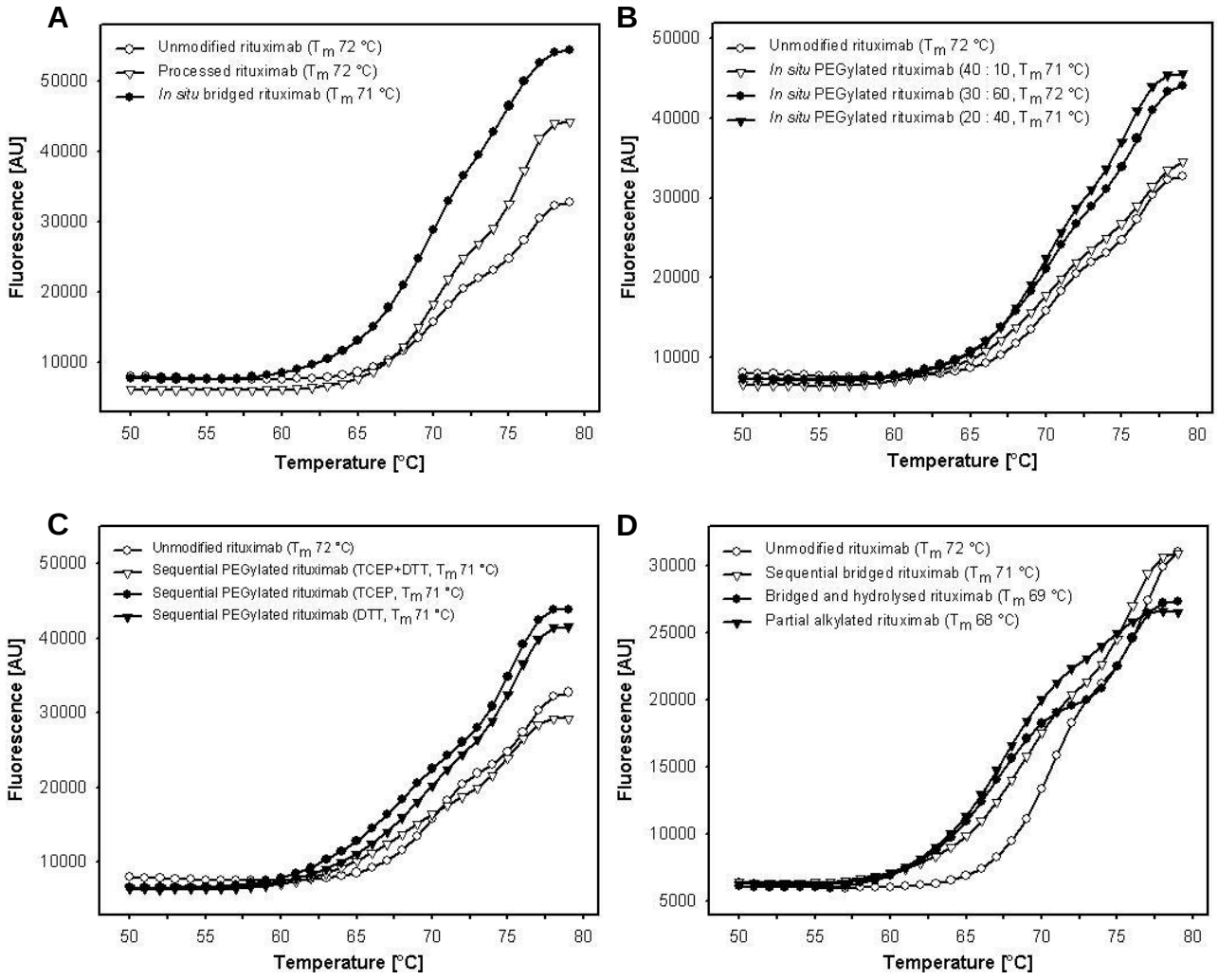


Fig. 2.115: Thermostability assay with rituximab analogues. Melting temperatures shown are the calculated average. (A) Controls and *in situ* bridged antibody. (B) *In situ* PEGylated antibody. Numbers in brackets are equiv used of benzeneselenol : *N*-PEG5000-dithiophenol-maleimide **31**. (C) Sequential PEGylated antibody. (D) Samples with various cystine modifications.

In general the melting temperature and behaviour of the antibody compares well with previously published data.[458] The two step-unfolding is based on the low stability of the C_H2 domain, which loses its structure before the remaining molecule starts to unfold. The detected increase in fluorescence starts at lower temperatures in all samples of the modified antibody, and this effect is less pronounced when PEG chains had been introduced. A potential explanation is that the loss of stability is induced by the insertion of a hydrophobic group (the ma-

leimide), which was partially lessened by the presence of the polymer. Alternatively, the PEG chains may protect the antibody from unfolding, or simply hinder the access of the fluorescent dye to hydrophobic regions. Nevertheless, the average melting temperature of most samples is almost identical with the unmodified antibody. The two exceptions are the rituximab analogue with hydrolysed maleimide bridges and the alkylated sample. While the first effect might be attributable to the charged nature of the open maleimide ring, the second is clearly based on the loss of the disulfide bonds. In both cases the changes were not large, 3 °C and 4 °C respectively, especially compared to the drop in melting temperature by 8 °C upon loss of a (intramolecular) disulfide bond,[459] or its increase by up to 11 °C after addition of a (again intramolecular) cystine,[225] which has been found in other systems. With this regard, the performed assay may not have been ideal, as the intermolecular disulfide network of an antibody has only a small influence on the structural stability of its individual domains. However, re-bridged antibody samples showed a better performance than that which had lost its disulfide bonds.

Besides stability the aggregation behaviour of an antibody, naked or conjugated, is one of its most important biophysical properties though it is rarely commented on in the relevant literature. Aggregation is problematic for several reasons; it influences the yield and storage capacities of antibody production and modification, and resulting aggregates are often highly immunogenic and so avoiding their formation in clinical grade material is critical.[460] The gold standard at the moment for the detection of potential aggregates is size exclusion chromatograpy – multi angle light scattering (SEC-MALS). Here particles with different molecular weights are first separated by SEC and their oligomeric state analysed in sequence of their elution by MALS. This is possible as the light scattered by a particle is directly proportional to its molecular mass multiplied by the concentration. Coupling the system to an absorbance and/ or refractive index detector enables the accurate determination of the concentration, as well as the calculation of the mass distribution of the sample over its mono- and multimeric species. Therefore it can be used to reveal the presence and quantity of aggregates.

To determine the potential of the herein developed chemical methods to induce aggregation, rituximab was bridged via the *in situ*, sequential and stepwise process and subjected to SEC-MALS analysis alongside controls. Two spectra are shown (Fig. 2.115) as examples for the raw data and the analysis is summarised in Table 2.16. The assay was performed with the help of Dr Lone Friis (MRCT).

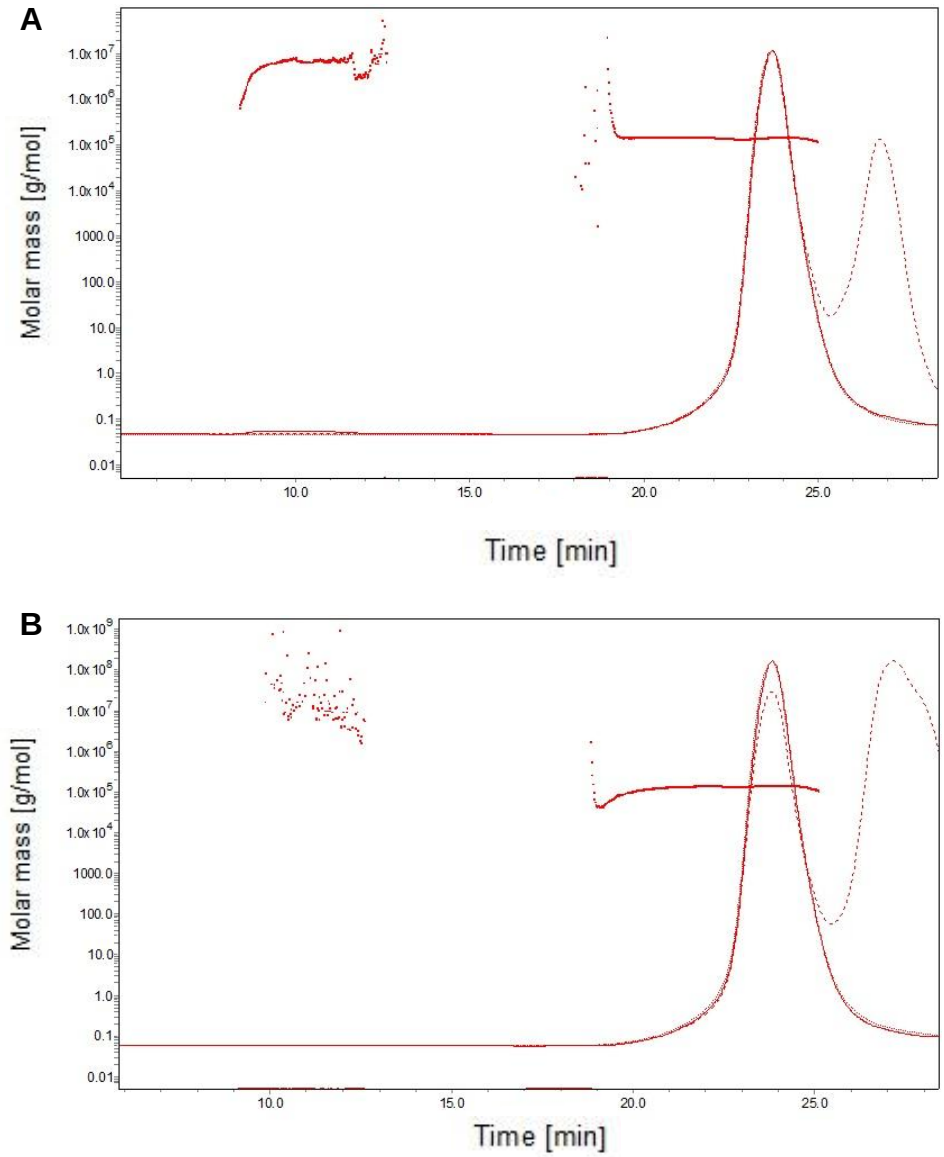


Fig. 2.115: Examples of the SEC-MALS analysis of rituximab and maleimide bridged analogues. Solid line = UV absorbance at 280 nm. Dashed line = refractive index. Dotted line = multi angle light scatter. (A) Spectrum of the unmodified antibody. (B) Spectrum of the sequential bridged antibody.

Table 2.16: Aggregation behaviour of rituximab and bridged analogues. N.t. = not detectable. Mn = mean molecular mass. “Signal” refers to the scattered light.

	Monomer				Aggregates			
Treatment	MW/ Mn	Fraction signal [%]	MW [kDa]	Fraction mass [%]	MW/ Mn	Fraction signal [%]	MW [kDa]	Fraction mass [%]
untreated	1.002	99.80	137	82.9	n.t.	0.2	n.t.	n.t.
processed	1.003	100	133	93.0	n.t.	n.t.	n.t.	n.t.
in situ bridged	1.003	99.99	136	92.7	1.015	0.01	3,924	n.t.
sequential bridged	1.004	99.99	134	99.9	1.006	0.01	6,520	n.t.
stepwise bridged	1.003	99.93	134	100	1.105	0.07	25,160	n.t.

The read-out of the refractive index detector showed that in addition to the antibody and its aggregates, another molecular species without optical activity was present. As it generated no UV absorbance and caused no light scatter, it was assumed that it had to be the polysorbate 80 preservative, which had not been removed completely when the clinical formulation of rituximab was exchanged for PBS. The analysis revealed that effectively no aggregates were present before or after modification. The main fraction was nearly perfectly monodisperse. This was suggested by the ratio of the molecular weight and the mean molecular weight (MW/ Mn) being close to 1, the generation of almost the entire scattered light signal by this fraction and the calculated mass clearly indicating a monomer. This mass is around 7% lower than the one expected from the amino acid sequence, however this is possible due to the globular and compact structure of the antibody domains. Only the mass fraction attributed to the monomer was a bit low for some of the samples. This could possibly be explained by a slightly incorrect positioned baseline, which was difficult to establish in the presence of the additional polysorbate 80 signal. Furthermore, the small peaks that were observed in the region of potential aggregates generated not enough signal intensity to account for any mass in the overall distribution. Against this background it seems safe to assume that the mass fraction of the monomer corresponds to its signal fraction, indicating the presence of only negligible amounts of aggregates.

In conclusion, modification of clinical grade rituximab with 3,4-substituted maleimides afforded in complete conversion nearly fully active products, without a

decrease in the antibody stability or the generation of aggregates. Depending on the choice of the process and the reducing agent, different modification numbers were accessible as well as a certain degree of site-specificity. In terms of these parameters and within the limitation of the explored model systems, the novel chemistry developed herein outperforms the methods that are currently used. Thus next generation maleimides might be the key to the synthesis of safer and more potent ADCs and other immunoconjugates in the future.

3 Summary and Outlook

3.1 Summary

The aim of this PhD project has been the development of a new method for the preparation of bioconjugates and the demonstration of its utility in a range of proteins with different target profiles, especially with regard to their disulfide bond connectivity. Already in a small molecule test-setting the envisaged reaction of 3,4-substituted maleimides with two thiols turned out to be not only feasible, but rapid and highly efficient. Dihalomaleimides reacted with two equivalents of a cysteine analogue within minutes, yielding a prototype of a maleimide bridged disulfide bond in complete conversion. The high fidelity of the reaction was preserved when carried out in aqueous buffers. This is an important property for a new methodology as water is almost always the solvent of choice for protein work.

The findings of these early experiments were fully transferrable to the small peptide hormone somatostatin. A sequential protocol, that is reduction followed without intermediate purification by bridging, furnished the maleimide bridged peptide in 100% yield. The observed reaction rates were comparable to those of plain maleimides.[95] Another useful feature of the developed chemistry was revealed when the product was treated with a large excess of thiol-reducing agents. Under these conditions the bridging reaction is reversible and the maleimide is cleaved from the protein. Such a mechanism can be desirable in a number of applications such as ADCs,[38] the (strept-)avidin-biotin system,[142] or structural and receptor studies.[58] To broaden the scope of this investigation a number of *N*-functionalised substituted maleimides were prepared. Acceptable yields of these compounds were obtained in one-step synthesis, either by reaction of the dibromomaleic anhydride under acidic conditions, or with a modified version of the Mitsunobu reaction. No negative impact of even large *N*-substituents, such as a 5 kDa PEG chain, was observed on the speed or efficiency of the conjugation reaction to somatostatin. Most important, all of the prepared analogues of the peptide hormone exhibited full biological activity in patch-clamp experiments, revealing that the insertion of maleimide into a disulfide bond can be well tolerated.

To reduce the negative effects that even temporary reduction of a disulfide bond can have, somatostatin was used for the development of *in situ* protocols where the substituted maleimide is added before the reducing agent. Dibromomaleimide displayed a too high cross-reactivity with TCEP to be of use in this process, however dithiomaleimides, especially dithiophenolmaleimide, were found to combine good reactivity with high resistance towards the phosphine. These compounds were used to bridge somatostatin *in situ* with either TCEP or benzeneselenol as the reducing agent. The reactions proceeded with near to complete conversion reached within minutes and again *N*-functionalised dithiomaleimides could be employed without loss of reactivity.

Although not necessary in a simple system such as somatostatin, the developed *in situ* protocol quickly turned out to be essential during the modification of insulin. The auto-reductive behaviour of the hormones' cysteines after reduction, made a sequential approach impossible. An optimisation process was carried out carefully to modify and functionalise insulin successfully via its cystines. Measures taken included lowering of the pH, the use of dibenzeneselenolmaleimides (rather than dithiophenolmaleimides), and the stepwise addition of the reducing agent 2-mercaptoethanol to the reaction mixture. This furnished (although in low yields when purified) double maleimide bridged, PEGylated or spin labelled insulin. The reaction did not allow for the isolation of the mono-modified hormone, but it has been shown that the attachment of two PEG chains as opposed to one increases the stability of insulin much more substantially.[295] In addition, a doubly spin labelled version has the potential to reveal more structural information about the analogue than with a single label attached.[305] Surprisingly, a performed digest study indicated the modification of the buried A6 – A11 disulfide bond, alongside the solvent accessible A7 – B7 cystine. This suggests, changes in the structure of the conjugated hormone, and this is supported by recorded CD spectra. Additional characterisation of the product would be necessary to determine their usefulness.

In contrast to insulin, the modification of an anti-CEA ds-scFv antibody fragment via its engineered disulfide bond was easily accomplished. A sequential protocol with DTT and dibromomaleimides, and alternatively an *in situ* method with benzeneselenol and dithiophenolmaleimides, furnished the maleimide bridged and a number of functionalised anti-CEA analogues in rapid reactions with full

conversion. Another advantage of the *in situ* process was revealed by the absence of dimers, which were seen in the sequential modified material. All ds-scFv analogues showed an increase in their biological activity compared to the unmodified anti-CEA, and displayed the desired functionality in a range of biochemical and biophysical assays. As good stability of the maleimide bridge under *in vivo* conditions is vital for many of the envisaged applications, this parameter was tested. A modified antibody fragment was incubated in human plasma and the stability of the bridge was found to be excellent, with no observed loss of the maleimide and retention of more than 85% of activity over 7 days.

Finally the modification of full antibodies based on substituted maleimide chemistry was developed step by step. Initially anti-FLAG tag mouse antibodies were used, and although successful PEGylation and spin labelling could be shown as well as retention of the activity, the limited amounts of available material made the optimisation of the process impossible. In contrast enough anti-MSP1 antibody was available to better understand the processes involved in the modification of these multiple disulfide containing protein complexes. These insights enabled the substantial reduction of the amounts of reagents necessary for efficient bridging, PEGylation and spin labelling. Unfortunately, the high tendency of this antibody to form aggregates made further characterisation of the reaction products difficult, which was partially reflected in the apparent loss of activity during ELISA tests with the prepared anti-MSP1 analogues.

Nevertheless, both projects had laid the foundation for the work with a more favourable system – rituximab. Here three different protocols were developed; sequential modification, based on DTT or TCEP, *in situ* modification with benzeneselenol, and a stepwise process where the excess of TCEP was removed before reaction of the fully reduced antibody with the substituted maleimides. Different conjugation patterns were observed with the different methods. Despite some issues arising due to the presence of organic solvents in the reaction mixtures, good conversion rates could be achieved while maintaining the quaternary structure of rituximab. Optimisation of the processes enabled the synthesis of relatively homogeneous products with the *in situ* and stepwise protocols, and even a limited amount of site-specificity for certain types of disulfide bonds was observed. Full binding activity, preservation of the stability and the

presence of negligible amounts of aggregates after the reaction were found in various assays with maleimide bridged and functionalised rituximabs.

3.2 Critical evaluation of the developed method

Overall a number of clinically relevant peptides and proteins were successfully modified via the envisaged “functionalisation by re-bridging”. A number of positive properties of the developed chemistry can be concluded from these experiments.

A consistent parameter was the high speed with which the re-bridging of reduced disulfides proceeded. This is certainly one of the reasons for the full conversion rates observed in almost all reactions (sometimes not reflected in the purification yields, depending on the system) together with the generation of very few side products. The synthesised bioconjugates are homogeneous if a single accessible disulfide bond is present and still relatively uniform when more cystines are available. The maleimide bridge was stable in human plasma for extended periods of time but can still be cleaved by a large excess of thiols, such as found inside living cells, implying good potential as a drug delivery system. Although no NMR or crystal structures were obtained, the preservation of the biological activity in most of the model systems yielded indirect evidence that the insertion of the maleimide into a disulfide bond was structurally well tolerated. All bridging reagents used, including the *N*-functionalised maleimides, are synthetically easy accessible in a few steps making their preparation inexpensive. This also means that a great variety of functional groups can be quickly tested in a given system, as they only have to be attached to the same moiety (the maleimide) and conjugated via a single protocol to the protein(s) of interest. Other benefits include simple product purification, due to the limited water solubility of the substituted maleimides (see below), no introduction of additional chiral centres, and the optical properties of dithiomaleimides, which allow some reactions to be followed by UV/ Vis spectroscopy.

Another, less quantifiable advantage, is the flexibility and adaptability of the chemistry introduced. A good example for this is the developed *in situ* protocol, which has permitted the modification of insulin via its disulfide bonds for the first time. The change of the leaving groups together with adaption of the reducing agent to the properties of the targeted protein cystines, enabled the conjugation

reaction to be carried out successfully. This implies that it should be possible for many protein targets to find an adequate pairing of substituted maleimide and reducing agent. To this end many more leaving groups than halides, thiols and selenols, as well as other reducing agents are conceivable.[216, 461, 462]

Besides these advantages the functionalisation of disulfide bonds with maleimides has a number of drawbacks.

The main limitation for the application of the introduced chemistry is of course the dependence on the presence of a disulfide bond in the protein of interest. The majority of polypeptides do not contain this structural feature (see 1.4). However, secreted proteins typically contain one or more cystines[198] and most targets of therapeutic interest fall into this category.[239] These include many peptide hormones,[463] the interferons,[170] the insulin-like growth factors,[306] as well as cystine containing proteases such as papain[464] to name a few. Another obstacle is the accessibility of the disulfide bond, as around 50% are buried in a hydrophobic environment.[46] Both limitations can be overcome by the genetic engineering of an additional disulfide bond into the target. However, this would add a labour intensive step to the production process and the results of such efforts have had varying results in the past.[224]

Difficulties in the modification reaction itself may arise from the limited water solubility of some of the substituted maleimides. From the perspective of protein chemistry, the reaction mixtures prepared during this project usually contained a medium to very high amount of organic solvent (10–45%), and some of the experiments with rituximab clearly demonstrated that this can have a negative impact on the results. A relatively simple solution could involve the introduction of carboxylic or hydroxyl groups, as well as small PEG chains, to the maleimide substituent or in fact, into linker between the bridging moiety and the functional group. Another issue is the hydrolysis of the maleimide bridges (see 1.3.1). Although this generates a mix of products after the reaction, the process can be controlled by various parameters (see 2.3.4). Indeed the stability of the conjugate is actually increased by the cleavage of the maleimide ring. Finally, minor disadvantages include the malodorous nature of the employed thiols and selenol reagents, the fact that in some protocols quite an excess of reagents is necessary, which is partially offset by their simple synthesis, and that the high reac-

tivity of the maleimide compounds precludes the *in vivo* application of the developed chemistry. Interestingly the maleimide motif has been found to be the active moiety in a few natural products, where it is protected from premature reaction by bulky groups surrounding it.[125] A similar mechanism might be possible with well designed substituents of the bridging moiety.

