Disulfide Bridging Poly(ethylene glycol) Reagents For Site-specific Protein Conjugation

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Dedicated to Ramya
Plagiarism Statement

This thesis describes the research conducted in the School of Pharmacy, University of London between 22.10.2003 and 22.10.2006 under the supervision of Prof. Steve Brocchini. I certify that the research described is original and that any parts of the work that have been conducted in collaboration are clearly indicated. I also certify that the text printed in this thesis is prepared by me and have clearly indicated any part of this thesis that has already appeared in publications.

Sibu Balan

(Signature)  
01 May 2007  
(Date)
ABSTRACT

Disulfide bonds are ubiquitous in proteins and are broadly involved with protein structure, function and stability. Most therapeutic proteins which act extracellularly have disulfides. Frequently at least one disulfide is accessible to protein disulfide reductants. Disulfide bridging-poly (ethylene glycol) (DB-PEG) reacts with the two sulfurs derived by reduction of such an accessible disulfide and inserts a 3-carbon bridge between the thiols thus attaching the PEG to the protein.

This thesis investigates the site-specificity and application of the bis-functional DB-PEG for the monoPEGylation of therapeutic proteins with accessible disulfides. The hypotheses of the project were i) some protein disulfides can be modified with DB-PEG with the protein retaining its structural features and biological activity, ii) upon reaction, the 3-carbon bridge forms between the cysteine thiols, iii) DB-PEGylation can be efficient with high yields of the PEGylated protein and iv) since many therapeutic proteins possess accessible disulfides that mostly maintain protein stability, this conjugation strategy will have a broad applicability.

DB-PEG was prepared by a simple synthetic route and their reactivity and bis-thiol specificity was studied using reduced glutathione. The characteristics of this PEGylation approach are i) a requirement to reduce the protein disulfides and ii) reaction efficiency depended on the disulfide bond position within the protein. Molecular modelling was done to study the 3-carbon disulfide bridged protein structure. The use of the DB-PEG to conjugate PEG to proteins was studied with L-asparaginase, interferon α-2b, leptin and somatostatin. Evaluation of the biological activities of the conjugates indicated that the observed activities were predominantly due to the steric shielding of PEG. The DB-PEG was found to be stoichiometrically efficient. Disulfide reduction without loss of protein structure was possible and the DB-PEG was specific to the thiols generated from a reduced disulfide bond.
RESEARCH PUBLICATIONS

Journal Publications


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1. Balan S., Zloh M., Shaunak S. and Brocchini S. Disulfide PEGylated leptin. Accepted as an oral presentation for the Pre-satellite meeting of the Pharmaceutical Sciences World Conference at Amsterdam (April 2007).
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<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CYS</td>
<td>cysteine</td>
</tr>
<tr>
<td>DB</td>
<td>disulfide bridging</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N' dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DCU</td>
<td>dicyclohexyl urea</td>
</tr>
<tr>
<td>DFT</td>
<td>density functional theory</td>
</tr>
<tr>
<td>DMAP</td>
<td>N,N' dimethylamino pyridine</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>HOMO</td>
<td>highest occupied molecular orbital</td>
</tr>
<tr>
<td>GB/SA</td>
<td>generalised Born/surface area</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage- colony stimulating factor</td>
</tr>
<tr>
<td>GRS</td>
<td>glutathione refolding solution</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>oxidised glutathione</td>
</tr>
<tr>
<td>EMCV</td>
<td>Encephalomyocarditis virus</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFN α-2a</td>
<td>interferon α-2a</td>
</tr>
<tr>
<td>IFN α-2b</td>
<td>interferon α-2b</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>LUMO</td>
<td>lowest unoccupied molecular orbital</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix assisted laser desorption ionisation</td>
</tr>
<tr>
<td>mPEG</td>
<td>methoxy poly (ethylene) glycol</td>
</tr>
<tr>
<td>MEC</td>
<td>molar extinction coefficient</td>
</tr>
<tr>
<td>MMMC</td>
<td>Monte Carlo multiple minimum</td>
</tr>
</tbody>
</table>
MS  mass spectrometry
MW  molecular weight
MWCO molecular weight cut off
NOESY nuclear overhauseur effect spectroscopy
NHS N-hydroxysuccinimide
NMR nuclear magnetic resonance
PAGE polyacrylamide gel electrophoresis
PEG poly (ethylene glycol)
PEG-ALD PEG propionaldehyde
PEG-MAL PEG maleimide
PEG-NHS PEG N-hydroxysuccinimide derivative
PEG-SBA succinimidyl butanoate
PEG-SC PEG succinimidylcarbonate
PEG-SCM succinimidyl carboxymethylated PEG
PEG-SPA PEG succinimidyl propionate
PEG-SS PEG succinimidyl succinate
PEG-VS PEG vinylsulfone
pI isoelectric point
PNBC paranitrobenzyl chloroformate
rcf relative centrifugal force
RMSD root mean square deviation
RNAs e ribonuclease
RP-HPLC reversed phase HPLC
rpm revolutions per minute
SDS sodium dodecyl sulphate
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC-HPLC size exclusion HPLC
SEM standard error of mean
STAT transcription factors
TAK tyrosine kinase
TCEP tris(2-carboxyethyl)phosphine
TFA trifluoroacetic acid
TOCSY total correlation spectroscopy
TOF time of flight
TSP trimethylsilyl-(2H4)-propionate
UV ultra violet
Chapter 1

Introduction to ‘disulfide bridging’ PEGylation
1.1 PROTEIN PEGYLATION

The covalent conjugation of poly (ethylene glycol) (PEG) to proteins is widely known as ‘PEGylation’. It is a clinically proven method to improve the biopharmaceutical efficacy of protein based medicines (Harris and Chess, 2003). Parenterally administered therapeutic proteins are rapidly cleared from the systemic circulation by enzymatic digestion and renal elimination (Poole et al. 1990). Therefore, frequent administration of parenteral doses is required to maintain the protein’s therapeutic effective concentration in the systemic circulation. This often puts the patient at the risk of toxicities due to immunogenicity. PEGylation has been clinically proven to address some of the limitations of therapeutic proteins such as their short \textit{in vivo} half-life and immunogenicity (Caliceti and Veronese, 2003).