3.3 Comparison with current methods

Not only is the performance of a new methodology important in order to become a well established and widely used process, but also how well it compares with the methods currently in use. At the moment these are modification via lysines (amines), free cysteines and non-natural amino acids. In addition, the method for disulfide bond modification developed by the Brocchini group should be taken for comparison. Three separate stages of a bioconjugation protocol need to be considered.

First, before any chemistry can be carried out, it is necessary to assess how difficult it is to provide the required protein or its mutated analogue. Here lies the great strength of targeting lysine residues, since practically every protein contains a large number of this residue. In contrast the introduction of a free cysteine is often necessary and associated with a number of problems (see 1.4). This point is also the biggest issue when working with non-natural amino acid specific chemistry, as the preparation of the genetically altered protein material is usually labour intensive and context-dependent (see 1.1). Disulfide targeting methods depend on the presence of one or more solvent accessible cystines, as discussed above, but otherwise do not require protein engineering.

Secondly, the performance of the method used to modify the target protein. Many parameters can vary between the different processes but the primary advantages and disadvantages can be quickly summarised. With lysine modification full conversion can be easily achieved with small amounts of reagent but site-specificity is extremely limited. The targeting of both free cysteines as well as non-natural amino acids on the contrary generates homogeneous products. However, complete conversion is rare despite the larger amounts of reagents often being employed. Bioconjugation via disulfide bonds seems ideal in this respect, as the two available methods are highly site-specific and good to full

conversion can be achieved in the presence of low concentrations of the reactants. The herein developed method outperforms the use of the enone-sulfonyl reagents mainly in that it is faster, which enables its application to modify multi-disulfide bond containing systems, and offers the option of an *in situ* protocol, greatly decreasing the time of the reaction and unwanted side reactions.
The third point, which has to be considered, is the properties of the products. This can be an issue when lysine modification has been carried out, as a decrease in the activity of the heterogeneous mixture is frequently observed, and the associated loss of surface charges as well as over-labelling can lead to problems with solubility. In contrast the other three methods usually yield products with excellent activity. This is due to the site-specificity of the chemistry, and as a considerable amount of rational design has often been invested in the protein material and/ or the reagents. With regard to the preparation of biotherapeutics, disadvantages here include the instability of thiosuccinimide products (see 1.3.1) under *in vivo* conditions, and the introduction of a chiral centre by the chemistry established by Brocchini and colleagues. Substituted maleimide chemistry alone offers the possibility of reversing the conjugation, while being stable in human plasma, but only if hydrolysis of the 5-membered ring is carefully controlled.

In summary the new platform developed during this project performs very well in all three aspects, as long as the protein of interest contains a solvent accessible disulfide bond. Therefore it complements the currently established methods for protein modification, and represents an enhancement of the idea of the functionalisation of disulfide bonds by re-bridging introduced by Brochini and co-workers. Furthermore, substituted maleimide-based cystine bridging also combines a number of unique advantages, and seems to be well suited for industrial processes.

3.4 Current use and outlook

The focus of the work presented in this thesis has been the development of the methodology itself. The disadvantage of this is of course that many interesting opportunities which arose during this project, for example the further development of somatostatin analogues or the *in vivo* analysis of tumours with labelled

anti-CEA, had to be left alone for the time being. But thanks to the versatility of the introduced chemistry, a number of groups at different institutions have already started to use “next generation maleimides” in a variety of projects. To illustrate these developments three examples should be briefly described.

Rudolf and colleagues at the University of Lodz have synthesised metallocarbonyl complexes with iron of mono- and dibromomaleimide (Fig. 3.1).[465] These compounds can be used to monitor protein-protein interactions, such as those of a hormone with its receptor, due to the properties of the metal complex as a good marker for IR-spectroscopy.

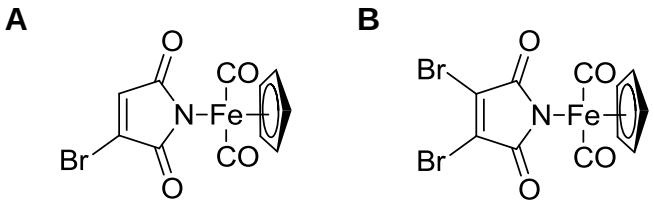
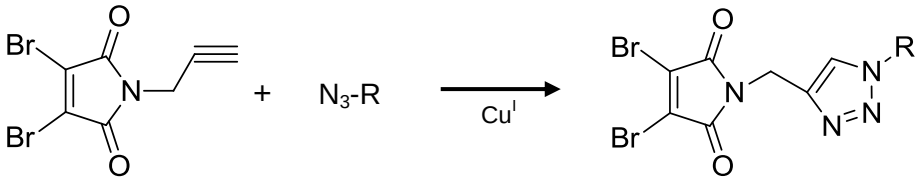


Fig. 3.1: Metallocarbonyl complexes prepared by Rudolf *et al*.[465] (A) Mono- and (B) dibromomaleimide.

The monobromomaleimide derivative was used to label and thereby inhibit the cysteine protease papain, and the signal properties of this material were examined. Upon addition of an excess of TCEP the activity of the protein could be partially regenerated (up to 35%), introducing the application of halomaleimides as reversible enzyme inhibitors.

In a series of publications Prof Haddleton and his group (University of Warwick) have demonstrated a striking way to prepare polymer carrying 3,4-substituted maleimides.[466-468] Initially attempts were made to use dibromomaleimide as a chain starter in polymerisation reactions, but the compound was found to be involved in side reactions with the reactants of the employed systems. To solve this problem the maleimide was *N*-functionalised with an alkyne and the corresponding PEG-based polymer bearing an azide prepared. Both compounds were then “clicked” together in the presence of copper to furnish the activated polymer in good yield (Scheme 3.1).



Scheme 3.1: Preparation of polymer-bearing dibromomaleimides with “click” chemistry as described by Jones *et al.*[468] R = PEG-based polymer.

This material was then used to modify salmon calcitonin, a small peptide hormone with similar efficiency as observed for somatostatin herein. An intriguing property of this system is the biocompatibility of the used “click” chemistry (see 1.1), which should make it possible to insert the *N*-azide-dibromomaleimide into a protein disulfide bond, and install any desired functionality subsequently.
In follow-up work the Haddleton group demonstrated that dithiophenolmaleimide is in contrast to dibromomaleimide, compatible with atom transfer radical polymerisation (ATRP). Thus various branched *N*-PEG-dithiophenolmaleimides were synthesised, with the maleimide included in the polymerisation reaction as a functional initiator. The reactivity of this material was again shown by PEGylation of salmon calcitonin. The whole process is highly valuable as it allows growing the polymer in a very controlled way and its simultaneous activation with a functional group. Such an approach saves material, makes difficult purification steps obsolete, and solves problems with double-functionalisation in the case of polymers with identical ends.

The last example of a current application of substituted maleimides has been the result of an ongoing and very fruitful collaboration with Mr Vishal Sanchania (UCL ISMB, supervised by Prof Gabriel Aeppli, London Centre of Nanotechnology). The idea of an EPR-based diagnostic method, called ‘spinostics’, was tested in this project, utilising the anti-CEA antibody fragment. Its theory has been outlined earlier (see 2.8.6) and should be repeated here only briefly; The concept is based on the attachment of a spin label in a highly immobilised fashion to an antibody. This would couple the tumbling motion of the label closely to those of the protein. Upon binding to the antigen the rate of tumbling of the now larger complex changes, which would be accompanied by a change in the EPR signal (Fig. 3.2). If this is proportional to the amount of antigen present (i.e. the

amount of complexes formed) a standard curve could be generated and used to determine the quantity of the antigen in an unknown sample, such as patient blood.

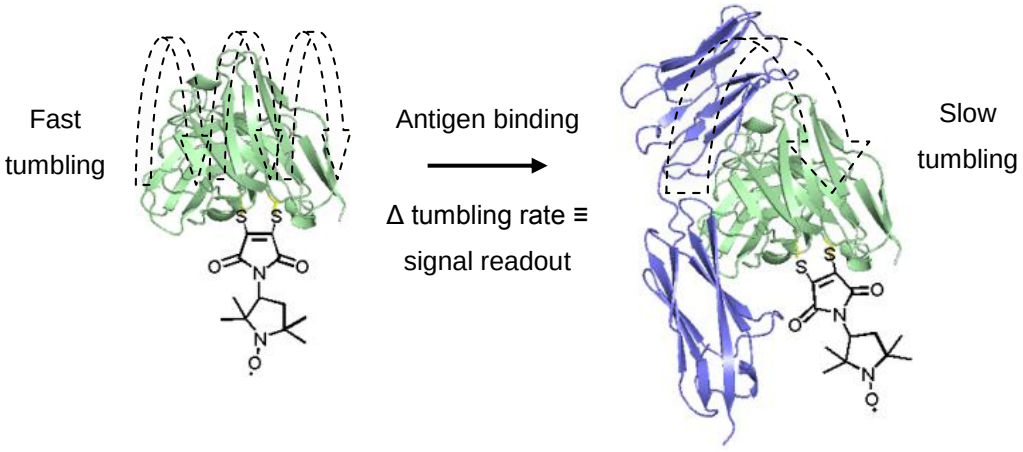


Fig. 3.2: Concept of 'spinostics'. Green = anti-CEA. Blue = NA1. Cartoons are derived from a published crystal structure of the antibody.[418,419]

The envisaged system was successfully realised (manuscript in preparation). The linker free connection of the PROXYL spin label directly to the maleimide moiety, as well as the insertion into the rigid structure of a disulfide bond, furnished the necessary tight coupling of the label to the antibody fragment. Addition of NA1 or CEA resulted in clear changes of the EPR readout, and was dependent on the amount of antigen present in the solution. The same effect was observed not only in PBS but also human plasma, and full blood, and titration curves could be generated in all three media.

Although similar experiments have been performed before,[437, 438] this is the first example with a positive result. The developed solvent based biosensor requires only minimal sample preparation, enables detection in an opaque solution (blood), and allows for a high sample throughput. Due to the potential of this technology a patent application has been filed with the kind support of UCL Business.[441] The major problem at the moment is the low sensitivity (around 100 nM) of the system but technical advances in the EPR sector are expected to offer simple solutions to this issue.

The three presented projects have already shown the successful application of "next generation maleimides" to various problems. Other ongoing work in the

Baker group currently focuses on the potential of different substituents and reducing agents, as well as the utilisation of the compounds' photochemical properties.[469] It should also be mentioned that a series of patents,[470-473] filed in connection to the substituted maleimide platform, has laid the foundation for the formation of Thiologics (http://www.thiologics.com, again with the support of UCL Business), a company aiming at the synthesis of ADCs and improved biologicals.

In conclusion the supposed "functionalisation by re-bridging" of protein disulfide bonds with 3,4-substituted maleimides has been successfully realised. The developed method for bioconjugation can be seen as an enhanced version of the technology pioneered by Brocchini and co-workers, and offers a real alternative to the conjugation chemistry currently in use. Novel maleimide derivatives have already found their way into academic as well as industrial labs, and work to unlock their full potential to solve a variety of chemical and biological problems is well under way. It is hoped that the discoveries made herein will eventually lead to the development of novel biotherapeutics, diagnostic methods, and research tools.

4 Experimental Procedures

4.1 Material

mPEG300, mPEG5000, TCEP, DTT, 2-mercaptoethanol, GSH, 1,2-ethanedithiol, benzeneselenol, thiophenol, maleimide and dibromomaleimide were purchased from Sigma-Aldrich and used without further purification. 3-Amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (TPO) was purchased from Acros Organics and used without further purification.

Dichloromaleimide[391] was kindly provided by Dr James Baker (UCL Chemistry). *N*-methyl-monobromomaleimide[243] was kindly provided by Mr Ramiz Nathani (UCL Chemistry). *N*-fluoresceine-dibromomaleimide[396] and *N*-biotin-dibromo-maleimide were kindly provided by Dr Christopher Ryan (UCL Chemistry). *N*-phenyl-dibromomaleimide[114] was kindly provided by Dr Mark Smith (UCL Chemistry).

Lyophilized somatostatin, lyophilised insulin, monoclonal anti-FLAG M1 and M2 antibodies, lyophilised thermolysin and human plasma were purchased from Sigma-Aldrich. Carcinoembryonic antigen was purchased from Calbiochem. The HRP/STREP conjugate was purchased from Invitrogen. Clinical grade rituximab (MabThera) was purchased from Roche.

The anti-CEA single chain antibody fragment and the CEA construct NA1 were kindly provided by Dr Berend Tolner (UCL Cancer Institute). The anti-MSP1 chimeric antibody and its recombinant MSP1-19 antigen were kindly provided by Dr David Matthews (MRCT). The FLAG peptide was synthesised by Cambridge Peptides LTD and kindly provided by Mr Vishal Sanchania (UCL ISMB).

4.2 General Methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 instrument operating at ambient temperature and a frequency of 500 MHz for ^{1}H and 125 MHz for ^{13}C. ^{1}H NMR spectra were referenced to the $CDCl_3$ (7.26 ppm) signal and ^{13}C NMR spectra also to the $CDCl_3$ (77.67 ppm) signal. Coupling constants are in Hz. Infrared spectra were recorded on a PerkinElmer Spectrum 100 FT-IR spectrometer with frequencies given in reciprocal centimetres (cm^{-1}). Mass spectra and high resolution mass data were recorded on a VG70-SE mass spectrometer (EI mode and CI mode). Melting points were measured on a

Gallenkamp heating block and are not corrected. Compound synthesis was performed in an inert-gas environment unless stated otherwise.

LCMS was performed on protein samples using a Waters Acquity UPLC connected to Waters Acquity Single Quad Detector [column: Acquity UPLC BEH C18 1.7 μm 2.1 × 50 mm; wavelength 254 nm; mobile phase 95 : 5 water (0.1% formic acid) : MeCN (0.1% formic acid), gradient over 4 min to 5 : 95 water (0.1% formic acid) : MeCN (0.1% formic acid); flow rate 0.6 ml/ min; MS mode ES+/ -; scan range (m/z) = 95–2090 Da; scan time 0.25 s]. Data were obtained in continuum mode. Sample volume was 30 µl and injection volume was 3–7 µl with partial loop fill. The electron spray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 20–200 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 l/ h. Total mass spectra for protein samples were reconstructed from the ion series using the MaxEnt 1 algorithm pre-installed on the MassLynx software.

MALDI-TOF analysis was performed on a MALDI micro MX (Micromass). Data was obtained with a source voltage of 12 kV and a reflectron voltage (if applicable) of 5 kV at a laser wavelength of 337 nm. Samples were recorded as outlined below (see Table 3.1). Buffer salts were removed prior to analysis by dialysis for 24 h at 4 °C against deionised water with Slide-A-Lyzer MINI dialysis units (Thermo Scientific, 2 or 10 kDa MWCO). All proteins were spotted onto a MALDI plate after 1 : 1 mixture with the matrix (10 mg/ ml in 1 : 1 H_2O : MeCN). If necessary trifluoroacetic acid (TFA, 10 mg/ ml) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, 10 mg/ ml in acetone) were pre-spotted. Adrenocorticotropic hormone (ACTH, nominal weight 2465.1989 Da, 10 ng/ ml) was used for mass calibration in reflectron mode.

Relative quantification of MS data was carried out by normalisation of all identifiable peptide or protein signals (starting material, product, side and degradation products) to 100% according to their unmodified signal strength (relative ion count). It should be noted that the resulting data represents an approximation as the signal intensity depends not only on the population of an individual molecule species but also on its ionisation properties. This method of quantification is validated to a certain extend by the complete conversion of starting material to the product(s) as observed in most cases during this project.

EPR spectra were recorded on a Bruker EMX instrument (X-band, 9.5 GHz, 0.3 Tesla) using a Superhigh Q resonator and an EMX Premium-X bridge. Samples were prepared in 1 mm inner diameter capillaries (Brand) and fixed in 4 mm diameter tubes in the resonator. Effective volume was 10–20 µl and spectra were recorded at ambient temperature.

Absorbance measurements were carried out on a Carry Bio 100 UV/ Vis spectrophotometer (Varian) equipped with a temperature-controlled 12x sample holder in quartz cuvettes (1 cm path length, volume 75 µl) at 25 °C. Samples were baseline corrected and slits set to 5 nm. Protein solutions were scanned from 450–250 nm and concentration calculated using either the published or calculated (based on the amino acid sequence via the ProtParam tool of the ExPASy proteomics server; http://expasy.org/sprot/) molar extinction coefficients with Lambert Beers law. λ_{max} were obtained in the same range of wavelengths at a compound concentration of 152.6 µM.

The protein concentration of full antibody solutions was measured with a NanoDrop (Thermo Scientific). The device was blanked with the protein buffer, samples analysed in the IgG setting and the average from at least 6 readings calculated.

Fluorescence data was obtained on a Carry Eclipse (Varian) machine equipped with a temperature-controlled 4x sample holder in quartz cuvettes at 25 °C. Blank buffer was used as zero fluorescence; slits were set to 5 nm and scan speed was average. Absorbance scans were used to determine ideal excitation wavelengths and sample concentrations diluted to obtain a maximal fluorescence signal below 1000 AU.

Glycine-SDS-PAGE was performed following standard lab procedures.[474] Proteins from 20 kDa to 80 kDa were separated on 16% gels and proteins above 80 kDa were separated on 12% gels. In both cases a 4% stacking gel was used and a broad-range MW marker (10 kDa – 250 kDa, BioLabs) was co-run to estimate protein weights. For comparison a sample of the untreated protein (lane marked AB in the figures showing antibodies) and a sample with the protein mixed with organic solvents and the maleimide reagent in the highest concentration used in the experiment (lane marked C in the figures) were also usually loaded. Samples were mixed 1 : 1 with a running buffer (1M Tris-HCl, pH 6.8, 30% w/v glycerol, 10% w/v SDS, Coomassie G-250) and not thermal denatured

unless stated otherwise. Tricine-SDS-PAGE was performed following published procedures.[409, 475] Proteins and peptides below 20 kDa were separated on 16% gels with a 10% spacer gel and a 4% stacking gel. All gels were stained following a modified literature protocol,[476] where 0.12% w/v of the Coomassie G-250 and R-250 dyes were added to the staining solution instead of only the G-250 dye.

All buffer solutions were prepared with double-deionised water and filter-sterilised. Ultrapure DMF and DMSO were purchased from Sigma-Aldrich and stored under dry conditions. Opened bottles of benzeneselenol were kept under argon and replaced when the solution had turned orange. Urea was re-crystallised from hot ethanol before experimentation.

4.2.1 MALDI-TOF protocols

Suitable protocols to visualise individual proteins and conjugates by MALDI-TOF MS were developed and are summarised in Table 3.1.

Table 3.1: MALDI-TOF MS protocols. CHCA = α-cyano-4-hydroxycinnamic acid. DHB = 2,5-dihydroxy benzoic acid. SA = sinapinic acid. Ref + = reflectron positive, ref - = reflectron negative, lin - = linear negative, lin + = linear positive.

Sample	Matrix	Mode	Pre-spotting	Laser	Pulse	Detector	Suppression
somatostatin	SA	ref +	-	500	2000	2750	800
PEG-somatostatin	SA	ref +	TFA	500	2000	2750	800
insulin	SA	ref +	-	500	2000	2750	800
PEG-insulin	SA	lin -	TFA	390	2000	2750	800
digested insulin	SA	ref -	-	500	2000	2750	400
anti-CEA	CHCA	lin -	-	500	2000	2750	8000
anti-CEA-PEG5000	CHCA	lin -	TFA	500	3000	2750	8000
FLAG peptide	DHB	ref +	-	400	2000	2750	600
anti-FLAG antibodies	SA	lin +	TFA	500	3000	2750	8000
anti-FLAG antibodies-PEG5000	SA	lin +	TFA	500	3000	2750	8000
anti-MSP1 antibody	SA	lin +	TFA	350	3000	2750	8000
anti-MSP1 antibody-PEG5000	SA	lin +	TFA	500	3000	2750	8000
rituximab	SA	lin -	TFA	500	2000	2750	8000
rituximab-PEG5000	SA	lin -	-	500	3000	2750	8000
rituximab fragments	SA	lin +	-	500	3000	2750	8000

4.2.2 Preparation of proteins

Lyophilized somatostatin (1 mg, 83% peptide content, 98% purity) was dissolved in 2 ml of buffer (50 mM sodium phosphate, pH 6.2, 40% MeCN, 2.5% DMF) to yield a stock solution of 0.5 mg/ ml which was stored at 4 °C. The individual concentration was determined every 14 days with triplicate absorbance measurements (ε_{280} = 5,500 M^{-1} cm^{-1}) for each batch. The stock solution was

diluted in the same buffer before the experiments to yield a final concentration of 152.6 µM (0.25 mg/ ml).

Lyophilized insulin was weighed prior to experiments and dissolved in an appropriate amount of buffer (50 mM sodium phosphate, pH 7.4 or other, 40% MeCN, 2.5% DMF) to yield a concentration of 86.1 µM (0.5 mg/ ml). Insulin solutions were stored at 4 °C no longer than 2 days.

Anti-CEA and NA1 were supplied in PBS (pH 7.4) in varying concentrations and stored in aliquots at –20 °C. The antibody fragment was diluted in PBS (pH 7.4) and DMF (final amount 10%) to yield a concentration of 70.0 µM (1.87 mg/ ml) prior to experimentation. An extinction coefficient of ε_{280} = 48,735 M^{-1} cm^{-1} was used to calculate protein concentrations. NA1 was diluted or concentrated in PBS (pH 7.4). An extinction coefficient of ε_{280} = 25,840 M^{-1} cm^{-1} was used to calculate protein concentrations.

The monoclonal M2 anti-FLAG antibody (mouse IgG1) was supplied in buffer (150 mM NaCl, 10 mM sodium phosphate, pH 7.4, 50% glycerol) at a concentration of 6.3 µM (1.0 mg/ ml). Aliquots were stored at –20 °C. Glycerol was removed prior to experimentation by dialysis in Slide-A-Lyzer MINI dialysis units (10 kDa MWCO, Thermo Scientific) against glycerol-free buffer at 4 °C over night.

Alternatively monoclonal M1 (mouse IgG2b) and M2 anti-FLAG antibodies were supplied in buffer (150 mM NaCl, 10 mM sodium phosphate, pH 7.4, 0.02% sodium azide) at a concentration of 6.3 µM (1.0 mg/ ml). Aliquots were stored at –20 °C. DMF was added to a final concentration of 20% to the antibody solution prior to experimentation.