Protein PEGylation was first conceived by Frank. F. Davis in the late 1970s while at the Rutgers University, USA (Davis, 2002). His studies showed that PEGylation reduced the antigenic properties of proteins like bovine serum albumin (BSA) and catalase, thereby prolonging their \textit{in vivo} half-lives (Abuchowski et al. 1977a; Abuchowski et al. 1977b). \textit{E.coli} L-asparaginase, an antileukaemic protein which causes hypersensitivity reactions when administered parenterally to humans (Schein et al. 1969; Killander et al. 1976), was observed to exhibit low immunogenicity after PEGylation (Veronese et al. 1996; Graham, 2003). PEGylated proteins like PEG-L-asparaginase (Oncaspar® for acute lymphoblastic leukaemia) and PEG-adenosine deaminase (Adagen® for combined immuno-deficiency disease) were the first products developed for safe clinical use.

More recently, recombinant human proteins such as interferon (IFN) and granulocyte-colony stimulating factor (G-CSF) have been PEGylated to improve their pharmacokinetics. These proteins have a short \textit{in vivo} half-life due to their relatively small molecular size (~ 20 kDa). Despite being ‘humanised’ these proteins can elicit immune response on repeated dosage (Porter, 2001), so PEGylation is important to limit the frequency of dosing and the total number of doses administered. PEGylation improves the pharmacokinetics of proteins by 1) increasing the molecular size, 2) providing protection from proteolytic enzymes and 3) preventing recognition by immune system (Harris and Chess, 2003). PEGylated recombinant proteins available for clinical use are PEG-IFN α2b (PEG-Intron®, Schering-Plough Corp.) and PEG-IFN α-2a (PEGASYS®, Roche Inc.) for hepatitis C and PEG-G-CSF (Neulasta®, Amgen Inc.) for neutropenia.
1.2 PEGYLATION CHEMISTRY

PEG is a nontoxic and non-immunogenic compound widely used in the cosmetic and pharmaceutical industries as a formulation additive. The monomethoxy terminated PEG (mPEG) with molecular weights ranging between 2 to 40 kDa is frequently used as a precursor that can be chemically functionalised for protein conjugation. Typically, the coupling of mPEG to the protein requires prior activation of its hydroxy group terminal with a functional moiety. Derivatisation can be easily accomplished with conventional reagents such as cyanuric chloride or N-hydroxy succinimide (Zalipsky, 1995). Purification of the prepared PEG reagent can be easily accomplished by precipitation of the PEG in solvents such as acetone or diethyl ether. Most conjugations utilise the protein as a nucleophile. Hence PEG reagents tend to be electrophilic.

The main advantages that PEG enjoys over other synthetic polymers are its 1) aqueous and organic solubility, 2) non-toxicity, 3) non-immunogenicity, 4) uncharged nature and 5) ease of synthesis (Nucci et al. 1991; Delgado et al. 1992; Harris and Zalipsky, 1997). Commercially, mPEG is prepared by anionic ring opening polymerisation which is initiated with methoxide ions (Roberts et al. 2002). Trace contamination with water molecules during the polymerisation process can cause the formation of diol PEG (HO – PEG – OH) which is a common occurrence. Typically, mPEG has a narrow molecular weight distribution (Mw/Mn) between 1.01 (< 5 kDa) and 1.10 (> 50 kDa) depending on the molecular weight (Roberts et al. 2002). PEG binds 2 to 3 molecules of water per one unit of ethylene oxide monomer. This property along with its flexibility in aqueous solution is the reason for its large apparent hydrodynamic volume compared to a protein of the same molecular mass (Roberts et al. 2002).

1.2.1 PEGylation reagents

The methoxy group terminal of the mPEG is not reactive. Efficient derivatisation of the hydroxy group terminal with an electrophilic group can be achieved with various reagents (Zalipsky, 1995). Cyanuric chloride was the activating reagent used by Davis et al for their initial PEGylation studies (Abuchowski et al. 1977a & b). Since then, PEGylation has advanced and many reviews discuss the chemistry and limitations of protein PEGylation (Zalipsky, 1995; Kodera et al. 1998; Veronese, 2001; Roberts et al. 2002; Veronese and Pasut, 2005).
1.2.1.1 Conventional amine reactive PEGs

Based on the type of reaction, the PEGylation reagents available for coupling to amine residues can be classified as 1. aroylating reagents, 2. acylating reagents and 3. alkylating reagents (Zalipsky, 1995). The first generation amine PEGylation reagents were mostly acylating reagents with the exceptions of PEG dichlorotriazine and PEG tresylate being aroylating and alkylating reagents respectively (Roberts et al. 2002). The acylating PEG reagents are electrophilic and undergo reaction with the nucleophiles present in the protein. The reaction of acylating reagents with amine groups result in either a carbamate (urethane) or an amide bond. The reactive amine sites on a protein are the amine groups present in lysine, arginine, histidine and the N-terminal amino acid (Table 1.1). The most common sites on the protein for PEGylation include alpha/epsilon amino groups of lysine, the N-terminal amine group, histidine and the thiol group of cysteine. Even though the ideal pH for the amine group reactivity is above 8.5, conjugation can occur at near neutral pH (Wong, 1991; Hermanson, 1996). Many of these reagents also undergo competitive hydrolysis reactions, so their conjugation efficiency can be low.