Lyophilised FLAG peptide with an additional cysteine (sequence DYKDDDDKC, MW 1,116 Da) was diluted in water/ 10% DMF to yield a concentration of 1.4 mM (1.53 mg/ ml).

The chimeric anti-MSP1 (human IgG1 with mouse variable regions) antibody was supplied in PBS (pH 7.4) at a concentration of 20.0 µM (3.0 mg/ ml) and stored in aliquots at 0 °C. DMF was added to a final concentration of 20% to the antibody solution prior to experimentation.

Clinical grade rituximab (human IgG1 with mouse variable regions) was supplied in 154.0 mM NaCl, 34.3 mM sodium citrate dihydrate, 0.53 mM polysorbate 80 at a concentration of 70.0 µM (10 mg/ ml). The buffer was exchanged for

PBS (pH 7.4) or 50 mM sodium phosphate, pH 6.8, 1 mM EDTA by ultrafiltration (100 kDa MWCO, Sartorius). Aliquots (5–6 mg/ ml) were stored at –20 °C and diluted to 23.7 µM (3.44 mg/ ml) prior to experimentation and organic solvent (DMF unless otherwise stated) added to the desired concentration (usually 20%).

4.2.3 Compound stock solutions

In all experimental descriptions of protein-based work the statet equiv of reagents are proportional to the amount of protein present. I.e. the amount of target protein is always considered to be 1 equiv. Stock solutions of chemical compounds and reducing reagents were prepared at 100x concentration (relative to the target protein) when 1–10 equiv were added to the proteins and at 400x or 1000x concentration if more than 10 equiv were added. Solutions of benzeneselenol were prepared immediately before the experiment and not reused. Stock solutions were stored no longer than 24 h (at 4 °C).

To dissolve the maleimide reagents and reducing agents in the protein buffer, corresponding to the tested protein, an increased amount of DMF was often necessary. The following list compiles conditions used if the buffers contained MeCN (i.e. in experiments with somatostatin and insulin):

No additional DMF was used with *N*-PEG300-dibromomaleimide, *N*-PEG5000-dibromomaleimide, *N*-PEG5000-dithiophenolmaleimide, *N*-PEG5000-dibenzeneselenolmaleimide, sodium iodide, 2-mercaptoethanol, TCEP, DTT, 1,2-ethanedithiol, GSH and L-cysteine.

5% additional DMF (final concentration 7.5%) was used with maleimide, monobromomaleimide, dibromomaleimide, diiodomaleimide, *N*-methyl-monobromomaleimide, *N*-methyl-dibromomaleimide, dicysteinemaleimide, di-2-mercaptoethanolmaleimide, *N*-biotin-dibromomaleimide and protected L-cysteine.

10% additional DMF (final concentration 12.5%) was used with dichloromaleimide, di-2-mercaptopyridinemaleimide, dithiophenolmaleimide, fluoresceinamine and thiophenol.

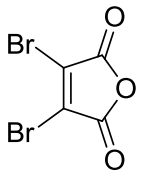
N-TPO-dibromomaleimide, *N*-TPO-dithiophenolmaleimide, *N*-TPO-dibenzeneselenolmaleimide, *N*-fluorescein-dibromomaleimide, dibenzeneselenolmaleimide and benzeneselenol were dissolved in DMF only.

400x and 1000x stock solutions of compounds were additional DMF was necessary were prepared in DMF only.

In cases where the buffer of the protein contained no MeCN (all antibodies) the compound stock solutions were prepared in DMF only, except for those containing *N*-PEG5000-dibromomaleimide, *N*-PEG5000-dithiophenolmaleimide, 2-mercaptoethanol, TCEP, DTT and GSH which were prepared in buffer only.

4.3 Synthesis

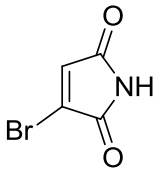
4.3.1 Dibromomaleic anhydride (**2**)[400]



To maleic anhydride (1.50 g, 15.3 mmol) and aluminium chloride (22.4 mg, 0.17 mmol) was added bromine (4.90 ml, 30.6 mmol). The reaction mixture was heated in a sealed tube to 120 °C for 20 h. The solution was allowed to cool to ambient temperature, mixed with ethyl acetate (40 ml), filtered twice and the solvent removed *in vacuo* from the filtrate to afford the product as a light orange crystalline powder (3.85 g, 99%).

^{1}H NMR (500 MHz, DMSO): no signals; ^{13}C NMR (125 MHz, DMSO): δ = 163.2 (C), 125.3 (C); IR (solid, cm^{-1}): 1868 (w), 1783 (s), 1729 (s), 1594 (m); MS (EI) *m/z,* (%): 259 (^{81,81}M+, 50), 257 (^{79,81}M+, 100), 255 (^{79,79}M, 52), 105 (20); Mass calc. for $C_4{}^{79}Br_2O_3$: 253.8214. Found: 253.8293; m.p. 102–106 °C (ethyl acetate) (Literature: 105–115 °C).

4.3.2 Monobromomaleimide (**4**)[243]

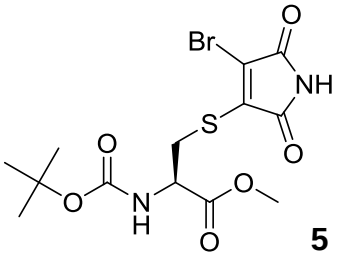


To maleimide (1.00 g, 10.2 mmol) in chloroform (15 ml) was added a solution of bromine (0.55 ml, 11.2 mmol) in chloroform (5 ml) dropwise. The reaction mix-

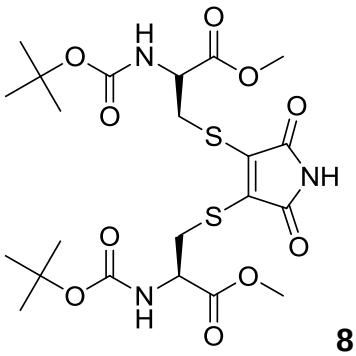
ture was refluxed for 2 h at 60 °C, left to cool to ambient temperature overnight and put on ice. The solid precipitate was filtered off and washed with cold chloroform (50 ml) to afford white crystals of crude 2,3-dibromosuccinimide (1.95 g, 7.56 mmol). The crude succinimide was dissolved in THF (50 ml) and a solution of triethylamine (1.15 ml, 8.32 mmol) in THF (10 ml) was added over 5 min at 0 ^{o}C. The reaction mixture was allowed to warm to ambient temperature and stirred for 96 h. The solution was filtered, washed with ethyl acetate (2 x 100 ml) and water (3 x 100 ml), dried over magnesium sulfate and the solvent removed *in vacuo*. The solid was dissolved in chloroform and re-crystallised overnight on ice to yield the product as a pale yellow powder (1.17 g, 88%).

^{1}H NMR (500 MHz, MeOD): δ = 7.01 (s, 1H, CH); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 171.1 (C), 167.9 (C), 134.3 (C), 132.5 (CH); IR (solid, cm^{-1}): 3233 (m), 1724 (s), 1716 (s); MS (EI) *m/z,* (%): 177 (^{81}M+, 100), 175 (^{79}M+, 100), 132 (100), 104 (95); Mass calc. for $C_4H_3O_2N^{79}Br$: 174.9269. Found: 174.9263; m.p. 148–150 $^{\circ}$C (chloroform) (Literature: 148–151 °C).

4.3.3 N-Boc-L-Cys-OMe-bromomaleimide (**5**)[243] and di(N-Boc-L-Cys-OMe) maleimide (**8**)



5



8

Procedure in methanol: To dibromomaleimide (12.3 mg, 0.05 mmol) and sodium acetate (19.7 mg, 0.24 mmol) in methanol (3 ml) was added *N*-(tert-butoxycarbonyl)-L-cysteine methyl ester (41.1 µl, 0.20 mmol) in methanol (3 ml). The reaction mixture was stirred for 1 h at ambient temperature. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 1 : 1) to afford product **5** as a yellow powder (6.10 mg, 29%) and product **8** as a bright

yellow powder (13.2 mg, 48%). Other conditions explored are described in 2.1.2.

Procedure in aqueous solution: To *N*-(tert-butoxycarbonyl)-L-cysteine methyl ester (32.0 µl, 0.16 mmol) in buffer (12.6 ml, 150 mM NaCl, 100 mM sodium phosphate, pH 8.0, 5.0% DMF) was added dibromomaleimide (43.6 mg, 0.17 mmol) in DMF (0.30 ml, final concentration of DMF 7.5%). The reaction was stirred at ambient temperature for 1 h. The yellow precipitate was dissolved by addition of ethyl acetate (25 ml), the organic phase washed with water (3 x 20 ml) followed by a 10% (w/v) lithium chloride solution (5 x 25 ml) and dried over magnesium sulfate. The solvent was removed *in vacuo* and the residue was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 1 : 1). Fractions containing the mono- or bis-addition product were collected and the solvent was removed *in vacuo*. Impure products were further purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5–2.0% methanol) to afford product **5** as a yellow powder (5.21 mg, 7%) and product **8** as a bright yellow crystalline powder (30.3 mg, 35%). Other conditions explored are described in 2.1.2.

*Data for product **5**:*

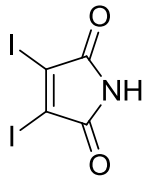
^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.52 (s, 1H, NH), 5.28 (d, 1H, *J* = 7.9, CH-N***H***), 4.71–4.64 (m, 1H, NH-C***H***-CO), 3.82–3.95 (m, 2H, S-C***H$_2$***-CH), 3.78 (s, 3H, O-CH_3), 1.43 (s, 9H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 170.6 (C), 165.7 (C), 163.4 (C), 155.1 (C), 143.1 (C), 120.8 (C), 80.9 (C), 53.8 (CH), 53.1 (CH_3), 33.2 (CH_2), 28.3 (CH_3); IR (solid, cm^{-1}): 3346 (w), 2926 (w), 1771 (w), 1730 (s); MS (ES) *m/z,* (%): 409 (^{81}M+H, 100), 407 (^{79}M+H, 94), 377 (12), 309 (16); Mass calc. for $C_{13}H_{17}O_6N_2SBr^{79}$: 407.9991. Found: 407.9912; m.p. 146–148 °C (dichloromethane) (Literature: 145–147 °C).

*Data for product **8**:*

λ_{max} (50 mM sodium phosphate, pH 6.2, 40% MeCN, 2.5% DMF)/ 395 nm (ε/ dm^3 mol^{-1} cm^{-1}) 3810; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.84 (s, 1H, NH), 5.81 (d, 2H, *J* = 7.3, CH-N***H***), 4.71–4.65 (m, 2H, NH-C***H***-CO), 3.71–3.84 (m, 4H, S-C***H$_2$***-CH), 3.76 (s, 6H, O-CH_3), 1.43 (s, 18H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ =

170.9 (C), 165.6 (C), 155.1 (C), 137.2 (C), 80.4 (C), 53.9 (CH), 52.9 (CH_3), 33.8 (CH_2), 28.4 (CH_3); IR (solid, cm^{-1}): 3363 (w), 2927 (w), 1778 (w), 1717 (s), 1694 (s); MS (ES) *m/z,* (%): 586 (M+H+Na, 10), 490 (40), 345 (18); Mass calc. for $C_{22}H_{33}O_{10}N_3S_2$[+H]: 563.1607. Found: 563.1505; m.p. 78–80 ^{o}C (dichloromethane).

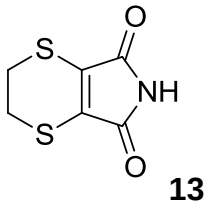
4.3.4 Diiodomaleimide[390]



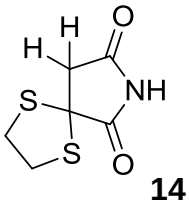
To dibromomaleimide (0.50 g, 1.97 mmol) in acetic acid (50 ml) was added sodium iodide (0.89 g, 5.91 mmol). The reaction mixture was heated at reflux for 2 h. The reaction was allowed to cool to ambient temperature before water (50 ml) was added and the solution cooled at 4 °C for 15 h. The yellow precipitate was filtered off and air dried to afford the product as an orange crystalline powder (415 mg, 60%).

^{1}H NMR (500 MHz, MeOD): no signals; ^{13}C NMR (125 MHz, MeOD): δ = 169.3 (C), 119.5 (C); IR (solid, cm^{-1}): 3244 (s), 2944 (m), 2833 (m), 1756 (w), 1708 (w); MS (EI) *m/z,* (%): 349 (M, 83), 179 (100); Mass calc. for $C_4H_{12}O_2N$: 348.8091. Found: 348.8102; m.p. 238–241 $^{\circ}$C (water) (Literature: 254–255 °C).

4.3.5 Pyrrole-5,7-dione (**13**) and nonane-6,8-dione (**14**)



13



14

To dibromomaleimide (43.6 mg, 0.17 mmol) and sodium bicarbonate (71.4 mg, 0.85 mmol) in methanol (57 ml) was added 1,2-ethanedithiol (15.7 µl, 0.19 mmol) in methanol (3 ml), at 0 °C over 30 min. The reaction was stirred for 24 h at 4 °C. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate,

gradient elution from 9 : 1 to 7 : 3). The impure product was further purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.2–1.0% methanol) to give product **13** as a yellow powder (7.10 mg, 22%) and product **14** as a yellow powder (10.1 mg, 22%).

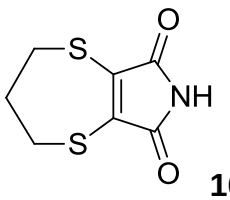
*Data for product **13**:*

^{1}H NMR (500 MHz, MeOD): δ = 3.47 (s, 4H, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 169.4 (C), 132.2 (C), 27.2 (CH_2); IR (solid, cm^{-1}): 3184 (m), 2925 (w), 1766 (w), 1696 (s); MS (EI) *m/z,* (%): 187 (M, 100), 144 (10); Mass calc. for $C_6H_5NO_2S_2$: 186.9762. Found: 186.9756; m.p. 123–127 °C (dichloromethane).

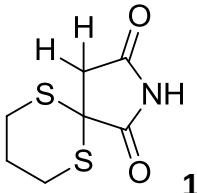
*Data for product **14**:*

^{1}H NMR (500 MHz, MeOD): δ = 3.62–3.56 (m, 4H, CH_2), 2.87 (s, 2H, CH_2-CO); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 176.6 (C), 172.7 (C), 71.2 (C), 51.8 (CH_2), 42.4 (CH_2), 42.1 (CH_2); IR (solid, cm^{-1}): 3238 (w), 2926 (w), 1780 (w), 1715 (s); MS (EI) *m/z,* (%): 189 (M, 71), 118 (100), 90 (79); Mass calc. for $C_6H_7NO_2S_2$: 188.9918. Found: 189.1503; m.p. 115–118 °C (dichloromethane).

4.3.6 Pyrrole-6,8-dione (**16**) and decane-1,3-dione (**17**)



16



17

To dibromomaleimide (43.6 mg, 0.17 mmol) and sodium bicarbonate (71.4 mg, 0.85 mmol) in methanol (57 ml) was added 1,3-propanedithiol (18.8 µl, 0.19 mmol) in methanol (3 ml) at 0 °C. The reaction was allowed to warm to ambient temperature after 1 h and stirred for 24 h. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 7 : 3). The crude product was purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.2–0.5% methanol) to afford product **16** as a yellow powder (2.00 mg, 6%) and product **17** as a yellow powder (27.1 mg, 78%).

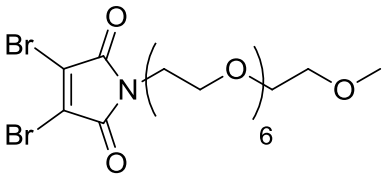
*Data for product **16**:*

^{1}H NMR (500 MHz, MeOD): δ = 4.83 (t, 4H, S-CH_2), 2.31 (quin, 2H, C***H***$_2$-CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 171.4 (C), 138.1 (C), 33.0 (CH_2), 31.1 (CH_2); IR (solid, cm^{-1}): 3229 (m), 2908 (w), 1754 (w), 1702 (s); MS (EI) *m/z,* (%): 201 (M, 100), 168 (82); Mass calc. for $C_7H_7NO_2S_2$: 200.9918. Found: 200.9913; m.p. 128–131 °C (dichloromethane).

*Data for product **17**:*

^{1}H NMR (500 MHz, MeOD): δ = 2.76 (s, 2H, CO-CH_2), 2.76–2.72 (m, 2H, S-C***H***$_2$), 2.71–2.66 (m, 2H, S-C**H**$_2$), 2.25-2.19 (m, 2H, CH_2-C***H***$_2$); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 178.8 (C), 175.4 (C), 70.9 (C), 45.5 (CH_2), 28.7 (CH_2), 24.8 (CH_2); IR (solid, cm^{-1}): 3217 (m), 2906 (w), 1779 (w), 1702 (s); MS (CI) *m/z,* (%): 204 (M+H, 100), 176 (50), 132 (46); Mass calc. for $C_7H_9NO_2S_2$: 203.0075. Found: 203.0015; m.p. 119–122 °C (dichloromethane).

4.3.7 *N*-PEG300-dibromomaleimide (**24**)

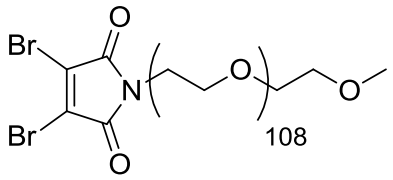


All glassware used was oven dried before the reaction. To a solution of triphenylphosphine (194 mg, 0.74 mmol) in THF (5 ml) was added dropwise diisopropyl azodicarboxylate (146 µl, 0.74 mmol) at –78 °C. The reaction was stirred for 5 min before mPEG300 (200 mg, 0.59 mmol) in anhydrous THF (4 ml) was added dropwise. The reaction was stirred for 5 min, neopentyl alcohol (45.8 mg, 0.52 mmol) in THF (1 ml) was added and after 5 min more, dibromomaleimide (189 mg, 0.74 mmol) in THF (2 ml) was added. The reaction was stirred for 10 min, the cold bath removed and stirred for 20 h at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5–5.0% methanol). The crude product was further purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gra-

dient elution from 7 : 3 to 2 : 8) to afford the product as a yellow oil (137 mg, 40%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.76 (t, 2H, *J* = 5.4, N-CH_2-C***H***$_2$), 3.64–3.52 (m, 24H, CH_2-O), 3.49 (t, 2H, *J* = 5.3, N-CH_2), 3.32 (s, 3H, O-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 163.8 (C), 129.5 (C), 72.0 (CH_2), 70.7–70.5 (9x CH_2), 70.1 (2x CH_2), 67.5 (CH_2), 59.1 (CH_3), 39.0 (CH_2); IR (solid, cm^{-1}): 3496 (w), 2869 (m), 1786 (m), 1720 (s), 1594 (m); MS (CI) *m/z,* (%): 580 (^{81}M+H, 12), 578 (^{81,79}M+H, 23), 576 (^{79}M+H, 12), 279 (100), 84 (61); Mass calc. for $C_{19}H_{31}{}^{79}Br_2O_9N$[+H]: 576.0444. Found: 576.0437.

4.3.8 *N*-PEG5000-dibromomaleimide (**25**)

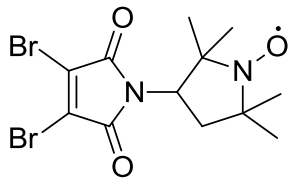


All glassware used was oven dried before the reaction. To a solution of triphenylphosphine (154.6 mg, 0.59 mmol) in a mixture of THF (8 ml) and DCM (3 ml) was added dropwise diisopropyl azodicarboxylate (116.0 µl, 0.59 mmol) at –78 °C. The reaction was stirred for 5 min before mPEG5000 (2.95 g, 0.59 mmol) in dichloromethane (7 ml) was added dropwise. After 5 min neopentyl alcohol (26.5 mg, 0.30 mmol) in a mixture of THF (1 ml) and DCM (1 ml) was added followed by dibromomaleimide (150 mg, 0.59 mmol) in anhydrous THF (2 ml) 5 min later. The reaction was stirred for 5 min, the cold bath removed and stirred for 20 h at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5–5.0% methanol). The crude product was re-purified by very slow flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5–6.0% methanol) to afford the product as a slightly green crystalline powder (417 mg, 13%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 3.58 (br s, xH, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 163.8 (C), 129.5 (C), 70.6 (CH_2)*; IR (solid, cm^{-1}): 3517 (w), 2872

(s), 1977 (w), 1727 (m), 1641 (w); m.p. 51–55 °C (dichloromethane). *multiple CH_3 visible by DEPT.

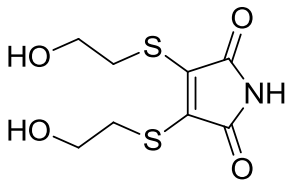
4.3.9 *N*-TPO-dibromomaleimide (**27**)



To 3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (120 mg, 0.79 mmol) in dry diethyl ether (30 ml) was added dibromomaleic anhydride (200 mg, 0.79 mmol) in dry diethyl ether (30 ml). The reaction was stirred for 30 min at ambient temperature, the solvent removed *in vacuo* and the residual material dissolved in 10 ml acetic anhydride. Anhydrous sodium acetate (32.3 mg, 0.39 mmol) was added and the reaction heated to 90 °C for 3 h. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 7 : 3) to afford the product as a brown solid (103 mg, 33%).

^{1}H NMR (500 MHz, $CDCl_3$, treated with hydrazobenzene*): δ = 4.51 (dd, 1H, *J* = 11.3, 8.8, CH), 2.92 (appt. t, 1H, *J* = 12.2, CH_2), 1.84 (dd, 1H, *J* = 11.6, 8.7, CH_2), 1.38 (s, 3H, CH_3), 1.28 (s, 3H, CH_3), 1.26 (s, 3H, CH_3), 1.11 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$, treated with hydrazobenzene*): δ = 164.4 (C), 129.7 (C), 57.1 (CH), 35.7 (CH_2), 27.2 (C), 25.6 (C), 25.2 (2x CH_3), 21.6 (2x CH_3); IR (solid, cm^{-1}): 2975 (w), 2934 (w), 1785 (w), 1722 (s), 1603 (m); MS (EI) *m/z,* (%): 397 (^{81,81}M+, 12), 395 (^{79,81}M+, 22), 393 (^{79,79}M, 12), 309 (100); Mass calc. for $C_{12}H_{15}O_3N_2{}^{79}Br_2$: 392.9444. Found: 392.9440; m.p. 171–176 ^{o}C (ethyl acetate). *In order to obtain detailed NMR spectra it was necessary to reduce the radical prior to NMR-spectroscopy. This was done by treatment of the purified spin label with a 3x excess (by mass) of hydrazobenzene for 60 min.[146] The presented data is thus an excerpt of the mixture of the spin label, hydrazobenzene and their reaction products.

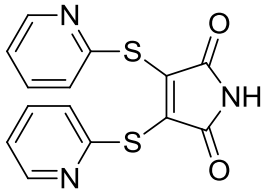
4.3.10 Di-2-mercaptoethanolmaleimide (**28**)



To 2-mercaptoethanol (684 µl, 9.77 mmol) in buffer (100 ml, 150 mM NaCl, 100 mM sodium phosphate, pH 8.0, 5.0% DMF) was added dibromomaleimide (1.00 g, 3.94 mmol) in DMF (2.5 ml, final concentration DMF 7.5%). The reaction was stirred for 30 min at ambient temperature and lithium chloride (20.0 g) was added. The aqueous reaction mixture was extracted with ethyl acetate (7 x 150 ml). The organic layers were combined, dried over magnesium sulphate, the solvent removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 1 : 1 to 1 : 9). Fractions containing the product were collected and the solvent was removed *in vacuo*. The impure product was further purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5–10.0% methanol) to afford the product as a yellow solid (518 mg, 53%).

λ_{max} (50 mM sodium phosphate, pH 6.2, 40% MeCN, 2.5% DMF)/ 318 nm (ε/ dm^3 mol^{-1} cm^{-1}) 1855; ^{1}H NMR (500 MHz, MeOD): δ = 3.74 (t, 4H, *J* = 6.4, HO-C***H***$_2$), 3.41 (t, 4H, *J* = 6.3, S-CH_2); ^{13}C NMR (125 MHz, MeOD): δ = 168.5 (C), 137.2 (C), 62.3 (CH_2), 34.4 (CH_2); IR (solid, cm^{-1}): 3344 (s), 2500 (m), 2078 (w) 1721 (w), 1646 (w); MS (EI) *m/z,* (%): 250 (M, 43), 232 (100), 161 (37); Mass calc. for $C_8H_{11}O_4NS_2$: 250.0207. Found: 250.0212; m.p. 46–50 °C (methanol).

4.3.11 Di-2-mercaptopyridinemaleimide (**29**)

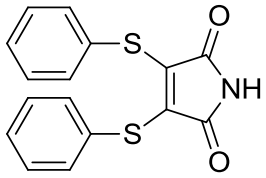


To dibromomaleimide (300 mg, 1.17 mmol) and sodium acetate (480 mg, 5.85 mmol) in methanol (15 ml) was slowly added 1*H*-pyridine-2-thione

(276 mg, 2.48 mmol) in methanol (4 ml). The reaction was stirred for 15 min at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5–3.0% methanol) to afford the product as a dark yellow powder (190 mg, 51%).

λ_{max} (50 mM sodium phosphate, pH 6.2, 40% MeCN, 2.5% DMF)/ 395 nm (ε/ dm^3 mol^{-1} cm^{-1}) 3508; ^{1}H NMR (500 MHz, MeOD): δ = 8.37 (d, 2H, *J* = 6.8, N-CH), 7.70 (t, 2H, *J* = 6.9, C-CH-C***H***), 7.38 (d, 2H, *J* = 7.2, C-CH), 7.26 (t, 2H, *J* = 6.7, N-CH-C***H***); ^{13}C NMR (125 MHz, MeOD): δ = 168.5 (C), 154.7 (C), 150.9 (CH), 140.0 (C), 139.0 (CH), 126.8 (CH), 123.7 (CH); IR (solid, cm^{-1}): 2926 (m), 2734 (w), 1771 (w), 1726 (s), 1619 (m); MS (CI) *m/z,* (%): 316 (M+H, 5), 152 (10), 126 (34), 112 (100); Mass calc. for $C_{14}H_9O_2N_3S_2$[+H]: 316.0214. Found: 316.0223; m.p. 70–72 ^{o}C (dichloromethane).

4.3.12 Dithiophenolmaleimide (**30**)[246]

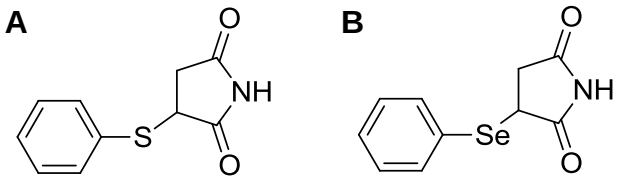


To dibromomaleimide (80.0 mg, 0.31 mmol) and sodium hydrogencarbonate (130 mg, 1.55 mmol) in methanol (6 ml) was slowly added benzenethiol (66.6 µl, 0.65 mmol) in methanol (1 ml). The reaction was stirred for 15 min at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 7 : 3) to afford the product as bright yellow crystals (72.6 mg, 75%).

λ_{max} (50 mM sodium phosphate, pH 6.2, 40% MeCN, 2.5% DMF)/ 412 nm (ε/ dm^3 mol^{-1} cm^{-1}) 2245; ^{1}H NMR (500 MHz, MeOD): δ = 7.27–7.22 (m, 6H, Ar-H), 7.16–7.14 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, MeOD): δ = 169.3 (C), 137.6 (C), 135.4 (C), 132.4 (CH), 130.1 (CH), 129.1 (CH); IR (solid, cm^{-1}): 3285 (m), 3059 (w), 2924 (w), 1774 (m), 1715 (s); MS (CI) *m/z,* (%): 314 (M+H, 100), 206 (13),

111 (12); Mass calc. for $C_{16}H_{11}O_2NS_2$[+H]: 314.0231. Found: 314.0309; m.p. 102–104 °C (chloroform) (Literature: 123–126 °C).

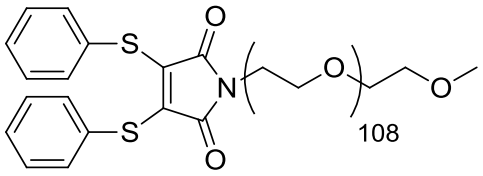
4.3.13 Thiophenolsuccinimide and benzeneselenolsuccinimide[403, 404]



To dithiophenolmaleimide (40.0 mg, 0.13 mmol) and sodium hydrogencarbonate (53.5 mg, 0.6 mmol) in methanol (15 ml) was slowly added benzeneselenol (54.0 µl, 0.52 mmol) in methanol (1 ml). After 3 h at ambient temperature maleimide (73.4 mg, 0.76 mmol) in methanol (2 ml) was added and the reaction stirred for additional 2 h. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 1 : 1) to afford product **A** and **B** as an inseparable mixture (7.90 mg). From NMR a ratio of 4 : 1 can be calculated (indicating a yield of 17% and 4% respectively).

^{1}H NMR (mixture of A and B, 500 MHz, $CDCl_3$): δ = 8.17 (s, 1H, NH, **A**), 8.09 (s, 1H, NH, **B**), 7.64 (dd, 2H, *J* = 7.0, 1.2, Ar-H, **A**), 7.53 (dd, 2H, *J* = 7.5, 2.0, Ar-H, **B**), 7.42–7.32 (m, 6H, Ar-H), 4.09 (dd, 2H, *J* = 9.2, 3.6, CH), 3.27 (dd, 2H, *J* = 9.4, 3.3, CH_2), 2.76 (dd, 2H, *J* = 5.7, 3.6, CH_2); ^{13}C NMR (mixture of **A** and **B**, 125 MHz, $CDCl_3$): δ = 176.7 (C), 175.6 (C), 174.9 (C), 174.3 (C), 136.5 (CH), 134.4 (CH), 130.4 (C), 129.8 (CH), 129.7 (CH), 129.6 (CH), 129.5 (CH), 125.2 (C), 45.4 (CH), 38.1 (CH_2), 37.3 (CH_2), 36.4 (CH); MS (mixture of A and B, EI) *m/z,* (%): 255 (M (**A**), 11), 207 (M (**B**), 21), 157 (46), 135 (100); Mass calc. for (**A**) $C_{10}H_9O_2NS$: 207.0348. Found: 207.0341; Mass calc. for (**B**) $C_{10}H_9O_2NSe$: 254.9793. Found: 254.9791; additional data on both compounds has been published elsewhere.[403, 404]

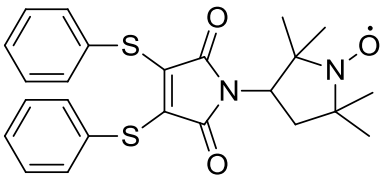
4.3.14 *N*-PEG5000-dithiophenolmaleimide (**31**)



All glassware used was oven dried before the reaction. To a solution of triphenylphosphine (168 mg, 0.64 mmol) in a mixture of THF (8 ml) and DCM (3 ml) was added dropwise diisopropyl azodicarboxylate (126 µl, 0.64 mmol) at –78 °C. The reaction was stirred for 5 min and mPEG5000 (1.60 g, 0.32 mmol) in DCM (7 ml) was added dropwise. After 5 min neopentyl alcohol (56.3 mg, 0.64 mmol) in a mixture of THF (1 ml) and DCM (1 ml) was added and dithiophenolmaleimide (200 mg, 0.64 mmol) in anhydrous THF (3 ml) was added 5 min later. The reaction was stirred for 10 min, the cold bath removed and stirred for 20 h at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5–10.0% methanol). The crude product was purified by flash chromatography on TLC grade silica gel (methanol : dichloromethane, gradient elution from 0.0–10.0% methanol) to afford the product as a bright yellow crystalline powder (1.24 g, 73%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.27–7.25 (m, 4H, Ar-H), 7.24–7.22 (m, 4H, Ar-H), 7.20–7.18 (m, 2H, Ar-H), 3.63 (br s, xH, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 166.7 (C), 135.7 (C), 131.9 (CH), 129.1 (C), 129.0 (CH), 128.4 (CH), 70.6 (CH_2)*; IR (solid, cm^{-1}): 3498 (w), 2881 (s), 1959 (w), 1711 (m); m.p. 57–59 °C (dichloromethane). *multiple CH_3 visible by DEPT.

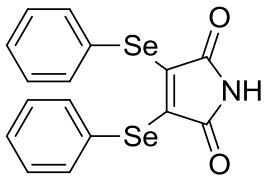
4.3.15 *N*-TPO-dithiophenolmaleimide (**32**)



To *N*-TPO-dibromomaleimide (100 mg, 0.26 mmol) and sodium hydrogencarbonate (107 mg, 1.28 mmol) in methanol (35 ml) was slowly added benzenethiol (52.2 µl, 0.51 mmol) in methanol (5 ml). The reaction was stirred for 5 min at ambient temperature. The solvent was removed *in vacuo* and the residual was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 7 : 3) to afford the product as an orange powder (78.7 mg, 68%).

^{1}H NMR (500 MHz, $CDCl_3$, treated with hydrazobenzene*): δ = 7.35–7.19 (m, 10H, Ar-H), 4.29 (dd, 1H, *J* = 12.6, 7.2, CH), 3.00 (dd, 1H, *J* = 10.2, 7.6, CH_2), 2.04 (dd, 1H, *J* = 12.5, 7.4, CH_2), 1.31 (s, 3H, CH_3), 1.23 (s, 3H, CH_3), 1.21 (s, 3H, CH_3), 1.08 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$, treated with hydrazobenzene*): δ = 167.4 (C), 136.0 (C), 132.4 (CH), 131.9 (CH), 131.1 (CH), 129.4 (C), 56.3 (CH), 35.5 (CH_2), 27.3 (C), 25.7 (C), 24.9 (2x CH_3), 21.9 (2x CH_3); IR (solid, cm^{-1}): 2973 (w), 2930 (w), 1771 (w), 1708 (s), 1631 (w), 1582 (w), 1537 (w); MS (EI) *m/z,* (%): 453 (M, 17), 99 (100); Mass calc. for $C_{24}H_{25}O_3N_2S_2$: 453.1307. Found: 453.1292; m.p. 124–128 °C (dichloromethane). *In order to obtain detailed NMR spectra it was necessary to reduce the radical prior to NMR-spectroscopy. This was done by treatment of the purified spin label with a 3x excess (by mass) of hydrazobenzene for 60 min.[146] The presented data is thus an excerpt of the mixture of the spin label, hydrazobenzene and their reaction products.

4.3.16 Dibenzeneselenolmaleimide (**33**)

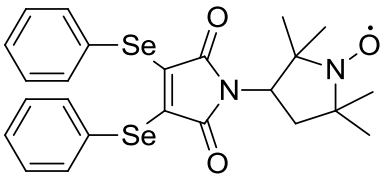


To dibromomaleimide (300 mg, 1.17 mmol) and sodium hydrogencarbonate (491 mg, 5.85 mmol) in methanol (38 ml) was slowly added benzeneselenol (236 µl, 2.22 mmol) in methanol (2 ml). The reaction was stirred for 30 min at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether : ethyl

acetate, gradient elution from 9 : 1 to 8 : 2) to afford the product as yellow crystals (367 mg, 81%).

^{1}H NMR (500 MHz, MeOD): δ = 7.42–7.39 (m, 4H, Ar-H), 7.34–7.30 (m, 2H, Ar-H), 7.28–7.25 (m, 4H, Ar-H); ^{13}C NMR (125 MHz, MeOD): δ = 169.9 (C), 140.6 (C), 135.2 (C), 130.4 (CH), 129.5 (CH), 127.9 (CH); IR (solid, cm^{-1}): 3346 (m), 2950 (w), 2481 (m), 2072 (m), 1757 (w), 1716 (m); MS (CI) *m/z,* (%): 409 (M, 9), 314 (100), 234 (33), 157 (100); Mass calc. for $C_{16}H_{11}O_2NSe_2$: 408.9115. Found: 408.9110; m.p. 123–125 °C (chloroform).

4.3.17 *N*-TPO-dibenzeneselenolmaleimide (**34**)

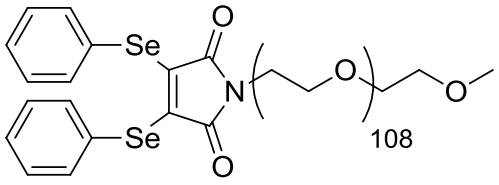


To *N*-TPO-dibromomaleimide (120 mg, 0.30 mmol) and sodium hydrogencarbonate (128 mg, 1.52 mmol) in methanol (30 ml) was slowly added benzeneselenol (67.7 µl, 0.64 mmol) in methanol (1 ml). The reaction was stirred for 40 min at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 1 : 1) to afford the product as an orange wax (33.4 mg, 20%).

^{1}H NMR (500 MHz, $CDCl_3$, treated with hydrazobenzene*): δ = 7.37–7.20 (m, 10H, Ar-H), 4.40 (dd, 1H, *J* = 12.4, 8.7, CH), 2.91 (dd, 1H, *J* = 10.6, 7.7, CH_2), 1.77 (dd, 1H, *J* = 12.4, 8.1, CH_2), 1.32 (s, 3H, CH_3), 1.27 (s, 3H, CH_3), 1.21 (s, 3H, CH_3), 1.06 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$, treated with hydrazobenzene*): δ = 167.9 (C), 138.7 (C), 134.7 (CH), 133.4 (CH), 132.6 (CH), 129.0 (C), 56.1 (CH), 35.5 (CH_2), 29.7 (C), 25.5 (C), 21.6 (2x CH_3), 19.7 (2x CH_3); IR (solid, cm^{-1}): 3054 (w), 2975 (w), 2930 (w), 1768 (w), 1705 (s), 1577 (w), 1533 (w); MS (EI) *m/z,* (%): 549 (M, 43), 476 (29), 157 (61), 99 (100); Mass calc. for $C_{24}H_{25}O_3N_2Se_2$: 549.0196. Found: 549.0190. *In order to obtain detailed NMR spectra it was necessary to reduce the radical prior to NMR-spectroscopy. This

was done by treatment of the purified spin label with a 3x excess (by mass) of hydrazobenzene for 60 min.[146] The presented data is thus an excerpt of the mixture of the spin label, hydrazobenzene and their reaction products.

4.3.18 *N*-PEG5000-dibenzeneselenolmaleimide (**35**)



All glassware used was oven dried before the reaction. To triphenylphosphine (96.6 mg, 0.37 mmol) in a mixture of THF (8 ml) and DCM (3 ml) was added dropwise diisopropyl azodicarboxylate (72.5 µl, 0.37 mmol) at –78 °C. The reaction was stirred for 5 min before mPEG5000 (921 mg, 0.18 mmol) in DCM (7 ml) was added dropwise. After 5 min neopentyl alcohol (32.4 mg, 0.37 mmol) was added in a mixture of THF (1 ml) and DCM (1 ml) followed after 5 min by dibenzeneselenolmaleimide (150 mg, 0.37 mmol) in anhydrous THF (3 ml). The reaction was stirred for 5 min, the cold bath removed and stirred for 20 h at ambient temperature. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (methanol : dichloromethane, gradient elution from 0.5–9.0% methanol) to afford the product as a yellow crystalline powder (168 mg, 17%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.41–7.40 (m, 4H, Ar-H), 7.30–7.26 (m, 4H, Ar-H), 7.23–7.20 (m, 2H, Ar-H), 3.58 (br s, xH, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 165.5 (C), 134.7 (C), 131.5 (CH), 129.3 (C), 128.8 (CH), 128.6 (CH), 70.6 (CH_2)*; IR (solid, cm^{-1}): 3483 (w), 2877 (s), 1973 (w), 1709 (w); m.p. 45–47 °C (dichloromethane). *multiple CH_3 visible by DEPT.

4.3.19 Reaction of *N*-Boc-L-Cys-OMe with halomaleimides

The reaction was carried out in three variants with each halomaleimide: (A) To *N*-(tert-butoxycarbonyl)-L-cysteine methyl ester (31.8 µl, 0.17 mmol, in 600 µl DMF) was added buffer (12 ml, 150 mM NaCl, 100 mM sodium phosphate, pH 8.0). Then 1.1 equiv of the halomaleimide (iodine: 59.2 mg, bromine:

43.5 mg, chlorine: 28.2 mg, in 300 µl DMF) were added and the reaction stirred at ambient temperature for 10 min. (B) To the halomaleimide (see above, in 300 µl DMF) was added buffer (12 ml, 150 mM NaCl, 100 mM sodium phosphate, pH 8.0). Then *N*-(tert-butoxycarbonyl)-L-cysteine methyl ester (31.8 µl, 0.17 mmol, in 600 µl DMF) was added and the reaction stirred at ambient temperature for 10 min. (C) To 1 equiv of the halomaleimide (see above, in 300 µl DMF) was added buffer (12 ml, 150 mM NaCl, 100 mM sodium phosphate, pH 8.0). Then *N*-(tert-butoxycarbonyl)-L-cysteine methyl ester (76.9 µl, 0.41 mmol, in 600 µl DMF) was added and the reaction stirred at ambient temperature for 10 min.

In each case the resultant yellow precipitate was dissolved by addition of ethyl acetate (20 ml) and the organic phase washed with water (3 x 25 ml), followed by a 10% (w/v) lithium chloride solution (5 x 25 ml) and dried over magnesium sulfate. The solvent was removed *in vacuo* and the residual material was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 1 : 1). The solvent was removed *in vacuo* from fractions that contained detectable material. The identity of the compounds was confirmed by ^{1}H and ^{13}C NMR. The mono-addition product and cystine were obtained as an inseparable mixture and the relative yields were estimated from the corresponding ^{1}H NMR spectra.

4.3.20 Treatment of dicysteinemaleimide with reducing agents

Procedure in methanol: To dicysteinemaleimide (50.0 mg, 0.09 mmol) in methanol (25 ml) was added TCEP (50.9 mg, 0.18 mmol) in methanol (5 ml). The reaction was stirred for 24 h at ambient temperature. The solvent was removed *in vacuo* and the remaining material purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 7 : 3) to recover dicysteinemaleimide in 98% yield.

Procedure in aqueous solution: To dicysteinemaleimide (50.0 mg, 0.09 mmol) in buffer (22 ml, 150 mM NaCl, 100 mM sodium phosphate, pH 8.0, 40% MeCN, 7.5% DMF) were added various reducing agents (TCEP: 80.8 or 255.4 mg, DTT: 137.1 mg, GSH: 54.3 mg, 1,2-ethanedithiol: 74.7 µl) in buffer (3 ml). The reaction was stirred at ambient temperature for indicated periods (see Table

2.3). Ethyl acetate was added (30 ml) and the organic phase was washed with water (3 x 50 ml). Both phases were dried *in vacuo*. The residual material of the aqueous phase was analysed by LCMS. The residual material of the organic phase was purified by flash chromatography on silica gel (petroleum ether : ethyl acetate, gradient elution from 9 : 1 to 1 : 1). The solvent was removed *in vacuo* from fractions that contained detectable material. The identity of the compounds was confirmed by ^{1}H, ^{13}C NMR, MS and LCMS.

4.4 Modification of somatostatin

4.4.1 Preparation of reduced somatostatin

To prepare reduced somatostatin the peptide solution was treated with 1.1 equiv of TCEP for 1 h at ambient temperature. To an aliquot a 5x excess of dibromomaleimide was added and completeness of bridging, indicating completeness of disulfide reduction, was confirmed by LCMS. If necessary more TCEP was added in 0.1 equiv steps, incubated for additional 10 min and the product tested via the same method.

To determine the speed of reduction, somatostatin was treated as described but the test reaction was carried out on an aliquot after 1 and 10 min indicating 50% and 95% disulfide bond cleavage respectively.

4.4.2 Bridging of somatostatin with dibromomaleimide

To reduced somatostatin was added 1.1 equiv of dibromomaleimide. The mixture was incubated at ambient temperature for 1 h and analysed by LCMS.

As a control reaction oxidised somatostatin was incubated with 10 equiv of dibromomaleimide and analysed for additional peaks over 28 days (storage at ambient temperature).

The stability of maleimide bridged somatostatin was monitored by LCMS over 8 week's storage at 4 °C.

4.4.3 Bridging of somatostatin with dihalomaleimides

To reduced somatostatin were added 1.1 equiv of dichloro-, dibromo-, or diiodomaleimide. The mixture was incubated at ambient temperature and analysed by LCMS after 1, 5, 10, 15, 20 and 25 min. After complete modification had

been observed the samples were stored at ambient temperature for 4 days and analysed for possible further reactions by LCMS.

4.4.4 Stopped-flow analysis of the somatostatin bridging-reaction

Ultra-fast kinetics for the reactions of halomaleimides with somatostatin were obtained with a stopped-flow device (Hi-Tech CW-61, Kinet Asyst) equipped with a 250 µl syringe (loaded with the halomaleimide compound) and a 2.50 ml syringe (loaded with the peptide).