<table>
<thead>
<tr>
<th>Amine functionality</th>
<th>pKa range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine’s ε-amine</td>
<td>9.3-9.5</td>
</tr>
<tr>
<td>Arginine’s guanidinyl group</td>
<td>&gt;12</td>
</tr>
<tr>
<td>Histidine’s imidazolyl nitrogen</td>
<td>6.7-7.1</td>
</tr>
<tr>
<td>α-amine of N-terminus</td>
<td>7.6-8</td>
</tr>
</tbody>
</table>

*Table 1.1. pKa ranges of the reactive amine groups in a protein molecule*

The most common acylating PEG reagents used for PEGylation are the N-hydroxysuccinimide (NHS) activated PEG derivatives. The NHS derivatives are categorised into first generation derivatives which include PEG succinimidyl carbonate (PEG-SC) 2 and PEG succinimidyl succinate (PEG-SS) and the second generation PEG-NHS derivatives prepared from carboxymethylated PEGs such as succinimidyl carboxymethylated PEG (PEG-SCM), succinimidyl propionate (PEG-SPA), succinimidyl butanoate (PEG-SBA) and ‘BRANCHED’ PEG-NHS 3 (Roberts et al. 2002). The first generation derivatives have the disadvantages of PEG diol contaminations and also of linkage instabilities (Roberts et al. 2002). PEG-SC 2 forms a weak degradable linkage with histidine residues whereas the PEG-SS has a degradable ester linkage within the PEG structure (Roberts et al. 2002). The second generation derivatives of PEG-NHS (PEG-SCM, PEG-SPA, PEG-SBA and ‘BRANCHED’ PEG-NHS 3) form stable amide linkages with the amine nucleophiles (Roberts et al. 2002).
PEGylation with the acylating amine reactive PEG yields randomly and non-specifically conjugated protein molecules or "PEGmers" (Roberts et al. 2002). These non-specific reagents are added in excess stoichiometry and react kinetically at all the exposed nucleophilic groups in the protein. While some proteins like BSA and catalase require non-specific multiple PEGylation to mask antigenic sites and thereby reduce the protein's innate antigenic properties (Abuchowski et al. 1977a & b; Ho et al. 1986), others like rhIFN and rhG-CSF require monoPEGylation to improve pharmacokinetic properties (Harris et al. 2001; Caliceti, 2004).

Although non-specific, NHS derivatives of PEG have been used successfully for the preparation of monoPEGylated growth hormone-releasing hormone analogues (Esposito et al. 2003), monoPEGylated IFN α-2a (Bailon et al. 2001; Foser et al. 2003; Dhalluin et al. 2005; Jo et al. 2006), monoPEGylated IFN α-2b (Wang et al. 2002; Youngster et al. 2002; Ramon et al. 2005), TNF-α (Yoshioka et al. 2004) and glucagon (Stigsnaes et al. 2006). MonoPEGylation of proteins is usually achieved by optimising the reaction conditions and reagent stoichiometry to minimise multiple PEG conjugation. This often results in significant protein not being PEGylated. The NHS derivative of PEG, PEG-NHS (succinimidyl carbonate) and the second generation NHS derivative of PEG, 'BRANCHED PEG-NHS' (Figure 1.2) have been used in the preparation of PEGylated α-IFNs (Kozlowski and Harris, 2001). PEGylated IFN α-2b prepared with PEG-NHS is marketed as PEG-Intron® (linear 12 kDa PEG) by Schering-Plough® and PEGylated IFN α-2a prepared with the 'BRANCHED' PEG-NHS as PEGASYS® ('branched' 40 kDa PEG) by Roche®.

![Figure 1.2. Chemical structures of PEG-NHS derivatives, PEG-SC and 'BRANCHED' PEG-NHS. These derivatives are used in the preparation of PEG-Intron® (Linear 12 kDa PEG-IFN α-2b) and PEGASYS® ('BRANCHED' 40 kDa PEG-IFN α-2a).](image)

Most reported studies conducted on the PEGylation of IFNs with NHS derivatives of PEG use 3-4 molar excess of the reagent to protein; reactions are typically carried out for 2 h at 4 °C in 50 mM sodium phosphate buffer, pH 6.5 (PEGIntron®) or 50 mM sodium borate buffer, pH 9.0 (PEGASYS®) (Bailon et al. 2001; Foser et al. 2003; Grace et al. 2005a; Ramon et al. 2005).
Chapter 1. Introduction to 'disulfide bridging' PEGylation

The NHS derivatives of PEG undergo rapid hydrolysis at pH 8.0 and 25 °C (Roberts et al. 2002). It is reported that PEGylation with NHS derivative of mPEG typically yields 45 – 50% of the monoPEGylated protein with 5 – 10% of diPEGylated protein and 40 – 50% unmodified protein (Bailon et al. 2001).

Due to steric shielding of the attached mPEG, reduced biological activity is typically observed with a PEGylated protein (Grace et al. 2005a). However, the monoPEGylated product obtained after PEGylation with the NHS derivatives of PEG is usually a mixture of positional isomers with varied activity (Table 1.2) (Monkarsh et al. 1997; Bailon et al. 2001; Foser et al. 2003; Grace et al. 2005a). The relative amount of each PEG-protein isomer depends upon the PEG's molecular weight, morphology, and the specific reaction conditions used for conjugation (Bailon et al. 2001; Wylie et al. 2001; Grace et al. 2005a). Isolation of each positional isomer tends to be a tedious process. The activity profile of each positional isomer of monoPEGylated α-IFNs PEGylated with PEG-NHS 2 and 3 is shown in Table 1.2. The observed reduction in the protein's biological activity among positional isomers could be due to the PEG interfering with the protein's receptor binding site (Grace et al. 2005a). The advantages of this PEGylation process are 1) mild reaction conditions and 2) short reaction period. The limitations are 1) requirement of high stoichiometry of reagent indicating reaction inefficiency, 2) formation of many protein-PEG positional isomers with varied activity indicating non-specificity and 3) PEG-SC 2 forms degradable linkage with histidine (Roberts et al. 2002).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PEGylated IFN α-2b (Grace et al. 1999)</th>
<th>PEGylated IFN α-2a (Bailon et al. 1999)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN type</td>
<td>IFN α-2b</td>
<td>IFN α-2a</td>
</tr>
<tr>
<td>PEG molecule</td>
<td>Single 12 kDa polymer</td>
<td>Branched 40 kDa polymer</td>
</tr>
<tr>
<td>Positional isomers</td>
<td>His^24 (&gt;50 %); Cys^1 (~13 %); Lys^13 (~7 %); Lys^3 (~5 %); remaining 20 % consists of Lys^41, Lys^21, Lys^16, Lys^11, Lys^134, His, Tyr^19, Ser^16</td>
<td>Lys^31; Lys^121; Lys^131; Lys^134; minor constituents not reported.</td>
</tr>
<tr>
<td>Antiviral activity</td>
<td>28 % for mixture; 37 % for His^34 isomer</td>
<td>7 % for mixture</td>
</tr>
</tbody>
</table>

Table 1.2. Positional isomers of monoPEGylated IFN α (adapted and modified from Luxon et al. 2002).

1.2.1.2 Site-specific N-terminal amine reactive PEG reagents

The non-specific amine PEGylation yields monoPEGylated protein positional isomers of varied activity (Luxon et al. 2002). Therefore, a site-specifically PEGylated product of homogenous nature and defined biological activity would be more optimal. Reductive alkylation of protein amino groups is a well known method of protein modification (Means, 1984). J.M Harris has
described the preparation and use of PEG propionaldehyde for site-specific PEGylation via reductive alkylation of protein N-terminal amino acids (Harris and Herati, 1993).

Kinstler et al have used mPEG propionaldehyde \( \mathbf{4} \) (PEG-ALD) to prepare predominantly N-terminally PEGylated G-CSF, GM-CSF (Kinstler et al. 1996; Kinstler et al. 2002). They reported that the PEG propionaldehyde derivative \( \mathbf{4} \) is specific for the N-terminal \( \alpha \)-amine (pKa - 7.8) at pH 5.0 due to its lower pKa compared to other nucleophilic reactive groups. The conjugation reaction occurs by reductive alkylation via formation of a Schiff's base \( \mathbf{5} \) which requires reduction in situ with sodium cyanoborohydride to yield a stable secondary amine linkage \( \mathbf{6} \) (Figure 1.3). Typically, the protein PEGylation reaction is conducted in sodium phosphate buffer, pH 5.0 in the presence of sodium cyanoborohydride as the reducing agent and with what is claimed a 5-fold molar excess of the PEG propionaldehyde \( \mathbf{4} \). Numerous studies with PEG propionaldehyde \( \mathbf{4} \) have been conducted on proteins like megakaryocyte growth and development factor (Guerra et al. 1998), IFN \( \beta \)-1a (Pepinsky et al. 2001; Baker et al. 2006); rhEGF (Lee et al. 2003) and octreotide (Na et al. 2005) which demonstrate that there is site-specific PEGylation. A product available for clinical use based on this technology is Neulasta\textsuperscript{\textregistered} (PEG-G-CSF, Amgen Inc.).

The claimed advantages of this process are 1) high yield of monoPEGylated protein (up to 92 %) and 2) N-terminal amine specificity (Kinstler et al. 2002). The disadvantages are 1) reaction has to be carried out in the presence of the toxic sodium cyanoborohydride, 2) excess PEG reagent stoichiometry indicating reaction inefficiency and 3) specific optimisation of reaction conditions like pH and temperature. Also, formation of up to three protein-PEG positional isomers has been reported with recombinant human epidermal growth factor (EGF) (Lee and Park, 2002).

![Figure 1.3](image_url). Reductive amination reaction of PEG-ALD \( \mathbf{4} \) with amine groups; reduction of the Schiff's base is usually achieved with sodium cyanoborohydride in the reaction buffer.
Chapter 1. Introduction to 'disulfide bridging' PEGylation

1.2.1.3 Site-specific thiol reactive PEGs

The thiol group of cysteine is a reactive nucleophile compared to most amines at neutral pH. The cysteine's thiol has a pKa range of 8.8-9.1. There can be a significant concentration of reactive thiolate at neutral pH values. Cysteine residues provide a site-specific target for conjugation reactions. Several thiol specific PEG derivatives have been synthesised of which the Michael acceptors, PEG-maleimide \( \text{PEG-MAL} \) and PEG-vinyl sulfone \( \text{PEG-VS} \) are generally used for cysteine conjugation (Figure 1.4). A 'forked' PEG-(MAL)\(_2\) has also been described for conjugation to the thiols of two Fab' fragments, the PEG thus acting as the heavy chain fragment of an antibody molecule (Harris and Kozlowski, 1999). PEG-MAL can undergo reaction with thiols even at lower pH values of 6-7, whereas PEG-VS requires a more basic pH of 7-8 for the reaction to occur (Roberts et al. 2002). One disadvantage observed is that both reagents can react with amino groups when the pH is raised higher than 8.5 (Veronese, 2001). Also, PEG-MAL reagent can undergo hydrolysis in aqueous buffers.

Site-specific attachment of PEG with these thiol specific reagents to the naturally occurring cysteine was demonstrated with G-CSF (Park, 2005) and α1 Protease Inhibitor (Cantin et al. 2002) with the conjugates being biologically active. Although site-specific and useful, no thiol specific PEGylated product has so far been developed for clinical use. The fundamental limitation of exploiting single cysteine thiol PEGylation is that only few proteins have naturally occurring unpaired cysteines.

![Figure 1.4. Chemical structures of the thiol reactive PEG reagents; PEG-MAL and PEG-VS.](image)

1.2.2 Other strategies for site-specific PEGylation

Several other strategies have been examined for the site-specific PEGylation of proteins (Veronese, 2001). They include reaction to specific residues like arginine with diketone derivatives of PEG (PEG phenylglioxal 9) (Figure 1.5A), carboxylic acid with PEG hydrazide 10 (Figure 1.5B) and hydroxyl groups with PEG isocyanate 11 (Figure 1.5C). Many of these PEG derivatives can be prepared directly from monomethoxy poly (ethylene glycol) \( \text{Zalipsky S.}, 1995 \). Although designed for site-specificity, only PEG hydrazide 10 displays site-specific
conjugation, whereas the PEG diketone 9 and PEG isocyanate 11 can react to any nucleophilic site. PEG hydrazide 10 can undergo reaction with carboxylic groups in the presence of N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide HCl (EDC.HCl) at a pH range of 4.5 – 5.0 where all the amines are protonated and unreactive (Veronese, 2001). Although site-specificity can be achieved, the presence of one or more carboxylic acids present in the protein would yield multiple sites of PEG attachment.