Reactions were performed at 20 °C and monitored via the increase of absorbance at a wavelength of 395 nm. The data was corrected for the absorbance of the peptide solution. Final concentrations in the mixing chamber were 34.7 µM reduced somatostatin and a 5x excess (173.5 µM) of the halomaleimide compound. For a single experiment the syringes were loaded, 10–15 short reactions (100 s) performed and then raw data recorded from 3 reactions for 12 min.

As the reaction followed no lower order kinetics, apparent rate constants were calculated using SigmaPlot (Systat Software). A two-exponential fit ($y = A - B^{(-x\ C)} - D^{(-x\ E)}$, with A = final absorbance, B = amplitude 1, C = k_{app1}, D = amplitude 2, E = k_{app2}) gave the best results indicating a two step process. Final rate constants were averaged from 3 experiments. The assay was performed with the help of Dr Tina Daviter (Birkbeck Department of Biological Sciences).

4.4.5 Cleavage of maleimide bridged somatostatin

Maleimide bridged somatostatin was synthesised as described. 100 equiv of DTT, 2-mercaptoethanol, GSH, TCEP or 1,2-ethanedithiol were added, the mixture incubated at ambient temperature and an aliquot analysed by LCMS after 10 min, 1, 4, 24 and 48 h. The samples were monitored especially for the formation of addition products of the reducing agents to the starting material and mixed disulfides between the thiols and the peptide. Reactions containing GSH were also analysed by MALDI-TOF after 48 hours.

Alternatively 10, 20 or 50 equiv of DTT or 2-mercaptoethanol were added to maleimide bridged somatostatin and the cleavage reaction analysed by LCMS.

4.4.6 Catalysed cleavage of maleimide bridged somatostatin

Maleimide bridged somatostatin was synthesised as described. 5 equiv of benzeneselenol or sodium iodide were added followed by 20 equiv of 2-

mercaptoethanol, the reaction incubated at ambient temperature and formation of un-bridged peptide and side products monitored by LCMS after 1, 5, 10, 15 and 20 min.

Alternatively 10 equiv of benzeneselenol were added to maleimide bridged somatostatin followed by 0, 2 or 5 equiv 2-mercaptoethanol and analysed as described. No cleavage was observed in the absence of thiol.

4.4.7 Modification of somatostatin with monobromomaleimides

To reduced somatostatin were added 0.5, 1 or 2 equiv of monobromomaleimide or *N*-methylmonobromomaleimide. The mixture was incubated at ambient temperature and monitored for the formation of mono- and bis-addition products by LCMS after 1, 10 and 60 min. The presence of the mono-addition product as opposed to a succinimide bridge was tested by addition of 2 equiv of maleimide to an aliquot and analysis by LCMS after 30 min at ambient temperature.

4.4.8 Formation of a succinimide bridge upon reaction of monobromomaleimide with somatostatin

To reduced somatostatin was added slowly 1 equiv of monobromomaleimide. The formation of products was analysed after 1 h at ambient temperature by LCMS. Two aliquots were removed and either 2 equiv of maleimide were added for 30 min at ambient temperature or 2 equiv of buffer solution were added and both samples analysed by LCMS.

4.4.9 Bridging of somatostatin with functionalised maleimide derivatives

To reduced somatostatin was added 1.1 equiv of *N*-PEG300-, *N*-PEG5000-, *N*-biotin-, *N*-fluorescein-, or *N*-TPO-dibromomaleimide. The mixture was incubated at ambient temperature for 1 h and analysed by LCMS. In the case of *N*-PEG5000-dibromomaleimide the conversion was calculated from the loss of the UV signal of reduced peptide over time compared to a non-reduced control. The identity of functionalised somatostatin was confirmed by MALDI-TOF MS.

4.4.10 Cleavage of functionalised somatostatin

N-Methylmaleimide-, PEG300-, PEG5000-, biotin-, fluorescein- and TPO-somatostatin were synthesised as outlined above. 100 equiv of 2-mercaptoethanol were added and the reaction analysed after incubation for

10 min, 1, 4, 24 or 48 h at ambient temperature by LCMS. Cleavage of PEG5000-somatostatin was calculated from the loss of UV signal of the compound compared to the signal of the starting material.

4.4.11 Hydrolysis of somatostatin derivatives

PEG300-, PEG5000-, biotin-, fluorescein-, TPO- and maleimide bridged somatostatin were synthesised as described and if necessary the pH adjusted by dialysis in Slide-A-Lyzer MINI dialysis units (2 kDa MWCO) against the new buffer at 4 °C over night. Initial hydrolysis was determined by LCMS and the samples monitored by the same technique over various times at different temperatures (4 °C, ambient temperature and 37 °C) and pHs (6.2 and 8.0). Fluorescein-somatostatin was kept in the dark and the fluorescence intensity was recorded for the intact and fully hydrolysed compound at an excitation wavelength of 488 nm and corrected for differences in the concentration. Hydrolysis of PEG5000-somatostatin samples was analysed by MALDI-TOF MS comparing at least 5 PEG signals with their corresponding +18 Da signal.

4.4.12 Stability of somatostatin derivatives

PEG300-, biotin-, fluorescein-, TPO- and maleimide bridged somatostatin were synthesised as described and incubated at 37 °C for 48 hours. Samples were analysed by LCMS for the appearance of cleaved or hydrolysed peptide, loss of label or otherwise prominent somatostatin-related signals. Fluorescein-somatostatin was kept in the dark during analysis.

4.4.13 Optical activity of fluorescein-somatostatin

Fluorescein-somatostatin was synthesised as described. Fluorescence spectra were obtained at a concentration of 0.5 µg/ ml and at an excitation wavelength of 488 nm alongside spectra of *N*-fluorescein-dibromomaleimide (0.5 µg/ ml), fluorescein-amide (50 µg/ ml) and unmodified somatostatin (25 µg/ ml).

4.4.14 EPR data on maleimide spin labels and TPO-somatostatin

TPO-somatostatin was synthesised as described and dialysed against the somatostatin buffer in Slide-A-Lyzer MINI dialysis units (2 kDa MWCO) over night at 4 °C to remove the small excess of spin label. Spin labels were prepared in DMSO and diluted in the same buffer as somatostatin. EPR data was obtained

with the assistance of Mr Vishal Sanchania (UCL ISMB) at a concentration of 150 µM of spin label and peptide. *In situ* generated TPO-somatostatin (see 4.5.13) was dialysed for 5 days but spectra were still dominated by the presence of an excess of free spin label.

4.4.15 Preparation of somatostatin derivatives for patch-clamp experiments

PEG5000-, fluorescein- and maleimide bridged somatostatin were prepared as described. Somatostatin and its analogues were dialysed for 24 h at 4 °C in buffer (50 mM sodium phosphate, pH 6.2) to remove the organic solvents. After dialysis the concentration was determined and the peptides stored at 4 °C. A final concentration of 20 µM somatostatin and analogues were used (dilutions were prepared in the extracellular patch-clamp solution, see below).

4.4.16 Cell culture

Cell culture and patch-clamp experiments were performed by Dr Muriel Nobles (UCL Medicine). Cell-culture methods and the generation of stable cell lines were carried out as reported.[477] HEK293 cells (a human embryonic kidney cell line) stably expressing Kir3.1 and Kir3.2A channels were maintained in minimum essential medium supplemented with 10% foetal calf serum and 727 µg of G418 (Invitrogen), at 37 °C in humidified atmosphere (95% O_2, 5% CO_2). Cells were transiently transfected with sst_2-DNA (Missouri S&T cDNA Resource Centre) along with pEGFP-N1 (Clontech) for visualization of transfected cells using epifluorescence. Transfections were performed with 5 µl of Fugene HD (Roche) and 800 ng sst_2-DNA and 40 ng EGFP-DNA per 97 µl of cell culture medium (containing no serum or antibiotics).

4.4.17 Electrophysiology

Whole cell patch-clamp current recordings were performed with an Axopatch 200B amplifier (Axon Instruments) using fire-polished pipettes with a resistance of 3-4 MΩ pulled from filamented borosilicated glass capillaries (Harvard Apparatus, 1.5 mm OD x 1.17 mm inner diameter). Data was acquired and analysed via a Digidata 1322A interface (Axon Instruments) and pCLAMP software (version 8.1, Axon Instruments). A fast perfusion system was used to apply somatostatin and analogues (Rapid Solution Changer, RSC-160, Bio-Logic France). Cells were clamped at –60 mV. The extracellular solution was: 80 mM NaCl,

60 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 0.33 mM sodium phosphate, 10 mM glucose, pH 7.4. The intracellular solution was: 10 mM NaCl, 110 mM potassium gluconate, 20 mM KCl, 1 mM $MgCl_2$, 2 mM magnesium-ATP, 2 mM EDTA, 0.3 mM GTP, pH 7.4. For each cell it was assessed if flow artefacts resulting from the pressure of drug application were present. This was done by applying bath solution from one of the sewer pipes at the beginning of the recordings. Tertiapin-Q, an inhibitor of GIRK current (Alomone), was used at a final concentration of 100 nM. Cells were incubated overnight with pertussis toxin (Sigma-Aldrich, 100 ng/ml), an inhibitor of Gi/o proteins. Drugs were prepared as concentrated stocks solutions and kept at –20 °C.

4.5 Development of *in situ* disulfide bridging protocols

4.5.1 Combinations of dibromomaleimide and TCEP

To somatostatin were added the following reagent combinations (dibromomaleimide : TCEP; first addition of the maleimide then the phosphine): 1 : 1, 1 : 10, 2 : 1, 10 : 1, 10 : 10 or 20 : 10. The reaction was incubated for 1 h at ambient temperature and analysed by LCMS for starting material, product and side products (multiple additions of dibromomaleimide and/ or TCEP to the peptide).
Alternatively 10 equiv of dibromomaleimide were added to 1 equiv of somatostatin. Then 1 equiv of TCEP was added and the reaction incubated 10 min at ambient temperature. An aliquot was removed, analysed by LCMS and another equiv of TCEP added to the peptide solution. This process was repeated until a total of 9 equiv of the phosphine had been added.

4.5.2 Bridging of somatostatin with various dithiomaleimides

To reduced somatostatin were added 1, 5 or 10 equiv of di-2-mercaptoethanol-, dicysteine-, di-2-mercaptopyridine- or dithiophenolmaleimide. The mixture was incubated at ambient temperature and monitored by LCMS after 1, 10 and 60 min or in the case of dicysteinemaleimide after 10 min, 1, 2, 4 and 6 h. MS samples were analysed for by-products such as mixed disulfides of the liberated thiols with somatostatin cysteines.
As a control oxidised somatostatin was incubated with 5 equiv of all dithiomaleimides at ambient temperature for 24 h and the mixtures analysed by LCMS. No formation of bridged maleimides or by-products was observed.

4.5.3 Cross-reaction of maleimide derivatives with TCEP

TCEP (51.7 mg, 0.2 mmol) was dissolved in 50 ml buffer (50 mM sodium phosphate, pH 6.2, 40% MeCN, 2.5% DMF) and 1 equiv of bridging reagent (in 1.25 ml DMF, final concentration 5%) was added. The reaction mixture was stirred for 10 min at ambient temperature and then 100 ml ethyl acetate was added. The organic phase was washed with saturated lithium chloride solution (4 x 150 ml), water (100 ml), saturated sodium chloride solution (100 ml) and dried over magnesium sulphate. The solvent was removed *in vacuo*, the weight of the residual material quantified and its identity confirmed by NMR.
In the case of di-2-mercaptoethanolmaleimide no DMF was present in the buffer and the aqueous phase was just extracted with 5 x 100 ml ethyl acetate. A control reaction without TCEP was performed alongside each sample and the yield normalised accordingly.

4.5.4 Combinations of dithiomaleimides and TCEP

To somatostatin were added 10 equiv of di-2-mercaptoethanolmaleimide or 5 equiv of dithiophenolmaleimide and the mixtures incubated at ambient temperature for 10 min. Then 1, 2, 5 or 10 equiv of TCEP were added and an aliquot analysed by LCMS after 1 and 2 h reaction at ambient temperature.
Alternatively 5 equiv of dithiophenolmaleimide were added to somatostatin followed by 3 equiv of TCEP after 10 min at ambient temperature. Aliquots were removed after 1, 5, 10, 20, 40 and 60 min and analysed by LCMS.

4.5.5 Interaction of dibromomaleimide with 2-mercaptoethanol

To reduced somatostatin were added 10 equiv of 2-mercaptoethanol. The mixture was maintained at ambient temperature for 5 min and 1, 5, 10 or 15 equiv of dibromomaleimide were added. The reaction was incubated at ambient temperature and samples analysed by LCMS after 1 min, 10 min, 1, 2, 4 and 24 h.

4.5.6 Cleavage of the somatostatin disulfide bond with benzeneselenol

To somatostatin were added the following reagent ratios (benzeneselenol : 2-mercaptoethanol): 10 : 0, 10 : 2 or 1 : 10. The samples were incubated at ambient temperature and aliquots removed after 1 h and 3 d, mixed with 10 equiv of

dibromomaleimide for 20 min and analysed by LCMS for the formation of bridged peptide.

4.5.7 *In situ* reactions with dithiophenolmaleimide and benzeneselenol

To somatostatin were added various amounts of dithiophenolmaleimide for 10 min at ambient temperature followed by various amounts of freshly prepared benzeneselenol to yield the following combinations (maleimide : selenol): 5 : 3, 5 : 5, 10 : 3 or 10 : 5. The reaction was maintained at ambient temperature and samples analysed by LCMS after 1, 5, 10, 20, 40, 60, 90 and 120 min.

4.5.8 *In situ* reactions with di-2-mercaptoethanolmaleimide and benzeneselenol

To somatostatin were added various amounts of di-2-mercaptoethanol-maleimide for 10 min at ambient temperature followed by various amounts of freshly prepared benzeneselenol to yield the following combinations (maleimide : selenol): 10 : 5, 10 : 20, 20 : 5 or 30 : 5. The reaction was maintained at ambient temperature and samples analysed by LCMS after 5 min, 30 min, 1, 2, 4 and 24 h.

4.5.9 *In situ* reactions with di-2-mercaptoethanolmaleimide and 2-mercaptoethanol

To somatostatin were added 10 equiv of di-2-mercaptoethanolmaleimide and incubated for 10 min at ambient temperature followed by 1, 3, 5, 20, 30 or 40 equiv of 2-mercaptoethanol. The reaction was incubated at ambient temperature and aliquots analysed by LCMS after 1, 2, 4, 24 and 48 h.

4.5.10 Comparison of various *in situ* protocols

Somatostatin was incubated at ambient temperature for 10 min with 5 or 10 equiv dithiophenolmaleimide or 20 equiv of di-2-mercaptoethanolmaleimide followed by addition of 3 equiv TCEP respectively 5 or 10 equiv of freshly prepared benzeneselenol. All three samples were analysed after 1, 5, 10, 20, 40 and 60 min at ambient temperature by LCMS.

4.5.11 *In situ* PEGylation of somatostatin

Somatostatin was mixed with 5 or 10 equiv of *N*-PEG5000-dithiophenolmaleimide, incubated at ambient temperature for 10 min and

3 equiv TCEP or 5 equiv of freshly prepared benzeneselenol were added. Aliquots were removed and analysed by LCMS after 1, 5, 10, 20, 40 and 60 min. The conversion was calculated from the loss of the UV signal of unmodified peptide over time compared to a non-reacted control. The identity of the product was confirmed by MALDI-TOF MS.
Alternatively the reaction with TCEP was repeated in the absence of MeCN and DMF in the peptide buffer as well as in the compound stock solutions and analysed as described.

4.5.12 Stability of maleimide spin labels to reducing agents

A *N*-TPO-dithiophenolmaleimide stock solution was diluted in somatostatin buffer to a concentration of 152.6 µM and 5 equiv of TCEP, thiophenol or benzeneselenol were added. The samples were quickly transferred into the sample holder and EPR spectra recorded at ambient temperature in 28 s intervals for 2 h. The experiment was performed with Mr Vishal Sanchania (UCL ISMB).

4.5.13 *In situ* spin labelling of somatostatin

Somatostatin was mixed with 5 or 10 equiv of *N*-TPO-dithiophenolmaleimide, incubated at ambient temperature for 10 min and 3 equiv TCEP respectively 5 equiv of freshly prepared benzeneselenol were added. Aliquots were removed and analysed by LCMS after 1, 5, 10, 20, 40 and 60 min. Both products were dialysed multiple times with Slide-A-Lyzer MINI dialysis units (2 kDa MWCO) against buffer for a total of 5 d at 4 °C to remove free spin label and analysed by EPR (see 4.4.14).

4.6 Modification of insulin

4.6.1 Reduction of insulin

Insulin was incubated at ambient temperature with 1, 2, 3, 5, 10, 20, 30 or 50 equiv of TCEP for 1 h. The samples were dialysed with Slide-A-Lyzer MINI dialysis units (2 kDa MWCO) and analysed by MALDI-TOF MS.
Alternatively insulin was reduced with 20 equiv of TCEP for 1 h at ambient temperature, 10 equiv of monobromomaleimide were added, incubated for 10 min and the sample dialysed and analysed.

4.6.2 Partial reduction of insulin

Insulin was treated with 1, 2 or 3 equiv of TCEP for 1 h at 4 °C followed by addition of 5 equiv of dibromomaleimide for 30 min at ambient temperature. Samples were dialysed and analysed by MALDI-TOF MS as described.

4.6.3 Testing of the TCEP mediated *in situ* protocol

Insulin was mixed with various amounts of dithiophenolmaleimide, incubated for 10 min at ambient temperature followed by addition of various amounts of TCEP yielding the following combinations (maleimide : TCEP): 1 : 1, 1 : 3, 3 : 2, 5 : 3, 5 : 5, 10 : 1, 10 : 5, 10 : 10, 10 : 20 and 20 : 5. Samples were incubated for 5 min, 30 min, 4 or 24 h at ambient temperature or at 4 °C. In a separate experiment 1 : 1 and 1 : 3 mixtures were incubated for 30 min at 50 °C and reacted with and without 7 M urea present. Alternatively the 1 : 3 mixture was also incubated for 30 min at 37 °C with and without 7 M urea present. In a separate experiment a 5 : 5 mixture was reacted in the presence and absence of 0.5% SDS. Stepwise addition of TCEP (in 10 aliquots after a 1 : 10 dilution in buffer) was tested with 3 : 1, 5 : 2, 5 : 5, 10 : 2, 10 : 5 and 20 : 5 mixtures of dithiophenolmaleimide : TCEP. All samples were dialysed and analysed by MALDI-TOF MS.

4.6.4 Testing of the benzeneselenol mediated *in situ* protocol with insulin

Insulin was mixed with various amounts of dithiophenolmaleimide, incubated for 10 min at ambient temperature followed by addition of various amounts of benzeneselenol yielding the following combinations (maleimide : selenol): 3 : 1, 5 : 2, 5 : 5 and 10 : 5. Samples were incubated for 5 min, 30 min or 4 h at ambient temperature, dialysed and analysed by MALDI-TOF MS.

4.6.5 Time course experiments of benzeneselenol mediated modification of insulin

To insulin were added 5 or 3 equiv of dithiophenolmaleimide followed by incubation at ambient temperature for 10 min and addition of 5 respectively 1 equiv of benzeneselenol. The mixture was further maintained at ambient temperature and aliquots were removed and immediately dialysed as described after 1, 5, 10, 20, 40 and 60 min. All samples were analysed by MALDI-TOF MS.

4.6.6 Optimisation of the benzeneselenol mediated *in situ* protocol

Insulin was mixed with various amounts of dithiophenolmaleimide, incubated for 10 min at ambient temperature followed by addition of various amounts of benzeneselenol yielding the following combinations (maleimide : selenol): 2 : 0.5, 3 : 1, 5 : 1, 5 : 2, 5 : 5, 10 : 1, 15 : 5, 18 : 4, 20 : 4, 22 : 4 and 25 : 4. All samples were incubated for 1 min at ambient temperature and immediately dialysed as described. In a separate experiment 3 : 1, 5 : 5 and 18 : 4 mixtures were reacted in the presence and absence of 0.5% SDS. The 18 : 4 sample was also reacted in the presence and absence of 1 M urea and both SDS and urea. All samples were analysed by MALDI-TOF MS.

4.6.7 *In situ* reaction-stop with maleimide

To insulin were added 20 equiv of dithiophenolmaleimide followed by incubation at ambient temperature for 10 min and then addition of 4 equiv of benzeneselenol. Aliquots were withdrawn after 5 min, 30 min, 1, 2 and 4 h, reacted for 10 min at ambient temperature with 50 equiv of maleimide and dialysed. All samples were analysed by MALDI-TOF MS.

4.6.8 *In situ* reaction stop with dibromomaleimide

To insulin, 18 equiv of dithiophenolmaleimide were added, followed by incubation at ambient temperature for 10 min and addition of 4 equiv of benzeneselenol. Aliquots were withdrawn after 1, 5, 10, 30, 60 and 120 min, reacted for 10 min at ambient temperature with 20 equiv of dibromomaleimide and dialysed. All samples were analysed by MALDI-TOF MS.

4.6.9 Reduction of insulin with thiophenol and benzeneselenol

To insulin, 2, 5, 10 or 20 equiv of either thiophenol or benzeneselenol were added. The mixture was incubated for 1 h at ambient temperature, dialysed and analysed by MALDI-TOF MS as described.

4.6.10 Quantification of dibromomaleimide required for *in situ* reaction-stop

To insulin, 18 equiv of dithiophenolmaleimide were added, followed by incubation at ambient temperature for 10 min and addition of 4 equiv of benzeneselenol. After 30 s 0, 2, 3, 4, 5 or 20 equiv of dibromomaleimide were added,

the reaction maintained for 10 min at ambient temperature and the dialysed. All samples were analysed by MALDI-TOF MS.

4.6.11 Timing of dibromomaleimide induced *in situ* reaction-stop

18 equiv of dithiophenolmaleimide were added to insulin, followed by incubation at ambient temperature for 10 min and addition of 4 equiv of benzeneselenol. 20 equiv of dibromomaleimide were added after 1, 5, 10, 30, 60, or 120 min, the reaction maintained for 10 min at ambient temperature and dialysed. The samples were analysed by MALDI-TOF MS.

4.6.12 Start-stop reactions

Two start-stop protocols were tested: 18 equiv of dithiophenolmaleimide were added to insulin, followed by incubation at ambient temperature for 10 min and addition of 4 equiv of benzeneselenol. The reaction was stopped after 1 min with 2 equiv of dibromomaleimide, incubated for 5 min and restarted with 4 equiv of benzeneselenol. 5 cycles were performed and aliquots withdrawn after each reaction stop dialysed and analysed by MALDI-TOF MS.