![Figure 1.5. PEG derivatives designed for site selectivity; a) PEG phenylglyoxal, b) PEG hydrazide and c) PEG isocyanate (adapted from Veronese et al., 2001)](image)

Yamamoto et al. have shown a unique method of protein PEGylation with the help of genetic engineering (Yamamoto et al., 2003). They prepared a TNF-α mutant in which all the lysines are replaced with non-reactive amino acids and further successfully PEGylated the N-terminal amino acid residue with a second generation NHS derivative of PEG, succinimidyl propionate (PEG-SPA). The conjugate that was obtained was observed to retain up to 80% of the protein’s in vitro biological activity. Insertion of a cysteine residue by recombinant engineering is also reported to be useful for site-specific conjugation with thiol-specific PEG-MAL 7 reagent as observed with the clinically relevant protein models, anti-Tac (Fv)-PE38 (Tsutsumi et al., 2000), trichosanthin (Wang et al., 2004), granulocyte-macrophage colony-stimulating factor (Doherty et al., 2005) and IFN α-2 (Rosendahl et al., 2005). Recently, Wu et al. have reported another interesting study utilizing both genetic engineering and thiol chemistry to prepare site-specifically conjugated human basic fibroblast growth factor. They replaced three of the four unpaired cysteine residues with serine residues and further PEGylated the free cysteine with PEG-MAL 7 to obtain a biologically active conjugate (Wu et al., 2006). There have also been other successful attempts to site-specifically PEGylate genetically engineered proteins with the help of specific enzymes (DeFrees et al., 2006; Sato, 2002). Insertion of unnatural amino acids into proteins to facilitate site-specific PEGylation has also been reported (Deiters et al., 2004). Although site-specificity was achieved in all these reported methods, the production and characterisation of the mutant proteins were technically difficult tasks. Also, inclusion of the cysteine thiol could possibly lead to problems like aggregation, disulfide mismatching and protein misfolding (Arakawa et al., 1993).
Protein-polymer conjugates have also been prepared by in situ polymerisation using the proteins itself as macroinitiators (Heredia et al, 2005, Nicolas et al, 2006). Heredia et al have prepared mono-BSA-poly (N-isopropylacrylamide) conjugate and mono-Lysozyme-poly (N-isopropyl acrylamide) conjugates in greater than 65 % yield. The bioconjugate thus prepared retained its bioactivity. Nicolas et al have also used the same method of in situ living radical polymerisation to prepare BSA and lysozyme bioconjugates with poly (ethylene glycol) methyl ether methacrylate (PEGMA) and dimethylaminoethyl methacrylate (DMAEMA). The advantage of this technique is that the conjugates prepared can be easily purified and characterised. The disadvantage is that it needs a specific amino acid in the protein such as a free cysteine to convert the protein to a macroinitiator.

1.3 PROJECT HYPOTHESIS – DISULFIDE THIOLS FOR PEGYLATION

Cysteine is the most conserved amino acid in proteins after tryptophan (Thornton, 1981). The thiol group present in cysteine is a reactive nucleophile and could be used for efficient site-specific conjugation. However, the presence of an unpaired natural and non-functional cysteine is rare in extra-cellular therapeutic proteins. An exception is G-CSF (Ishikawa et al. 1992). It is therefore usually not possible to exploit the selective conjugation chemistry of the cysteine thiols. However, extracellular proteins which act via cell surface receptors have an even number of cysteines that pair up as disulfide bonds (Thornton, 1981). A disulfide bond on reduction liberates two thiols which could be useful for conjugation purposes. However, the covalent disulfide ‘bridge’ is important for the protein to maintain its tertiary structure and biological activity (Thornton, 1981). The premise of this project is that it might be possible to exploit both the thiols derived by reduction of a native disulfide bond by ‘rebridging’ them back via a cross-linking thiol reactive PEG compound (Figure 1.6).

![3-carbon bridge between cysteine sulfurs](image)

Figure 1.6. Schematic representation of the modified protein disulfide 12. The hypothesis is that the newly formed bridge between the sulfur atoms of the disulfide would help the protein maintain its tertiary structure without disrupting its biological activity.
Chapter 1. Introduction to ‘disulfide bridging’ PEGylation

1.3.1 Which disulfide bond?

The formation of a disulfide bond (-S-S-) is a structurally important post-translational modification that occurs in a protein. Disulfides which are accessible typically contribute to the conformational stability of the protein rather than to its biological function (Thornton, 1981). In contrast, the disulfides that are located in a protein’s hydrophobic interior are important for the protein’s structural compactness and biological function (Resnick et al. 1959; Thornton, 1981; Klink et al. 2000). Although modification of a disulfide/s can result in loss of biological activity in some proteins (Zhou et al. 1998; Rajaratnam et al. 1999; Wedemeyer et al. 2000), modification of some accessible disulfides does not lead to a loss of protein structure or biological activity (Bewley et al. 1969; Todokoro et al. 1975; Morehead et al. 1984). This is because a protein’s conformation also depends on the intramolecular hydrogen bonding and hydrophobic interactions (Pace, 1992). Modification of an accessible and biologically non-functional disulfide that results in a bridge between the two sulfurs (Figure 1.6) to maintain the protein structure is hypothesised in this thesis as a way for site-specific PEGylation.

Reduction of a disulfide bond 17 without disrupting the tertiary structure of a protein is a fundamental pre-requisite for the proposed conjugation approach. Several thiol reducing reagents such as dithiothreitol (DTT 16), 2-mercaptoethanol (2-ME), cysteine are available for the reduction of protein disulfide bond. Other phosphine reducing agents such as TCEP HCl are also used for protein disulfide reduction. However, DTT 16 which is also known as Cleland’s reagent, is the most widely used disulfide bond reducing agent (Figure 1.7) (Cleland, 1964; Hermanson, 1996). DTT 16 at a concentration range of 10-100 mM (Hermanson, 1996) is found to be an efficient reducing agent for proteins with accessible disulfide bonds, for example, growth hormone (Bewley et al. 1969), G-CSF (Leon et al. 1999). Reduction of the inaccessible internally located disulfides in proteins, for example bovine pancreatic ribonuclease A (RNAse A) with DTT 16 typically requires denaturants such as guanidine, urea or sodium dodecyl sulfate (SDS) (Zhou et al. 1998). The use of a denaturant significantly disrupts the non-covalent interactions within the protein resulting in the loss of the tertiary structural conformation (Smith and Scholtz, 1996). In the cases of DTT 16 reduced and iodoacetamide alkylated growth hormone and L-asparaginase where no denaturants were used, the proteins were biological active which suggests no change in tertiary structure (Bewley et al. 1969; Todokoro et al. 1975).
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Figure 1.7. DTT reduction of disulfide bonds (Figure adapted and modified from Hermanson, 1996)

1.3.2 Bis-thiol specific compounds

A bis-alkylating compound 13 (Figure 1.8) which undergoes sequential reactions with nucleophiles was initially described by Lawton et al (Mitra and Lawton, 1979; Liberatore et al. 1990; del Rosario et al. 1990; Wilbur et al. 1994). This bis-sulfone compound 13 was designed to undergo alkylation reactions by elimination/Michael addition reactions with thiols. The bis-sulfone 13 was used for the site-specific labeling or attachment of diagnostic agents onto antibody and antibody fragments (Liberatore et al. 1990; del Rosario et al. 1990; Wilbur et al. 1994). Since the bis-sulfone compound 13 was insoluble in water, reactions had to be conducted in a mixed organic and aqueous solvent. The bis-sulfone compound 13 was originally intended for the site-directed modification of thiol groups in a disulfide-reduced protein. However, due to presence of the organic solvent, the compound was found to react with amine nucleophiles and intermolecularly (Liberatore et al. 1990; del Rosario et al. 1990).

Figure 1.8. Chemical structure of the bis-alkylating compound 13.

The hypothesis of this project is that the bis-alkylating compound 13 when coupled to an mPEG 1 molecule (typically 5 kDa or more) would be water soluble and therefore be more selective to thiols. Water solubility of bis-sulfone compound 13 is very important for reaction with protein. For exploiting the bis-sulfone compound 13 for conjugation purposes, reduction of the disulfide bonds is necessary. However, this conjugation approach requires the reduced protein to maintain its tertiary structure on disulfide reduction. Small amounts of organic solvents in the reaction solution would tend to denature the reduced protein. It is hypothesised that reduction of a protein in mild aqueous conditions would help the protein retain its tertiary structure for reaction with a water soluble bis-sulfone compound 13. Therefore, the proposed DB-PEG 15 consists of essentially three functionalities which are 1) an attached mPEG which would impart water
Chapter 1. Introduction to 'disulfide bridging' PEGylation

solubility, 2) an α, β Michael acceptor which initiates the bis-alkylation reaction and 3) a sulfonyl leaving group for the completion of reaction (Figure 1.9).

![Chemical structure of the proposed PEG bis-sulfone 14 and PEG mono-sulfone 15](image)

Figure 1.9. Chemical structure of the proposed PEG bis-sulfone 14 and PEG mono-sulfone 15 (SO₂R = leaving group; Y = linkage functionality).

The proposed PEG mono-sulfone 15 would form a 3-carbon bridge between the thiols of the cysteines (originally of the disulfide). This process is termed as ‘disulfide bridging' (DB) PEGylation (Figure 1.10). Since the 3-carbon bridge is short in length, a hypothesis is that the bridged disulfides would predominantly maintain the protein's tertiary structure and thus, its biological activity. PEG mono-sulfone 15 being specific to the thiols in a protein that can be reduced while maintaining its tertiary structure can avoid heterogeneity associated with the amine PEGylation or the side reactions observed with the non-aqueous soluble bis-alkylation compound 13.

![Schematic representation of the DB-PEGylation approach with disulfide-reduced proteins](image)

Figure 1.10. Schematic representation of the DB-PEGylation approach with disulfide-reduced proteins

The hypotheses for this project are:

(a) Clinically relevant proteins have accessible disulfide bonds (Figure 1.11). Some accessible disulfides could be reduced with a suitable reductant, for example DTT 16 without denaturing the protein.

(b) The water soluble, bis-alkylating PEG mono-sulfone 15 would display more selectivity towards thiols due to its water solubility.

(c) The thiols derived from the reduced disulfide could be chemically modified with the PEG mono-sulfone 15 to form a 3-carbon bridge between the cysteine sulfurs whilst the protein maintaining its conformation required for biological activity.

(d) This conjugation strategy may be applicable to many proteins with disulfide bonds.
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Figure 1.11. Some proteins with accessible disulfide bonds (disulfides shown in yellow); experimentally determined and modelled structures of a) leptin (pdb entry — 1AX8) b) IFN α-2a (pdb entry — IITF) and c) an antibody fragment (Fab) (pdb entry — ICBV) that binds to a trinucleotide ligand. The region of protein binding is shown in green.

1.4 OBJECTIVES OF THE PROJECT

Evaluation of the aforementioned hypotheses on disulfide bridging PEGylation will be accomplished with the following objectives:

1. Preparation of DB-PEG compounds and characterisation.

Initially, the proposed PEG bis-sulfone 14 and PEG mono-sulfone 15 (Figure 1.9) with molecular weights 5 kDa, 10 kDa and 20 kDa will be prepared. Characterisation of these compounds will be conducted by nuclear magnetic resonance (NMR) spectroscopy and reversed-phase HPLC. The site-specificity of these compounds will be characterised with reduced glutathione (GSH) 26 or cysteine.