Alternatively 10 equiv of dithiophenolmaleimide were added to insulin, incubated for 10 min at ambient temperature and the reaction started with 0.5 equiv of benzeneselenol. The reaction was stopped after 5 min with 0.2 equiv of dibromomaleimide and re-started by addition of another 0.5 equiv of benzeneselenol 5 min later. 12 cycles were performed. After the 4^{th} and 8^{th} cycle 2 equiv of dithiophenolmaleimide were added. Aliquots were withdrawn after every second cycle, dialysed and analysed by MALDI-TOF MS.

4.6.13 pH study of the *in situ* modification of insulin

Insulin was prepared as described in 4.2.2 but in 50 mM phosphate buffers (40% MeCN, 2.5% DMF) with a pH of 5.0, 6.5, 7.4 or 8.0. As the protein was not completely soluble at pH 5.0 this sample was omitted from the experiment. To the other samples 10 equiv of dithiophenolmaleimide were added, the mixture incubated for 10 min at ambient temperature and the reaction initiated by addition of 4 equiv of benzeneselenol. The reaction was stopped after 5 min or 30 min with 10 equiv of dibromomaleimide, samples dialysed and analysed by MALDI-TOF MS.

4.6.14 *In situ* generation of dibenzeneselenolmaleimide for the *in situ* modification of insulin

To insulin were added 10 equiv of dibromomaleimide and incubated for 10 min at ambient temperature. Then 20 equiv of benzeneselenol were added for 10 min followed by addition of more benzeneselenol: either 3 equiv were added and the reaction monitored over 40 min with aliquots withdrawn every 10 min or 0.5, 1, 2, 3, or 5 equiv were added and the reaction stopped with 5 equiv dibromomaleimide after 30 min or 0.5 equiv were added each 5 min with 6 repetitions and aliquots withdrawn every 10 min. In all cases the (removed) samples were dialysed and analysed by MALDI-TOF MS.

4.6.15 Condition screen for the dibenzeneselenolmaleimide-based *in situ* modification of insulin

Insulin was prepared in pH 6.5 or pH 7.4 buffer. 10 equiv of dithiophenolmaleimide or dibenzeneselenolmaleimide were added to both insulin preparations and incubated for 10 min at ambient temperature. The reaction was started by addition of 1 equiv of thiophenol, benzeneselenol, TCEP, DTT, 2-mercaptoethanol or L-cysteine and stopped after 10 min with 5 equiv of dibromomaleimide. All 24 samples were dialysed and analysed by MALDI-TOF MS.
Guided by the results the reactivity of 2-mercaptoethanol towards insulin was tested in a reduction study as described for thiophenol and benzeneselenol under 4.6.9.

4.6.16 Optimisation of the *in situ* modification of insulin: quantity of reducing agent

To insulin (in pH 6.5 buffer) were added 10 equiv of dibenzeneselenolmaleimide and the reaction mixture was incubated for 10 min at ambient temperature. Then 0.5, 1.0, 1.5, 2.0, 3.0 or 5.0 equiv of 2-mercaptoethanol were added and the reaction stopped with 5 equiv of dibromomaleimide after 10 min. The samples were dialysed and analysed by MALDI-TOF MS.

4.6.17 Optimisation of the *in situ* modification of insulin: reaction time

To insulin (in pH 6.5 buffer) were added 10 equiv of dibenzeneselenolmaleimide and the reaction mixture was incubated for 10 min at ambient temperature. The

reaction was initiated with 1.5 equiv of 2-mercaptoethanol and stopped with 5 equiv of dibromomaleimide after 1, 3, 5, 8, 10 or 15 min. The samples were dialysed and analysed by MALDI-TOF MS.

4.6.18 Optimisation of the *in situ* modification of insulin: quantity of bridging reagent

To insulin (in pH 6.5 buffer) were added 2, 5, 10, 15, 20 or 25 equiv of dibenzeneselenolmaleimide and the reaction mixture was incubated for 10 min at ambient temperature. The reaction was initiated with 1.5 equiv of 2-mercaptoethanol and stopped with 5 equiv of dibromomaleimide after 10 min. The samples were dialysed and analysed by MALDI-TOF MS as described.

4.6. 19 Optimisation of the *in situ* modification of insulin: ratio of compounds

To insulin (in pH 6.5 buffer) were added various amounts of dibenzeneselenolmaleimide, the mixture was incubated for 10 min at ambient temperature and the reaction started with various amounts of 2-mercaptoethanol. The final amounts of reducing and bridging agent were multiples of 1.5 equiv 2-mercaptoethanol and 10 equiv of dibenzeneselenolmaleimide up to 6x the indicated compounds (i.e. 9.0 equiv of reducing agent and 60 equiv of bridging reagent). The reaction was stopped after 10 min with 5 equiv of dibromomaleimide. All samples were dialysed and analysed by MALDI-TOF MS.

4.6.20 Timing of the *in situ* modification of insulin with a large excess of bridging reagent

To insulin (in pH 6.5 buffer) were added 25 equiv of dibenzeneselenolmaleimide. The reaction mixture was incubated for 10 min at ambient temperature and the reaction initiated by addition of 1.5 equiv of 2-mercaptoethanol. The modification reaction was stopped with 5 equiv of dibromomaleimide after 1, 5, 10, 15, 20 or 30 min and the reactions dialysed and analysed by MALDI-TOF MS.

4.6.21 Small amounts of 2-mercaptoethanol for a stepwise modification protocol

25 equiv of dibenzeneselenolmaleimide were added to insulin (in pH 6.5 buffer) and the reaction mixture was incubated for 10 min at ambient temperature. 0.3, 0.5, 0.7, 1.0, 1.5, or 2.0 equiv of 2-mercaptoethanol were added to the mixture,

reacted for 10 min and the reaction stopped by addition of 5 equiv of dibromomaleimide. All samples were dialysed and analysed by MALDI-TOF MS.

4.6.22 Optimisation of the stepwise *in situ* modification of insulin

25 equiv of dibenzeneselenolmaleimide were added to insulin (in pH 6.5 buffer) and the reaction mixture was incubated for 10 min at ambient temperature. The reaction was started by addition of 1.0 or 1.5 equiv of 2-mercaptoethanol and the same amount of reducing agent was added 5 times every 10 min. Aliquots were withdrawn before the addition of fresh 2-mercaptoethanol, stopped with 5 equiv of dibromomaleimide, dialysed and subjected to MALDI-TOF MS.

4.6.23 Optimisation of the two-step *in situ* modification of insulin

To insulin (in pH 6.5 buffer) were added 25 equiv of dibenzeneselenolmaleimide and the reaction mixture was incubated for 10 min at ambient temperature. Then various amounts of 2-mercaptoethanol were added to start the reaction followed by the addition of more reducing agent, again varying in its quantity, after 10 min. The reaction was stopped after 10 min more by the addition of 5 equiv of dibromomaleimide. Combinations of the two-step additions were 1.5/1.5, 1.5/1.0, 1.5/0.5, 1.0/1.5, 1.0/1.0, 1.0/0.7, 1.0/1.5, 1.0/0.3 and 1.0/0.2 equiv of 2-mercaptoethanol. All samples were dialysed and analysed by MALDI-TOF MS.

4.6.24 Optimisation of the three-step *in situ* modification of insulin

This experiment was carried out as described in 4.6.23 but a third addition step of reducing agent was added. This time the combinations were 1.0/0.0/0.0, 1.0/0.7/0.0, 1.0/0.7/0.1, 1.0/0.7/0.3, 1.0/0.7/0.5 and 1.0/0.7/0.7 equiv of 2-mercaptoethanol.

4.6.25 *In situ* spin labelling of insulin

25 equiv of *N*-TPO-dibenzeneselenolmaleimide were added to insulin (in pH 6.5 buffer) and the reaction mixture was incubated for 10 min at ambient temperature. Then 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 equiv of 2-mercaptoethanol were added and the reaction stopped after 10 min with 10 equiv of dibromomaleimide. All samples were dialysed and analysed by MALDI-TOF MS.

4.6.26 Optimised *in situ* spin labelling reactions of insulin

To insulin (in pH 6.5 buffer), 10 equiv of *N*-TPO-dibenzeneselenolmaleimide were added and the reaction mixture incubated at ambient temperature for 10 min. Next 0.5, 1.0, 1.5, 2.0, 3.0 or 5.0 equiv of 2-mercaptoethanol were added and the reaction stopped with 10 equiv of dibromomaleimide after 10 min. The samples were dialysed and analysed by MALDI-TOF MS.

4.6.27 Optimisation of the two-step *in situ* spin labelling reaction of insulin

To insulin (in pH 6.5 buffer), 10 equiv of *N*-TPO-dibenzeneselenolmaleimide were added and the reaction mixture was incubated for 10 min at ambient temperature. Then 1.5 equiv of 2-mercaptoethanol were added to start the reaction followed by the addition of varying amounts of more reducing agent after 10 min. The reaction was stopped after 10 min more by addition of 10 equiv of dibromomaleimide. The amounts of 2-mercaptoethanol added in the second step were 0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 equiv. All samples were dialysed and analysed by MALDI-TOF MS.

4.6.28 Optimisation of the three-step *in situ* spin labelling reaction of insulin

This reaction was carried out as described in 4.6.27 but after addition of 1.5 equiv followed by 1.0 equiv of 2-mercaptoethanol was added a third reaction step for 10 min before the reaction stop with 0.0, 0.3, 0.5, 0.75 or 1.0 equiv reducing agent. The samples were dialysed and analysed by MALDI-TOF MS.

4.6.29 *In situ* PEGylation of insulin and optimisation

PEGylation of insulin via the *in situ* protocol was optimised as outlined above. To insulin (in pH 6.5 buffer) were added in the presence of 25 equiv *N*-PEG5000-dibenzeneselenolmaleimide various amounts of 2-mercaptoethanol in 10 min intervals before reaction stop with dibromomaleimide. Tested amounts of reducing agent were 0.75, 1.0, 1.5 and 2.0 equiv in the first step, 0.75, 1.0 and 1.5 equiv in the second step and 0.5, 0.75 and 1.0 equiv in the last step. All samples were analysed by Tricine SDS-PAGE and MALDI-TOF MS.

4.6.30 Purification of PEGylated insulin

Doubly PEGylated insulin was prepared using the optimised *in situ* protocol: 2 ml of a 0.5 mg/ ml solution in pH 6.5 buffer were sequentially treated with 1.5,

1.5 and 1.0 equiv of 2-mercaptoethanol for 10 min each in the presence of 25 equiv of *N*-PEG5000-dibenzeneselenolmaleimide and the reaction stopped with 5 equiv of *N*-PEG5000-dibromomaleimide. Modified insulin was purified on a HiPrep 26/60 Sephacryl S-100 HR column (GE Healthcare, equilibrated in 150 mM NaCl, 50 mM phosphate, pH 7.4). Fractions containing the product were identified by MALDI-TOF MS, pooled and further purified by repeated filtration (30 kDa MWCO, Sartorius). The buffer was exchanged for sterile water in the same step and the material freeze-dried in aliquots. These were weighted and re-solubilised as described for the bridged insulin for CD spectroscopy or digest studies.

4.6.31 Purification of bridged insulin

Double bridged insulin was prepared via the optimised *in situ* protocol: 8 ml of a 0.5 mg/ ml solution in pH 6.5 buffer were sequentially treated with 1.0, 0.7 and 0.5 equiv of 2-mercaptoethanol for 10 min each in the presence of 25 equiv of dibenzeneselenolmaleimide and the reaction stopped with 5 equiv of dibromomaleimide. The reaction mixture was dialysed over night into deionised water in a Slide-A-Lyzer G2 dialysis cassette (3.5 kDa MWCO, Thermo Scientific), the insoluble particles sedimented by centrifugation (4,000 rpm, 10 min) and the pellet washed repeatedly with 1 ml of sterile water until no A chain side products could be observed by MALDI-TOF MS. The residual material was resuspended in buffer and filtered through a PD MiniTrap G-25 desalting column (GE Healthcare) following manufacturers' instructions to remove the insoluble B chain side products. The modified insulin was dialysed into water, freeze-dried and re-solubilised in a low salt buffer suitable for CD spectroscopy (see below) or in sterile water for a digest study. The concentration of doubly bridged insulin in these solutions was determined with a Bradford assay (Quick Start Bradford Kit, BioRad) following manufacturers' instructions for which a standard curve had been prepared with unmodified insulin (0.1–1.5 mg/ ml). The presence of maleimide, dibenzeneselenolmaleimide or *N*-PEG5000-dibenzeneselenolmaleimide was found to have no influence on the Bradford reaction.

4.6.32 Digest of insulin and analogues with thermolysin

Insulin and it's double bridged and PEGylated analogues were digested with thermolysin[410] to localise the position of the maleimide bridges. The protocol was optimised with the unmodified hormone in order to achieve a complete digest without the appearance of fragments from a potential self-digest of the protease. Some of the samples were treated after the digest with TCEP (10 equiv, 1 h) and/ or maleimide (in DMF, 10 equiv, 10 min) to clarify the data.

Insulin and its analogues were dissolved in sterile water at 0.5 mg/ ml and thermolysin added to a final concentration of 0.05 mg/ ml. All samples were incubated under constant agitation at 70 °C for 1 h and analysed by MALDI-TOF MS. The obtained fragmentation pattern was compared to a simulated digest prepared with the PeptideMass and PeptideCutter tools of the ExPASy proteomics server (see 4.2). Here the A and B chains of insulin were analysed independently while allowing a maximum of 5 missed cleavages, and the masses of potentially disulfide connected fragments combined.

4.6.33 CD spectroscopy with insulin and analogues

CD spectra of insulin analogues were acquired on a J-720 spectropolarimeter (Jasco) between 300–180 nm at ambient temperature (20.8 °C) with a scan speed of 50 nm/ min and a band width of 2.0 nm in a 1.0 mm cuvette. Data for double bridged and PEGylated insulin was recorded in a 10 mM NaCl, 10 mM phosphate, pH 7.4 buffer at concentrations of 0.05 and 0.17 mg/ ml respectively. For comparison and as controls, spectra of unmodified insulin at 0.1 and 0.2 mg/ ml as well as of mPEG5000 and *N*-PEG5000-dibenzeneselenol-maleimide at 0.39 mg/ ml were acquired. Data was recorded in triplicate and the background (buffer) subtracted before data analysis. The molar ellipticity was calculated as described[285] with a mean residue molecular weight of 114 g/ mol.[302]

4.7 Modification of an anti-CEA scFv antibody fragment

4.7.1 Proteins

Anti-CEA is single chain antibody fragment directed against the two most N-terminal (extracellular) Ig domains of human CEA which it binds with low nM affinity.[418] The original scFv is a mouse antibody isolated from a phage display and can be produced in large quantities in bacteria.[426, 478, 479] The construct

used in this work (internal name shMFELL2Cys) is a humanised version (28 amino acid substitutions)[416] comprising the variable domain of a heavy and a light chain respectively which are connected by a peptide linker $(Gly_4Ser)_4$ and has a MW of 26.7 kDa (246 amino acids). A His_6-tag has been added to the C-terminus to facilitate purification and an artificial disulfide bond was introduced opposite to the antigen binding site (Gly`H44Cys and Ala`L100Cys, Kabat numbering) to stabilise the protein. A crystal structure of the parental antibody is available (PDB code: 1QOK).[418] This crystal structure was used to model the cartoons of unmodified and maleimide bridged anti-CEA as follows: Residues Gly44 and Ala100 were replaced in Chimera (University of California, San Francisco, version 1.5.3) with cysteines, their orientation corrected using the rotamer library of the software and one round of energy minimization (100 iterations, both cysteines fixed) applied with and without previous insertion of a disulfide bond. The material was kindly supplied by Dr Berend Tolner (UCL Cancer Institute) and was 95% pure as estimated from SDS-PAGE analysis.

NA1 is a 26.0 kDa fragment of CEA comprising its two N-terminal Ig domains and is bound by anti-CEA with nM affinity. The construct has a His_6-tag for purification via IMAC.[480] NA1 was also kindly supplied by Dr Berend Tolner (UCL Cancer Institute).

4.7.2 Reduction of anti-CEA with TCEP

Anti-CEA was treated with 1, 2, 3, or 5 equiv of TCEP for 24 h at 4 °C or with 1, 2, 3, 5, 10 or 20 equiv of TCEP for 1 h at ambient temperature. Monobromomaleimide was added in 2x excess over TCEP for 20 min and samples were analysed by LCMS.

4.7.3 Reduction study of anti-CEA

To anti-CEA, 50 equiv of TCEP, 2-mercaptoethanol or DTT were added and incubated for 2, 4 or 6 h. The reactions were maintained at ambient temperature and after the incubation time 100 equiv of monobromomaleimide were added and incubated for 20 min. All samples were analysed by LCMS.

4.7.4 Reduction of anti-CEA with DTT

Anti-CEA was treated with 1, 5, 10, 20 or 40 equiv of DTT for 2 h at ambient temperature after which a 2x excess of monobromomaleimide over DTT was added and incubated for 20 min and the reaction analysed by LCMS.

4.7.5 Optimisation of the reduction of anti-CEA with DTT

To anti-CEA, 10 or 20 equiv of DTT were added and the reaction was incubated for 10, 30, 60 or 90 min at ambient temperature. A 2x excess of dibromomaleimide over DTT was added and incubated for 20 min and the samples analysed by LCMS.

The same experiment was carried out under high-salt conditions for which the antibody fragment had been diluted in a PBS buffer containing an increased concentration of NaCl, so that the final salt concentration was 500 mM (instead of 137 mM).

4.7.6 Treatment of anti-CEA with thiophenol and benzeneselenol

Anti-CEA was treated with 20 equiv of thiophenol or benzeneselenol (with and without 4 equiv of thiophenol) for 10 min at ambient temperature. Then either 20 equiv of dibromomaleimide or 50 equiv of maleimide were added, the samples incubated for 20 min and analysed by LCMS.

4.7.7 Sequential bridging of anti-CEA

Anti-CEA was treated with 20 equiv of DTT at ambient temperature for 1 h. Then 30 equiv of dibromomaleimide were added and samples withdrawn after 5, 10 and 15 min and analysed by LCMS.

4.7.8 *In situ* bridging of anti-CEA

To anti-CEA were added various amounts of dithiophenolmaleimide followed by various equiv of benzeneselenol to yield the following combinations (maleimide : selenol): 5 : 2, 5 : 5, 10 : 10, 15 : 15, 20 : 10 and 20 : 20. As samples with 20 equiv of dithiophenolmaleimide became immediately cloudy, indicating problems with the solubility of the compound, these were omitted from analysis. The remaining reactions were kept at ambient temperature for 1 h and analysed by LCMS.

4.7.9 Timing of the *in situ* modification of anti-CEA

To anti-CEA, 15 equiv of dithiophenolmaleimide were added followed by 15 equiv of benzeneselenol. Aliquots were withdrawn after 5, 10, 20, 30, 45 and 60 min and subjected to LCMS.

4.7.10 Optimisation of the *in situ* modification of anti-CEA

To anti-CEA, 2 or 5 equiv of dithiophenolmaleimide were added followed by 5, 10, 15, 20 or 25 equiv of benzeneselenol. The reaction was maintained at ambient temperature for 20 min and analysed by LCMS.
Alternatively 2 equiv of dithiophenolmaleimide were added to anti-CEA followed by 5, 8, 10, 12 or 15 equiv of benzeneselenol. The reaction mixtures were incubated for 15 min at ambient temperature and then the same amount of benzeneselenol was added again. After 15 min more the samples were analysed by LCMS. The best combination was also tested with 1.2 and 1.5 equiv of dithiophenolmaleimide.

4.7.11 Sequential functionalisation and alkylation of anti-CEA

Anti-CEA was reduced with 20 equiv of DTT for 1 h at ambient temperature. Then 30 equiv of *N*-fluorescein-, *N*-biotin- or *N*-PEG5000-dibromomaleimide or alternatively 50 equiv of maleimide were added and the reactions analysed by LCMS after 10 min. In the case of anti-CEA-PEG, PEGylation was indicated by complete loss of the UV signal of the unmodified antibody compared to a non-reacted control. The identity of the product was confirmed by MALDI-TOF MS and SDS-PAGE.

4.7.12 *In situ* functionalisation of anti-CEA

To anti-CEA were added 15 equiv of *N*-PEG5000-dithiophenolmaleimide or *N*-TPO-dithiophenolmaleimide followed by addition of 15 equiv of benzeneselenol. The reaction was maintained for 20 min at ambient temperature and analysed by LCMS. In the case of PEGylation, successful conjugation was indicated by the complete loss of the UV signal of the unmodified antibody compared to a non-reacted control. The identity of the product was confirmed by MALDI-TOF MS and SDS-PAGE.

The PEGylation reaction was repeated but maintained for 60 min at ambient temperature and aliquots withdrawn and analysed by LCMS after 5, 10, 20, 30, 45 and 60 min.

4.7.13 FPLC analysis of maleimide bridged anti-CEA

Maleimide bridged anti-CEA was synthesised either sequential or *in situ* as described. Processed sscFv was prepared by mixing the antibody fragment with DMF and dithiophenolmaleimide (15 equiv) but no reducing agent and incubation for 1 h. The samples were purified on PD G-25 desalting columns and 0.3 mg loaded on a HiLoad 16/26 Superdex 75 column (GE Healthcare) equilibrated in PBS. Analysis was performed at ambient temperature with a flow rate of 1.5 ml min/ min and at a wavelength of 280 nm by Dr Berend Tolner (UCL Cancer Institute).

4.7.14 Control of dimerisation of modified anti-CEA

Maleimide bridged anti-CEA was synthesised via the sequential or the *in situ* protocol as described under low salt and high salt conditions (see 4.7.5). Aliquots of these samples were treated with 50 equiv of DTT for 1 h at ambient temperature and run alongside untreated samples on a SDS-PAGE. As controls unmodified anti-CEA, reduced anti-CEA (treated with 50 equiv of DTT for 1 h) and anti-CEA treated with 15 and 30 equiv of dithiophenolmaleimide and dibromomaleimide respectively were loaded. SDS-PAGE analysis was also performed for sequential and *in situ* functionalised anti-CEA.

4.7.15 ELISA with anti-CEA and analogues

To prepare samples of anti-CEA for ELISA the analogues of the antibody fragment were purified on PD G-25 desalting columns after modification and concentrations were determined by UV/Vis spectroscopy.