2. DB-PEG conjugation to protein/peptide and characterisation studies.

Disulfide bridging PEGylation will initially be evaluated with the anti-leukaemic agent, L-asparaginase. Reduction of the protein disulfides will be conducted with DTT 16 and analysed by Ellman’s assay. The reaction conditions for DB-PEGylation will be studied and optimised mainly with PEG mono-sulfone 15. Control reactions of PEG mono-sulfone 15 with native
(unreduced) L-asparaginase will be conducted and analysed by SDS-PAGE. The DB-PEGylated L-asparaginase will be characterised with SDS-PAGE, SEC-HPLC and MALDI-TOF mass spectrometry. The \textit{in vitro} biological activities such as the enzyme activity and antigenicity of the DB PEGylated L-asparaginase will be determined.

The applicability of the proposed PEG \textit{mono}-sulfone 15 for site-specific monoPEGylation of therapeutic proteins with multiple disulfide bonds will be evaluated with IFN \(\alpha\)-2b (immunomodulatory cytokine). The PEGylation approach will also be studied on proteins/peptides with single disulfide bonds such as leptin (OB protein) and somatostatin. The reaction conditions will be studied and optimised for each protein model. Structural and biological effects due to DB-PEGylation of IFN \(\alpha\)-2b will be assessed with protein modeling, circular dichroism (CD) spectroscopy and biological activity studies. The conjugation reaction will be analysed by tryptic digestion and mass spectrometry studies of IFN \(\alpha\)-2b and somatostatin.
Chapter 2

DB-PEG synthesis and reactivity
2.1 INTRODUCTION

The bis-sulfone compound 13a (Figure 2.1) described by Liberatore et al and del Rosario et al has been initially considered a tri-functional molecule (Wilbur et al. 1994). The functional group ‘X’ mainly prepared was a carboxylic acid (del Rosario et al. 1990; Liberatore et al. 1990), but bis-sulfone adducts with amine 13b and hydroxyl 13c moieties could also be prepared. Variation in ‘X’ allowed binding of either mPEG 1 or mPEG amine 24 via different functional groups ‘Y’ to the bis-sulfone compound 13 (e.g., amide, ester, carbonate, urethane) (Figure 2.1). The sulfonyle leaving groups (SO$_2$R) are at the β sites where alkylation occurs. Elimination of one of the sulfonyle leaving groups yields the PEG mono-sulfone 15. Mechanistically, the PEG mono-sulfone 15 is the reactive compound. PEG bis-sulfone 14 is the precursor that yields the reactive PEG mono-sulfone 15. The α, β alkenyl group in PEG mono-sulfone 15 is a Michael acceptor and is reactive to thiols. The first Michael addition of a thiol will result in the elimination of the second sulfonyle group to form the α, β’ alkenyl group. This undergoes another Michael addition to a second thiol to complete the 3-carbon bridging (Chapter 1, Figure 1.10). These interactive, sequential reactions are characteristic to the PEG mono-sulfone 15 and have not been reported for any other PEG reagents. Due to these sequential reactions, the bis-alkylating α, β alkenyl–α, β’ sulfonyle moieties could actually be considered as a single functional group.

![Figure 2.1. Chemical structures of the bis-sulfone 13 derivatives, PEG bis-sulfone 14 derivatives and the PEG mono-sulfone 15 derivatives that were prepared.](image)

The objectives of this chapter were to prepare and characterise PEG bis-sulfone 14 and PEG mono-sulfone 15 with different linkage functionalities ‘Y’. However, the protein PEGylation work described in thesis was mainly conducted with amide PEG bis-sulfone 14a and amide PEG mono-sulfone 15a.
2.2 MATERIALS

1. acetylbenzoic acid (Sigma-Aldrich cat. no. 177458, m.w – 164.16)
2. 4-nitroacetophenone (Sigma-Aldrich cat. no. N9608, m.w – 165.15)
3. 4-hydroxyacetophenone (Sigma-Aldrich cat. no. 54180, m.w – 136.15)
4. Paraformaldehyde (Sigma-Aldrich, cat. no. 158127, m.w – 30.03)
5. Piperidine hydrochloride (Sigma-Aldrich, cat. No. P46105, m.w – 121.61)
6. 4-methylbenzenethiol (Sigma-Aldrich, cat. no. T28525, m.w – 124.20)
7. Formalin – 37 % w/v aqueous solution (Sigma-Aldrich, cat. no. F1635)
8. Piperidine (Sigma-Aldrich, cat. no. 104094, m.w – 85.15)
9. Oxone® (Sigma-Aldrich, cat. no. 228036, m.w – 61478)
10. Thionyl chloride (Sigma-Aldrich, cat. no. 447285, m.w – 118.97)
11. Anhydrous dichloromethane (Sigma-Aldrich, cat. no. 270997)
12. O-(2-aminoethyl)-O′-methylpolyethylene glycol 5,000/10,000/20,000 Da (Sigma-Aldrich / NOF/ Nektar)
13. Anhydrous triethylamine (Sigma-Aldrich, cat. no. T0886, m.w – 101.19)
14. N,N′-Dicyclohexylcarbodiimide (Sigma-Aldrich, cat. no. 36650)
15. Reduced L-glutathione (Sigma-Aldrich, cat. no. G4251)
16. Oxidised L-glutathione (Sigma-Aldrich, cat. no. G4376)
17. Sodium dihydrogen orthophosphate dihydrate (Na₂H₂PO₄.2H₂O, Fisher Scientific UK cat. no. S/3760/53)
18. Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich-Fluka, cat. no. 03682)
19. 1 M sodium hydroxide standard solution (Sigma-Aldrich, cat. no. 319511)
20. Anhydrous Magnesium Sulfate (Sigma-Aldrich, cat. no. 63136)
22. Acetonitrile, HPLC grade (Fisher)
23. Water, HPLC grade
24. Anhydrous dichloromethane (Sigma-Aldrich)
25. Diethyl ether (BDH)
26. Absolute alcohol
27. Methanol, GPR (BDH)
28. Acetone, GPR (BDH)
29. Ammonium hydroxide solution
30. Trifluoroacetic acid (Sigma-Aldrich)
31. C₁₈ Phenomenex column for RP-HPLC (5 μm, 250 × 4.6 mm).
2.3 METHODS