ELISA plates were coated with full length human CEA diluted to a final concentration of 1 µg/ ml in PBS, incubated for 1 h at ambient temperature, washed and blocked over night at 4 °C with a 5% solution of Marvel milk powder in PBS (Premier Foods). Plates were washed and anti-CEA and its analogues were added after dilution to the indicated concentrations (typically 5, 1, 0.5, 0.1, 0.05 and 0.01 µg/ ml) in PBS. The assay was incubated at ambient temperature for 1 h, washed and the primary antibody (anti-tetra-His mouse IgG1, Quiagen, 1 :

1,000 in 1% Marvel solution) added. After 1 h the ELISA plates were washed again and the secondary antibody (ECL anti-mouse sheep IgG1 HRP linked, GE Healthcare, 1 : 1,000 in 1% Marvel solution) added and incubated for 1 h at ambient temperature. The plates were washed and freshly prepared substrate solution (one tablet of o-phenylenediamine in 25 ml 50 µM phosphate citrate buffer, Sigma-Aldrich) was added to each well. When a strong orange colour had developed the reaction was stopped by addition of 4 M HCl and the plates read at a wavelength of 490 nm. Controls were included in every ELISA, in which PBS had been added to some of the wells instead of CEA or instead of the antibody fragment. Each sample was tested in triplicate, and errors are shown as the standard deviation of the average.

4.7.16 SPR with anti-CEA and analogues

Maleimide bridged anti-CEA and anti-CEA-PEG5000 were prepared via the *in situ* protocol, purified on PD G-25 desalting columns and the concentrations were determined by UV/Vis spectroscopy.

The binding activity was then tested alongside unmodified anti-CEA via surface plasmon resonance on a Biacore T100 device by Dr Kim Vigor (UCL Cancer Institute). In brief a SA chip (coated with streptavidin) was loaded with 566 AU of biotinylated NA1 and serial dilutions of the anti-CEA fragment and its analogues were injected (400, 200, 100, 50, 25, 12.5 and 0 nM). The contact time was 120 s at a flow rate of 20 µl/ min followed by dissociation time of 600 s. The chip was regenerated with a 10 mM glycine solution for 60 s at a flow rate of 30 µl/ min. All sample runs were performed at 25 °C and binding parameters were calculated using the provided software package (Biacore T100 Evaluation Software V 2.0.3).

4.7.17 Fluorescence of anti-CEA-fluorescein

Anti-CEA-fluorescein was synthesised as described via the sequential protocol and the excess of *N*-fluorescein-dibromomaleimide was removed by purification on PD G-25 desalting columns. The concentration of the protein solution was determined by UV/Vis spectroscopy, the anti-CEA analogue diluted to 25 or 5 µg/ ml and the fluorescence recorded at an excitation wavelength of 488 nm alongside unmodified anti-CEA (350 µg/ ml).

4.7.18 Cell-binding assay with anti-CEA-fluorescein

Anti-CEA-fluorescein was synthesised via the sequential protocol and the excess of *N*-fluorescein-dibromomaleimide was removed by purification on PD G-25 desalting columns. The concentration of the protein solution was determined by UV/Vis spectroscopy.

Cell culture was performed by Mr Marco D'Alicarnasso (UCL Cancer Institute) and cells were grown at 37 °C in humidified atmosphere (95% O_2, 5% CO_2). Log-phase cultures of CAPAN-1 (CEA expressing cells,[422] cultured in DMEM, 20% FCS, 1% glutamate, 1% streptomycin) and A375 (negative control,[434] cultured in DMEM, 10% FCS, 1% glutamate, 1% streptomycin) cell lines were detached non-enzymatically, counted and diluted (3x 10^3 to 1x 10^5 per well) in a 96-well plate. Cells (in their respective media) were allowed to attach for 24 h in the incubator (at 37 °C in humid atmosphere, 5% CO_2), were gently washed twice with PBS and treated with 500 ng of the fluorescent antibody fragment (5 µg/ ml in PBS) for 1 h at ambient temperature. All samples were gently washed twice with PBS, wells filled with PBS and the fluorescence read at 518 nm (excitation 488 nm, exposure time 100 ms, slits 12 nm). Cells treated with non-fluorescent anti-CEA, untreated cells and PBS only were used to determine the background. Each sample was tested in quadruplicate and errors are shown as the standard deviation of the average.

4.7.19 Synthesis of anti-CEA-HRP

Anti-CEA-biotin was synthesised as described via the sequential protocol and the excess of *N*-biotin-dibromomaleimide was removed by purification on PD G-25 desalting columns. The concentration of the protein solution was determined by UV/Vis and adjusted to 20 µM. 15 µl of the antibody solution were mixed with 1, 2, 4, 6, 8, 10, 12 or 15 µl of a HRP/STREP solution (Invitrogen, 1.25 mg/ ml), the sample volume adjusted to 30 µl and incubated for 1 h at ambient temperature. Samples were analysed by SDS-PAGE.

4.7.20 ELISA with anti-CEA-HRP

Anti-CEA-biotin was synthesised via the sequential protocol and the excess of *N*-biotin-dibromomaleimide was removed by purification on PD G-25 desalting

columns. The concentration of the protein solution was determined by UV/Vis spectroscopy.

The biotinylated antibody was incubated with a 3x excess (in mass) of a HRP/STREP conjugate for 1 h at ambient temperature and the anti-CEA-HRP conjugate purified with PureProteome Nickel Magnetic Beads (Millipore) following manufacturer's instructions. The product was analysed by SDS-PAGE and quantified by its' OD_{280}. 10 µl of serial dilutions of the anti-CEA-HRP conjugate (1 : 10^1 to 1 : 10^5) in PBS were mixed with 90 µl ELISA substrate solution in a 96-well plate and absorbance read after reaction stop at 490 nm. For comparison serial dilutions of the HRP/STREP conjugate (1 : 10^2 to 1 : 10^6) and of the secondary antibody for the used ELISA (1 : 10^3 to 1 : 10^7) were also tested. A 1 : 500 dilution of an OD_{280} = 0.4 solution of the HRP-anti-CEA conjugate was found to give a good signal comparable to the ELISA mixture used.

A 96-well plate was coated with various amounts of full length CEA (0.125 mg/ ml to 4 mg/ ml in PBS), blocked and washed as described and incubated with 100 µl per well of a 1 : 500 dilution of a OD_{280} = 0.4 solution of the anti-CEA-HRP conjugate for 1 h at ambient temperature. Plate read-out was performed as described.

Alternatively a standard ELISA was performed with dilutions of an OD_{280} = 0.4 solution of the anti-CEA-HRP conjugate in place of the usual antibody solutions.

4.7.21 Two-step ELISA with anti-CEA-HRP on-plate formation

An ELISA plate was prepared as described and treated with the usual dilutions of biotinylated anti-CEA. One dilution series was reacted with the described mix of primary and secondary antibody. Another sample was treated with a 1 : 460 dilution of the HRP/STREP conjugate (in PBS, 1% Marvel, 20x estimated mass excess over the antibody) and a third one with a 1 : 4600 dilution of the HRP/STREP conjugate (in PBS, 1% Marvel, 2x estimated mass excess over the antibody). Incubation times were staggered so that they did not exceed 1 h at ambient temperature for any of the samples. Visualisation and read-out were performed as described.

4.7.22 PEGylation of anti-CEA via amine groups

To anti-CEA were added 2, 5 or 10 equiv of methoxypolyethylene glycol succinate *N*-hydroxysuccinimide (NHS-PEG5000, Sigma-Aldrich) and the mixture was incubated at ambient temperature for 30 min, 2 h or 4 h after which aliquots were withdrawn and the reaction stopped by addition of glycine (final concentration 1.7 mM). All samples were analysed by SDS-PAGE.

4.7.23 Optimisation of anti-CEA PEGylation via amine groups

Anti-CEA was treated with 2 equiv of NHS-PEG5000 for 30 min at ambient temperature after which 2 more equiv of the PEGylation reagent were added. This was repeated 6 times. Aliquots were removed before the addition of more NHS-PEG5000 and stopped by addition of glycine as described. All samples were analysed by SDS-PAGE.

Alternatively 12, 15 or 18 equiv of NHS-PEG5000 were added to anti-CEA, the reactions incubated at ambient temperature for 4 h, stopped via the addition of glycine and analysed by SDS-PAGE.

4.7.24 Activity of PEGylated anti-CEA analogous

Mono PEGylated anti-CEA was synthesised with *N*-PEG5000-dithiophenolmaleimide via the described *in situ* protocol. Multi PEGylated anti-CEA was prepared via reaction with NHS-PEG5000 (18 equiv for 4 h at ambient temperature). These two species were purified using nickel spin columns (Quiagen) according to manufacturers' instructions and imidazole was removed by buffer exchange into PBS with Vivaspin 500 ultrafiltration spin columns (5 kDa MWCO, Sartorius).

Mono PEGylated anti-CEA was also synthesised by reaction with NHS-PEG5000 according to the optimised protocol (2 equiv for 30 min at ambient temperature) and purified via size exclusion chromatography in PBS (HiLoad Sephadex 75 16/60, GE Healthcare).

The activity of the three PEGylated anti-CEA species was tested alongside the unmodified antibody fragment via the described ELISA assay.

4.7.25 EPR data on anti-CEA-TPO

Anti-CEA-TPO was synthesised via the *in situ* protocol and the excess of *N*-TPO-dithiophenolmaleimide was removed by purification on PD G-25 desalting columns twice. The protein as well as a solution of NA1 was concentrated via ultrafiltration (5 kDa MWCO) and the concentration of the solutions was determined by UV/Vis spectroscopy. EPR data was obtained by Mr Vishal Sanchania (UCL ISMB) at a concentration of anti-CEA-TPO of 10 µM and 120 µM (diluted in PBS). Binding activity of anti-CEA-TPO towards NA1 was tested by diluting the spin labelled antibody fragment (final concentration 10 µM) into a 123.8 µM solution of NA1 and recording of 144 scans. In a control experiment the antigen was replaced by the same amount of BSA and the same number of scans was obtained. Both experiments were repeated in human plasma and human blood instead of PBS.

4.7.26 Stability ELISA

Maleimide bridged anti-CEA and anti-CEA-PEG5000 were prepared via the *in situ* protocol, purified on PD G-25 desalting columns and stored at 4 °C for 7 d. After this time both compounds were prepared again, purified as described, the concentration determined by UV/Vis spectroscopy and binding activity tested alongside the stored compounds via ELISA.

4.7.27 Stability of maleimide bridged anti-CEA in human plasma

Maleimide bridged anti-CEA was prepared via the *in situ* protocol, purified on a PD G-25 desalting column and the concentration determined by UV/Vis spectroscopy.

70 µg of the anti-CEA analogue were added to 500 µl of human plasma and incubated at 37 °C for 1 h, 4 h, 24 h, 3 d, 5 d or 7 d. The antibody fragment was purified from plasma using nickel magnetic beads according to manufacturers' instructions, with a few exceptions: the beads were washed 4 times in wash buffer containing no imidazole and the protein eluted twice in 500 mM imidazole for 5 min. Imidazole was removed and the eluate concentrated by repeated washes in PBS in ultrafiltration spin columns (5 kDa MWCO). The protein solution was analysed by SDS-PAGE and LCMS.

As controls alkylated and unmodified anti-CEA were incubated in human plasma at 37 °C for 7 d and isolated and analysed as described.

4.7.28 Stability of maleimide bridged anti-CEA against reducing agents

Maleimide bridged anti-CEA was prepared via the *in situ* protocol, purified on PD G-25 desalting columns and the concentration was determined by UV/Vis spectroscopy.

The modified antibody fragment was treated with 100 equiv of 2-mercaptoethanol, DTT or GSH for 48 h at ambient temperature. Aliquots were withdrawn after 1, 2, 4, 24 and 48 h and analysed by LCMS. After 48 h, all samples were reacted with 200 equiv dibromomaleimide for 1 h and again subjected to LCMS.

4.7.29 Activity of anti-CEA analogues in human plasma

Maleimide bridged anti-CEA and anti-CEA-PEG5000 were synthesised via the *in situ* protocol and alkylated anti-CEA was synthesised via the sequential protocol. All analogues were purified on PD G-25 desalting columns and the concentration determined by UV/Vis spectroscopy.

37.5 µg of the antibody and its analogues were added to 500 µl of human plasma and incubated at 37 °C. 12 µl were withdrawn from each sample after 1 h, 4 h, 24 h, 3 d, 5 d and 7 d, diluted in 788 µl PBS (to yield an assumed concentration of 1.1 µg/ ml), flash frozen in liquid nitrogen and stored at –20 °C. After all samples had been collected an ELISA assay was performed as described. As a control a dilution of human plasma in PBS was co-run.

4.8 Modification of anti-FLAG antibodies

4.8.1 Reduction of the M2 anti-FLAG antibody (glycerol)

To the M2 anti-FLAG antibody were added 0, 1, 3, 5, 20 or 50 equiv of TCEP. The mixtures were maintained on ice for 24 h and reduction analysed by SDS-PAGE.

4.8.2 *In situ* bridging of the M2 anti-FLAG antibody (glycerol)

20 or 50 equiv of dithiophenolmaleimide were added to the M2 anti-FLAG antibody, the mixture was maintained on ice for 10 min and mixed with 20 or

50 equiv of TCEP respectively. Control samples contained only the reducing agent. The reaction was kept on ice for 24 h and analysed by SDS-PAGE.

4.8.3 *In situ* PEGylation of the M2 anti-FLAG antibody (glycerol)

To the M2 anti-FLAG antibody were added 20 or 40 equiv of *N*-PEG5000-dithiophenolmaleimide and the solution was incubated on ice for 10 min followed by addition of 10 or 20 equiv of TCEP respectively. The reaction was kept on ice for 24 h and analysed by SDS-PAGE and MALDI-TOF MS.

4.8.4 *In situ* spin labelling of the M2 anti-FLAG antibody (glycerol)

20 or 50 equiv of *N*-TPO-dithiophenolmaleimide were added to the M2 anti-FLAG antibody, the mixture was maintained on ice for 10 min and mixed with 20 or 50 equiv of TCEP respectively. The reaction was kept on ice for 24 h, analysed by SDS-PAGE and the excess of spin label was removed by dialysis against assay buffer for 2x 24 h at 4 °C. EPR spectroscopic analysis did not confirm successful spin labelling but revealed the presence of free spin label.
Alternatively 10, 20, 50 or 100 equiv of *N*-TPO-dithiophenolmaleimide were used with 5, 20, 50 or 100 equivalents of benzeneselenol respectively in the described method. EPR spectroscopic analysis did not confirm successful spin labelling and no free spin label was observed.

4.8.5 Reduction of M1 and M2 anti-FLAG antibodies (sodium azide)

The anti-FLAG antibodies were treated with 0, 20, 40, 60, 100, 150 or 200 equiv (M1) or 0, 20, 240 or 480 equiv (M2) of TCEP for 1 h at ambient temperature and all samples were analysed by SDS-PAGE. In the case of the M2 antibody a sample of the protein in the buffer containing glycerol was treated the same way with 20 equiv of TCEP and co-run.

4.8.6 *In situ* bridging of the M2 anti-FLAG antibody (sodium azide)

To the M2 anti-FLAG antibody were added 10, 20, 60 or 120 equiv of dithiophenolmaleimide followed by 10, 20, 60 or 120 equiv of TCEP respectively. The reaction was maintained at ambient temperature for 1 h and analysed by SDS-PAGE.

4.8.7 Stability of the spin label in the presence of sodium azide

N-TPO-dithiophenolmaleimide was diluted in the anti-FLAG antibody buffer containing 0.02% sodium azide (3 mM) to yield a concentration of 6.3 µM (thus the preservative was present in a 476x excess). The samples were quickly transferred into the sample holder and EPR spectra recorded at ambient temperature in 28 s intervals for 2 h.

The experiment was repeated in the presence of 630 µM TCEP (thus a 100x excess over the spin label). Both experiments were performed with Mr Vishal Sanchania (UCL ISMB).

4.8.8 *In situ* spin labelling of M1 and M2 anti-FLAG antibodies (sodium azide)

To the M1 and M2 anti-FLAG antibodies (50 µl) were added 100 equiv of *N*-TPO-dithiophenolmaleimide. Then 100 equiv of TCEP were added and the reaction incubated at ambient temperature for 1 h. The volume was increased to 100 µl with sodium azide free sample buffer, the excess of spin label removed by purification on PD G-25 desalting columns twice and the eluate concentrated with Vivaspin 500 ultrafiltration spin columns (10 kDa MWCO, Sartorius) to approximately 20 µl to yield a concentration of 17.6 µM (M1) and 23.0 µM (M2) as determined by NanoDrop. EPR measurements were performed by Mr Vishal Sanchania (UCL ISMB) as well as a spin count at which the number of spins present in the solution was calculated from the second integral of the first derivative spectrum. This yielded in comparison with the protein concentration a spin count of 2.04 (M1) and 1.01 (M2) spins, thus labels, per antibody molecule (on average).

4.8.9 Alkylation of the FLAG peptide

To the FLAG peptide, bearing an additional C-terminal cysteine were added 3 equiv of maleimide and the mixture was incubated at ambient temperature for 2 h. Completeness of the alkylation was confirmed by MALDI-TOF MS.

4.8.10 EPR binding study of spin labelled M1 anti-FLAG antibody

Spin labelled M1 anti-FLAG antibody was prepared as described. Unmodified or alkylated FLAG peptide was diluted in the protein solution to yield a final concentration of 100 µM as well as Ca^{2+} to a final concentration of 1 mM via addi-

tion of a concentrated calcium buffer solution. 1000 EPR spectra were recorded of both mixtures in 28 s intervals. The experiment was performed by Mr Vishal Sanchania (UCL ISMB).

4.8.11 ELISA with spin labelled M2 anti-FLAG antibody

Spin labelled M2 anti-FLAG antibody was prepared as described.

The ELISA plate was coated with *E. coli* ras like GTPase (synthesised by Mr Vishal Sanchania, UCL ISMB), bearing a N-terminal FLAG tag in decreasing concentrations of 20, 18, 16, 14, 12, 10, 8, 6, 4, 2 and 0 µg/ l (in PBS) at 4 °C over night. Wells were washed and blocked with a 1% BSA solution (in PBS) for 1 h at ambient temperature. The plate was washed and the spin labelled M2 anti-FLAG antibody (1 : 10,000 in the blocking solution) added and incubated for 2 h at ambient temperature. The assay was washed and the secondary antibody was added (peroxidase coupled anti-mouse rabbit IgG, Sigma-Aldrich, 1 : 10,000 in blocking solution). The substrate was prepared (1 mg of 3,3',5,5'-tetramethyl benzidin in 1 ml of DMSO, added to 9 ml of 50 µM phosphate citrate buffer and 2 µl of H_2O_2) and added for 30 min to the ELISA plate after 1 h incubation at ambient temperature and washing. The reaction was stopped with 2 M sulphuric acid and the plate was read at a wavelength of 450 nm. Uncoated wells served as controls. Each sample was tested in triplicate, and errors are shown as the standard deviation of the average. The experiment was performed by Mr Vishal Sanachania (UCL ISMB).

4.9 Modification of an anti-MSP1 antibody

4.9.1 Reduction of the anti-MSP1 antibody

To the anti-MSP1 antibody were added 0, 1, 2, 3, 5, 8, 10, 15, 20, 25, 30, 40, 50, 80 or 100 equiv of TCEP and the mixture was incubated for 1 h at ambient temperature. Reduction of the antibody was analysed by SDS-PAGE.

4.9.2 *In situ* bridging of the anti-MSP1 antibody

The anti-MSP1 antibody was mixed with 0, 2, 5, 10, 50 or 100 equiv of dithiophenolmaleimide followed by addition of the same amount of TCEP.

Alternatively the anti-MSP1 antibody was mixed with 0, 2, 4, 10, 20 or 50 equiv of dithiophenolmaleimide and half the amount of TCEP was added (0, 1, 2, 5, 10 or 25 equiv).
Alternatively 0, 5, 10, 25, 50 or 100 equiv of dithiophenolmaleimide were added to the anti-MSP1 antibody followed by one fifth of this amount of TCEP (0, 1, 2, 5, 10 or 20 equiv).
All samples were incubated at ambient temperature for 1 h and analysed by SDS-PAGE.

4.9.3 *In situ* PEGylation of the anti-MSP1 antibody

To the anti-MSP1 antibody were added 100 equiv of *N*-PEG5000-dithiophenolmaleimide followed by 100 equiv of TCEP or benzeneselenol. Both samples were also prepared with only the reducing agents present. The mixtures were maintained at ambient temperature for 1 h, aliquots removed for analysis by SDS-PAGE and the remaining sample purified with PureProteome Protein A Magnetic Beads (Millipore) according to manufacturers' instructions, with the following exceptions: Incubation time was at least 1 h under constant mixing and bound samples were washed 4x with PBS. The eluates were dialysed and analysed by MALDI-TOF MS.

4.9.4 Optimisation of the *in situ* PEGylation of the anti-MSP1 antibody

The anti-MSP1 antibody was mixed with 100 equiv of *N*-PEG5000-dithiophenolmaleimide and 100 equiv of TCEP were added. As a control a sample without the bridging agent was also prepared. The reaction was either incubated at ambient temperature for 2 h or at 37 °C for 1 h. Aliquots were removed for SDS-PAGE analysis and the remaining sample subjected to purification as outlined above and MALDI-TOF MS.

4.9.5 *In situ* spin labelling of the anti-MSP1 antibody

The anti-MSP1 antibody was reacted with 100 equiv of TCEP or benzeneselenol in the presence or without 100 equiv of the *N*-TPO-dithiophenolmaleimide spin label for 1 h at ambient temperature. The excess of reagents was removed on two PD G-25 desalting columns and samples subjected to analysis by SDS-PAGE and EPR (see 4.8.8).

4.9.6 Influence of the reducing agent on the *in situ* spin labelling reaction

100 equiv of the *N*-TPO-dithiophenolmaleimide spin label were added to the anti-MSP1 antibody followed by either 10 equiv or 100 equiv of TCEP or benzeneselenol. Controls included the antibody mixed with DMF or with DMF and spin label and samples with the reducing agent only. The reaction was incubated at ambient temperature for 1 h and analysed by SDS-PAGE.

4.9.7 Influence of the amount of spin label on the *in situ* spin labelling reaction

The anti-MSP1 antibody was mixed with 0, 15, 30, 45, 60 or 75 equiv of *N*-TPO-dithiophenolmaleimide followed by addition of 15 equiv TCEP. All samples were incubated at ambient temperature for 1 h and analysed by SDS-PAGE.

4.9.8 Optimisation of the *in situ* spin labelling of the anti-MSP1 antibody

To the anti-MSP1 antibody were added 0, 5, 10, 15, 20, 25 or 30 equiv of the *N*-TPO-dithiophenolmaleimide spin label. 25 equiv of TCEP were added to all samples except for a control with the spin label only and incubated for 1 h at ambient temperature. The reaction was analysed by SDS-PAGE.

4.9.9 Stepwise *in situ* spin labelling of the anti-MSP1 antibody

25 equiv of the *N*-TPO-dithiophenolmaleimide were added to the anti-MSP1 antibody. 25 equiv of TCEP were added to the mix in 1, 2 or 3 equal portions in the same number of equal intervals over 1 h at ambient temperature.
Alternatively both the spin label and the reducing agent were added divided into 1, 2 or 3 portions over the period of 1 h. Controls included samples without the maleimide reagent. All reactions were analysed by SDS-PAGE.

4.9.10 Optimised *in situ* PEGylation of the anti-MSP1 antibody

The anti-MSP1 antibody was mixed with 0, 5, 10, 15, 20, 25 or 50 equiv of *N*-PEG5000-dithiophenolmaleimide followed by 25 equiv of TCEP. Controls included reducing or PEGylation reagent only. All samples were incubated for 1 h at ambient temperature and analysed by SDS-PAGE.

4.9.11 Sample preparation for ELISA

PEGylated anti-MSP1 antibody was prepared by addition of 15 equiv or 100 equiv of *N*-PEG5000-dithiophenolmaleimide to the protein solution followed

by 25 equiv or 100 equiv of TCEP respectively. Controls included samples without reducing or bridging reagent. The reactions were incubated at ambient temperature for 1 h, aliquots removed for analysis by SDS-PAGE and the remaining sample purified with protein A magnetic beads as described. The buffer of the eluate was exchanged to PBS by ultrafiltration (5 kDa MWCO), aliquots dialysed for analysis by MALDI-TOF and the concentration of the PEGylated antibody determined.

Spin labelled anti-MSP1 antibody was synthesised by mixing the protein with 25 equiv of the *N*-TPO-dithiophenolmaleimide spin label followed by addition of 25 equiv TCEP. The reaction was maintained at ambient temperature for 1 h and analysed by SDS-PAGE. Controls included samples without reducing or bridging reagent. The spin labelled antibody was purified twice on PD G-25 desalting columns (into PBS), concentrated, the molarity determined and subjected to EPR analysis (see 4.8.8).

4.9.12 ELISA with anti-MSP1 analogues

96-well plates were coated with recombinant MSP1-19 antigen at a final concentration of 6 µg/ ml in PBS for 2 h at ambient temperature. Plates were washed and blocked for 1 h at 37 °C with a 3% solution of Marvel milk powder in PBS. After washing the anti-MSP1 analogues were added in dilutions of 2000, 400, 80 and 16 ng/ ml in PBS and the assay was incubated at 37 °C for 1 h. The plate was washed and a HRP-coupled antibody (anti-human κ light chain goat IgG1, Quiagen, 1 : 5,000 in 3% Marvel solution) added. After 1 h at ambient temperature the ELISA plate was washed and KBlue substrate solution (Neogen) was added to each well. When a visible blue colour had developed (in the dark) the reaction was stopped with Red Stop solution (Neogen) and the plate read at a wavelength of 650 nm. Controls included freshly thawed and diluted anti-MSP1 antibody and an IgG1 negative isotype control (TAG 55). Each sample was tested in duplicates, and errors are shown as the standard deviation of the average.

4.10 Modification of rituximab

4.10.1 Reduction of rituximab

The antibody was treated with 5, 10, 20, 40, 60, 80 or 100 equiv of TCEP for 1 h at ambient temperature and the extent of reduction was analysed by SDS-PAGE. Intact and fully reduced samples were combined separately and visualised by MALDI-TOF MS.

4.10.2 Initial modification studies

To rituximab were added either 3, 5, 10 or 20 equiv of dithiophenolmaleimide followed by 10 equiv of TCEP or 5, 20, 40 or 80 equiv of dithiophenolmaleimide followed by 40 equiv TCEP. The mixtures were incubated for 1 h at ambient temperature and analysed by SDS-PAGE. Alternatively the same reactions were carried out with *N*-PEG5000-dithiophenolmaleimide in place of dithiophenolmaleimide and analysed by SDS-PAGE and MALDI-TOF MS after purification with protein A magnetic beads.

4.10.3 *In situ* PEGylation of rituximab

To the antibody was added *N*-PEG5000-dithiophenolmaleimide followed by benzeneselenol to yield mixtures of 80 : 40, 60 : 30, 40 : 20, 40 : 40, 20 : 40, 10 : 40 and 10 : 30 of maleimide : selenol. The reaction was maintained for 1 h at ambient temperature and analysed by SDS-PAGE.

The three best combinations (10 : 40, 40 : 20 and 60 : 30) were repeated, the antibody purified with protein A magnetic beads as described and the product analysed by MALDI-TOF MS.

4.10.4 Initial sequential modification of rituximab

Rituximab was mixed with 40 equiv of TCEP and incubated for 1 h at ambient temperature. Then 5, 10, 20, 30, 40, 60 or 80 equiv of dithiophenolmaleimide were added, reacted for 1 h and the samples analysed by SDS-PAGE alongside a reduction control.

4.10.5 DMF-free sequential PEGylation of rituximab

The antibody (in PBS only) was reduced with 40 equiv of TCEP for 1 h at ambient temperature followed by addition of 5, 10, 20, 30, 40, 60 or 80 equiv of *N*-

PEG5000-dithiophenolmaleimide. Samples were analysed after 1 h by SDS-PAGE.

4.10.6 Alternative reduction studies of rituximab

Rituximab (in PBS only) was reduced with the following reagents and reaction times at ambient temperature and all samples analysed by SDS-PAGE: 5, 10, 20 or 50 equiv DTT or 2-mercaptoethanol for 1 h; 5, 10, 20 or 50 equiv DTT for 4 h; 3 or 5 equiv TCEP for 1 h followed by 10, 20 or 50 equiv DTT for 3 h; 60 equiv of immobilised TCEP (Thermo Scientific) for 1 h or 120 equiv of immobilised TCEP for 2 h under constant agitation.

4.10.7 Optimisation of the sequential PEGylation of rituximab

Rituximab (in PBS only) was reduced via the following protocols: 10 equiv TCEP for 1 h, 20 equiv DTT for 4 h or 5 equiv TCEP for 1 h followed by 10 equiv DTT for 3 h. The samples were reacted with 5, 10, 15, 20, 25 or 30 equiv *N*-PEG5000-dithiophenolmaleimid when TCEP had been used as the only reducing agent or *N*-PEG5000-dibromomaleimide when any DTT was present. All reactions were analysed by SDS-PAGE.

4.10.8 Sequential PEGylation of rituximab

Rituximab (in PBS only) was PEGylated via the following sequential protocols at ambient temperature: 10 equiv TCEP for 1 h, then 20 equiv *N*-PEG5000-dithiophenolmaleimide; 20 equiv DTT for 4 h, then 25 equiv *N*-PEG5000-dibromomaleimide; 5 equiv TCEP for 1 h, then 10 equiv DTT for 3 h, then 20 equiv *N*-PEG5000-dibromomaleimide. All samples were analysed by SDS-PAGE, purified with protein A magnetic beads and subjected to MALDI-TOF MS.

4.10.9 Initial stepwise modification of rituximab

The antibody (in PBS only) was reduced with 80 equiv TCEP for 1 h at ambient temperature. The excess reducing agent was removed on a PD G-25 desalting column and aliquots immediately reacted for 1 h with 5, 8 or 10 equiv of *N*-PEG5000-dithiophenolmaleimide. All samples and antibody reduction were analysed by SDS-PAGE.

4.10.10 Re-oxidation study of rituximab

Rituximab (in PBS only) was reduced with 80 equiv TCEP for 1 h at ambient temperature, the excess phosphine removed and the buffer exchanged for 50 mM phosphate, 1 mM EDTA, pH 6.8 on a PD G-25 desalting column. An aliquot was used for the determination of the antibody concentration by NanoDrop. Argon was bubbled through the sample and the solution kept at ambient temperature in the dark. Aliquots were carefully withdrawn under argon atmosphere after 5 min, 20 min, 40 min, 1 h, 2 h, 4 h, 20 h, 30 h and 40 h, reacted with 40 equiv of maleimide (in DMF to a final concentration of 20%) for 30 min and stored at 4 °C. 3.65 µg of the antibody samples were loaded alongside 1, 2 and 4 µg of the unmodified antibody on a SDS-PAGE and the extent of the re-formation of the disulfide bonds estimated by densitometry after PAGE and staining. Therefore, the gel was digitalised on an UVP Imaging Systems device, loaded into the Lab Works 4.5 software and the lanes and regions captured and corrected by hand. The area density was exported and the signals quantified on basis of a standard curve calculated from the unmodified antibody samples. For the final analysis it was assumed that both hinge region cystines had re-formed in fragments were both heavy chains were present.

4.10.11 Stepwise modification of rituximab

Reduced rituximab was prepared as described in 4.10.10 by reduction with TCEP, transferred into the anti-oxidising buffer (50 mM phosphate, 1 mM EDTA, pH 6.8) and kept under argon. The sample was then incubated at ambient temperature in the dark for 24 h and aliquots reacted with 4, 8, 12 or 16 equiv of *N*-PEG5000-dithiophenolmaleimide (in PBS) or dithiophenolmaleimide (in DMF to a final concentration of 20%) for 1 h. The reaction was analysed by SDS-PAGE alongside a reduction control and selected samples of PEGylated antibody purified with protein A magnetic beads and analysed by MALDI-TOF MS.

4.10.12 Optimisation of the organic solvent-based stepwise modification of rituximab

Reduced rituximab was prepared as described in 4.10.10 by reduction with TCEP, transferred into the anti-oxidising buffer (50 mM phosphate, 1 mM

EDTA, pH 6.8) and kept under argon. Aliquots were reacted with dibromomaleimide or dithiophenolmaleimide at ambient temperature under a variety of conditions and analysed by SDS-PAGE: The maleimide reagents were prepared in pure DMF, DMSO, MeCN or THF. The final amount of organic solvent in the reactions was varied (6%, 10% or 20%) as well as the amount of bridging compounds (4, 8, 12, 16 or 20 equiv) and the reaction times (30 min, 1 h, 16 h or 24 h). Selected samples were analysed by MALDI-TOF MS.

4.10.13 Digest of rituximab with papain

Rituximab was digested using components of the Pierce Fab Preparation Kit (Thermo Scientific) but a thiol-free protocol was established: Immobilised papain was activated with 10 mM DTT (in digest buffer: 50 mM phosphate, 1 mM EDTA, pH 6.8) under argon atmosphere and constant agitation (1,100 rpm) for 1 h at 25 °C in the dark. The resin was washed 4x with digest buffer (without DTT) and 0.5 ml of the antibody solution, which had been transferred into digest buffer via ultrafiltration (5 kDa MWCO), was added. The mixture was incubated for 18 h at 37 °C while shaking (1,100 rpm) in the dark. The resin was separated from the digest using a filter column, washed 3x with PBS and the digest combined with the washes. The buffer was exchanged completely for PBS using ultrafiltration columns (5 kDa MWCO), the volume adjusted to 1.5 ml and the sample applied to a NAb protein A column (Thermo Scientific) and incubated at ambient temperature under constant agitation for 1 h. The Fab fraction was eluted according to manufacturers' protocol, the column washed 3x with PBS and the Fc fraction eluted 4x with 0.2 M glycine-HCl, pH 2.5, which was neutralised with 10% of the volume of a 1 M Tris, pH 8.5 solution. The Fab fraction was combined with the washes and both Fab and Fc solutions were buffer-exchanged into PBS using ultrafiltration columns (10 kDa MWCO).

All digests were analysed by SDS-PAGE and did not reach completion. The concentration of Fab fragment was determined by UV/Vis using a molecular extinction coefficient of ε_{280} = 82,905 M^{-1} cm^{-1}. A typical yield of the Fab fragment in a digest of the unmodified rituximab was 35%.

4.10.14 Preparation of the Fc fragment of rituximab

The Fab-depleted fractions of two or more digests of rituximab were combined and the Fc fragment isolated on a HiLoad 16/26 Superdex 75 size exclusion column (GE Healthcare) equilibrated in PBS. SDS-PAGE analysis suggested purity greater than 95% of the material. The concentration was determined by UV/Vis using a molecular extinction coefficient of ε_{280} = 71,570 M^{-1} cm^{-1}.

4.10.15 Deglycosylation of rituximab and its fragments

Full length rituximab, digest mixtures and the purified Fc fragment were deglycosylated with N-glycanase (ProZyme). Antibody containing solutions were diluted to a concentration of 1 mg/ ml and 100 µg incubated with 25 mU enzyme at 37 °C for 24 h under constant agitation (in PBS). Completeness of the enzymatic reaction was confirmed by SDS-PAGE where a clear mass-shift of the Fc fragment or the antibody heavy chain (after reduction with TCEP) was visible. Deglycosylation of the Fc fragment was further confirmed by LCMS.

4.10.16 Localisation of the PEG chains in rituximab

PEGylated rituximab was prepared either *in situ* (10 : 40 equiv of *N*-PEG5000-dithiophenolmaleimide : benzeneselenol, 1 h) or sequential with TCEP (10 equiv TCEP 1 h, then 20 equiv *N*-PEG500-dithiophenolmaleimide, 30 min) or DTT (20 equiv DTT 4 h, then 25 equiv *N*-PEG5000-dibromomaleimide, 30 min) and the products were purified on a NAb protein A column as described. The PEGylated antibodies were digested with immobilised papain, the Fab and Fc fragments separated on a Nab protein A column and analysed by MALDI-TOF MS alongside fragments of the unmodified antibody. Efficiency of all intermediate steps was confirmed by SDS-PAGE.

4.10.17 Localisation of the maleimide bridges in rituximab

Maleimide bridged rituximab was prepared either *in situ* (with 20 : 20, 10 : 40 or 40 : 20 ratios of dithiophenolmaleimide : benzeneselenol, 1 h) or sequential with TCEP (10 equiv TCEP 1 h, then 20 dithiophenolmaleimide, 30 min) or DTT (20 equiv DTT 4 h, then 25 equiv dibromomaleimide, 30 min) or stepwise (16 equiv of dithiophenolmaleimide in DMSO added for 30 min to the reduced and purified antibody). The excess of reagents were removed via PD G-25 de-

salting columns, 4 equiv of GSH added for 30 min and the samples digested with immobilised papain as described. The digest mixtures were subjected to deglycosylation and analysed by LCMS without further purification. The efficiency of all intermediate steps was confirmed by SDS-PAGE.

4.10.18 PEGylation of individual rituximab fragments

The purified Fab and Fc fragments of rituximab were subjected at 37.3 µM and 18.7 µM respectively to the optimised *in situ* and sequential PEGylation procedures as outlined under 4.10.16. Fragment PEGylation was visualised alongside reduction controls by SDS-PAGE.

4.10.19 Reduction study of the Fab fragment of rituximab

The purified Fab fragment of rituximab was diluted to 37.3 µM and treated with 1, 2, 3, 4 or 5 equiv of TCEP for 1, 2 or 4 h at ambient temperature. Then 10 equiv of maleimide (in DMF to a final concentration of 20%) were added for 30 min and samples were analysed by SDS-PAGE after thermal denaturation.

4.10.20 Reduction study of the Fc fragment of rituximab

The purified Fc fragment of rituximab was diluted to 18.7 µM and treated with 2, 4, 6, 8, 10 or 50 equiv TCEP for 1, 2 or 4 h at ambient temperature. Then 20 equiv of maleimide (in DMF to a final concentration of 20%) were added for 30 min and samples were analysed by SDS-PAGE after thermal denaturation.

4.10.21 PEGylation of a mix of the rituximab Fab and Fc fragments

The purified Fab and Fc fragments of rituximab were mixed in a 2 : 1 ratio to a final concentration of the “full antibody” of 18.7 µM. The mixture was PEGylated either *in situ* with 2, 5 or 10 equiv of *N*-PEG5000-dithiophenolmaleimide and 30 or 60 equiv benzeneselenol or via the TCEP-based sequential protocol with 2, 4, 6, 8, 10 or 15 equiv TCEP followed by 20 equiv of the PEGylation reagent after 1 h. All samples were analysed alongside reduction controls and single fragment reactions (see 4.10.18) by SDS-PAGE after thermal denaturation.

4.10.22 Functionalisation of rituximab

Maleimide bridged and spin labelled rituximab were prepared using an *in situ* method (30 : 60 equiv of benzeneselenol : dithiophenolmaleimide or *N*-TPO-

dithiophenolmaleimide, 1 h). Fluorescent rituximab was generated by a sequential protocol (20 equiv DTT 4 h, then 30 equiv *N*-fluorescein-dibromomaleimide in DMF to a final concentration of 20%, 30 min). All samples were purified with protein A magnetic beads and analysed by SDS-PAGE. The fluorescence of rituximab-fluorescein was recorded at a wavelength of 518 nm (excitation 488 nm) and a concentration of 50 ng/ ml. The average number of spin labels per antibody was calculated as outlined under 4.8.8 by Mr Vishal Sanchania (UCL ISMB).

4.10.23 Flow cytometry with modified rituximab

PEGylated rituximab was synthesised as outlined under 4.10.3 and 4.10.8 and functionalised antibody was synthesised as described under 4.10.22. Processed antibody was prepared by subjecting rituximab to the established *in situ* bridging conditions without addition of benzeneselenol. All antibody samples were purified with protein A magnetic beads, concentrated and the concentration determined with the NanoDrop (0.22 mg/ ml to 0.39 mg/ ml).

Cell culture was performed by Ms Carolina Fernandes (MRCT). Log phase cultures of Raji cells (a B cell line)[445] were grown (in RPMI 1640 +GlutarMAX, 25 mM HEPES, at 37 °C in humid atmosphere, 5% CO_2), harvested and transferred into buffer (PBS, 4% FCS, 0.02% sodium azide) by centrifugation and plated at 50,000 cells per well in 96 well plates. Cells were treated with 50 µl of 10, 5 or 1 µg/ ml primary antibody (the rituximab samples) in buffer for 1 h at 4 C°. As controls Raji cells were also treated with unmodified/ unprocessed rituximab (positive control), an isotype control (mouse chimeric IgG1 κ, 1 µg/ ml, negative control), the secondary antibody only (goat FITC-conjugated anti-human IgG $F(ab)_2$, Jackson ImmunoResearch, negative control, 50 µl buffer during primary antibody incubation), and buffer only (in both steps, live gate control). The plate was washed and the secondary antibody was added (1 µl solution in 50 µl buffer per well). Fluorescently labelled rituximab was added in this step to cells which had previously been treated with buffer only. The samples were incubated for 1 h at 4 °C in the dark, washed and fixed in 2% formaldehyde (in PBS) for 10 min at ambient temperature. The cells were washed again, resuspended in 200 µl buffer and the plate loaded into the flow cytometer (Guava easyCyte 8HT, Millipore).

Data was acquired (5,000 events, excitation wavelength 495 nm, emission wavelength 521 nm) and analysed using the installed software (guaraSoft, In-Cyte 2.2.2). Settings were adjusted using the unstained cells, positive and negative controls and samples, which had been prepared in duplicates, read accordingly. Fluorescent staining was analysed after gating for live cells (forward scatter vs. side scatter). Small shifts in the fluorescent cell population over the antibody dilutions confirmed that saturation had not been reached in the two lower concentrations. The experiment was performed with Ms Carolina Fernandes (MRCT).

4.10.24 Preparation of rituximab analogues with various cystine modifications

Three different rituximab analogues were synthesised in preparation of a stability test (see below): Maleimide bridged rituximab was prepared by reduction of the antibody with 20 equiv DTT for 4 h at ambient temperature and addition of 25 equiv dibromomaleimide (in DMF to a final concentration of 20%) for 30 min. In analogy bridged and hydrolysed antibody was synthesised by addition of *N*-phenyl-dibromomaleimide instead of dibromomaleimide and incubation of the material at 37 °C for 16 h. Partial alkylated rituximab was prepared as described in the literature (method B).[229] In brief the antibody was transferred into a 25 mM NaCl, 25 mM sodium borate, 1 mM EDTA, pH 8.0 buffer, treated with 2.75 equiv TCEP for 2 h at 37 °C, cooled to 4 °C and reacted with 4.4 equiv of maleimide for 30 min.

All rituximab analogues were purified after the reaction on PD G-25 desalting columns (into PBS) and the concentration was determined by NanoDrop.

4.10.25 Thermal stability of modified rituximab

The thermal stability of all rituximab analogues prepared for the activity test (see 4.10.23), with the exception of the fluorescent antibody, was analysed alongside the specially synthesised samples (see 4.10.25) in a thermal shift assay. Unmodified and processed rituximab served as controls. The concentration of the antibody analogues was adjusted to 600 nM or 150 nM and mixed with a prediluted (1 : 100 in PBS) hydrophobic fluorescent dye (Sypro Orange, Sigma-Aldrich) in a 1 : 10 ratio of dye : antibody solution. 40 µl were transferred into a 96-well plate, which was briefly centrifuged (1,000 rpm) and sealed. The ther-

mal shift assay was performed in a Mx 3005P qPCR machine (Stratagene) by heating the samples from 25 °C to 95 °C at a speed of 1 °C per min. The increase in fluorescence was recorded (excitation wavelength 472 nm, emission wavelength 570 nm) with the installed MxPro Software, the data exported and fitted to a sigmoid curve shape from which a simple melting temperature T_m was calculated. The experiment was performed with Ms Carolina Fernandes (MRCT) and the help of Dr Alexander Brown (MRCT).

4.10.26 SEC-MALS with modified rituximab

Maleimide bridged rituximab was synthesised with the optimised protocols: *in situ* with 10 : 40 equiv of dithiophenolmaleimide : benzeneselenol for 1 h, sequential with 20 equiv DTT for 4 h, then 30 equiv dibromomaleimide for 30 min or stepwise by adding 16 equiv of dithiophenolmaleimide in DMSO (final 10%) for 30 min to the reduced antibody. A processed sample was prepared by mixing the antibody with 20 equiv of dithiophenolmaleimide in 20% DMF for 1 h. All samples were purified on PD G-25 desalting columns into Gibco 1x PBS (Life Technology, Ca^{2+} and Mg^{2+} free), the concentration determined with the NanoDrop and analysed by SDS-PAGE and MALDI-TOF MS.

The used SEC-MALS system consisted of a WTC-05055 size exclusion column in line with a Dawn HELEOS-II 18-angle MALS light scattering detector and an Optilab T-rEX refractive index detector (both Wyatt Technology). 150 µg of sample were injected into the SEC column and the UV absorption at 280 nm, the differential refractive index and the scattered light acquired for 40 min at a flow rate of 0.5 ml/ min at 25 °C. Data analysis was performed after determination of the baseline based on the UV absorption with the installed software (Astra 5.3.4.18). The experiment was performed with Dr Lone Friis (MRCT).

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