2.3.1 Preparation of PEG bis-sulfone 14a

Initially, the carboxylic acid bis-sulfone 13a was prepared from p-acetylbenzoic acid 21 by Mannich reaction followed by thiol addition and oxidation (del Rosario et al. 1990; Liberatore et al. 1990). The carboxylic acid bis-sulfone 13a was used for coupling to mPEG amine 24 (Figure 2.2) which was accomplished by in situ activation using either thionyl chloride (SOCl₂) or dicyclohexylcarbodiimide (DCC).
2.3.2 Preparation of PEG mono-sulfone 15a

The carbonyl α-proton in PEG bis-sulfone 14a is acidic in nature. Deprotonation results in the elimination of one of the leaving sulfone groups as p-tolyl sulfinic acid 25 (Figure 2.3). This yields the PEG mono-sulfone 15a. Therefore, the elimination reaction in neutral to mildly basic buffer (pH 7 - 8) was studied by 1H-NMR and reversed phase-HPLC (RP-HPLC) analyses. Initially, the ‘amide’ PEG bis-sulfone 5,500 Da 14a was used for the elimination studies. For the 1H-NMR analysis, amide PEG bis-sulfone 14a ‘5,500 Da’ was used since the proton NMR peaks with low molecular weight (mw) mPEGs was more distinct than the proton peaks with high mw PEGs. The higher mw mPEGs also tend to show broad elution peaks during RP-HPLC analysis.

![Figure 2.3. Preparation of PEG mono-sulfone 15a](image)

2.3.3 Preparation of ester PEG bis-sulfone 14b

Carboxylic acid bis-sulfone 13a was also used for the preparation of the ‘ester’ PEG bis-sulfone 14b (Figure 2.4). Most PEGylation compounds described in the literature are derived directly from mPEG 1 (Zalipsky, 1995). Therefore, the preparation of ester PEG bis-sulfone 10 kDa 14b was conducted to determine its reactivity and applicability for protein PEGylation. Coupling of mPEG 1 to the carboxylic acid bis-sulfone 13a was accomplished by *in situ* activation using DCC in the presence of catalytic amounts of a tertiary amine base (DMAP).

![Figure 2.4. Preparation of ester PEG bis-sulfone 14b](image)
2.3.4 Preparation of urethane PEG bis-sulfone 14c

The amine bis-sulfide 32 was prepared with the starting compound p-nitroacetophenone 29 via Mannich reaction, thiol addition and reduction reactions (Figure 2.5). This compound was coupled to mPEG molecule 1 (5,000 Da or more) to obtain the urethane PEG bis-sulfide 34. This compound on oxidation with Oxone® would yield the urethane PEG bis-sulfone 14c. Urethane PEG bis-sulfone 14c was characterised for its elimination in buffer (pH 7.8) and reactivity to thiols.

![Figure 2.5. Preparation of urethane PEG bis-sulfone 14c](image)

2.3.5 Preparation of carbonate PEG bis-sulfone 14d

Hydroxy bis-sulfone 13c was also prepared from p-hydroxyacetophenone 35 via Mannich reaction, thiol addition and oxidation reactions (Figure 2.6). The compound 13c allowed to react with p-nitrobenzyl chloroformate activated mPEG 38, in the presence of a tertiary amine base (DMAP) gave the ‘carbonate’ PEG bis-sulfone 14d. Synthetic help to prepare some of the precursor molecules (e.g. 33 and 13c) was provided by an Erasmus and a MPharm student.

![Figure 2.6. Preparation of carbonate PEG bis-sulfone 14d](image)
2.3.6 Site-specificity assessment using glutathione reactions

Reduced glutathione (GSH) 26 has a primary amine group in the \( \gamma \)-glutamic acid residue and a thiol group in the cysteine residue (Figure 2.7A). The presence of both amine and thiol nucleophiles in GSH 26 would make it possible to evaluate the site-specificity of the amide PEG bis-sulfone 14a. The reaction product of amide PEG bis-sulfone 14a with 2 eq. of GSH molecules 26 was an adduct 28 of 2 glutathione molecules connected by a 3-carbon bridge attached with a PEG molecule (Figure 2.7C). GSH 26 is a low molecular weight peptide (313 Da). Therefore, separation of the reaction product 28 from any excess GSH 26 was possible by simple desalting using a PD-10 Desalting column. The reaction product 28 was then compared to the structurally similar oxidised glutathione (GSSG) 27 and also to GSH 26. GSSG 27 is basically two GSH molecules 26 connected via a disulfide bond (Figure 2.7B).

\[
\begin{align*}
&\text{A} \\
&\text{B} \\
&\text{C}
\end{align*}
\]

Figure 2.7. Chemical structures of GSH 26, GSSG 27 and GS-PEG-SG 28

2.3.7 NMR Spectroscopy

NMR spectra of all the prepared compounds were obtained using a Bruker Avance 400 MHz spectrometer, while the spectra of GSH 26, GSSG 27 and GS-PEG-SG 28 were acquired using a Bruker Avance 500 MHz. Both instruments were equipped with a 5 mm BBO probe including Z-axis pulse field gradients. Assignments for the \( ^1\text{H}-\text{NMR} \) spectra of peptides were achieved using standard sequential assignment procedures of protein spectra. Two dimensional \( [^1\text{H},^1\text{H}] \) TOCSY spectra were acquired employing 60 ms mixing time and two dimensional \( [^1\text{H},^1\text{H}] \) NOESY spectra employing mixing times from 100 – 300 ms. NMR spectra were processed using Bruker NMR Suite 3.5 and Amix Viewer 3.1.5 (Bruker Biospin GMBH). Chemical shifts were referenced to the residual solvent signals when samples were dissolved in an organic solvent and to sodium trimethylsilyl-(2H4)-propionate (TSP) when samples were in \( \text{D}_2\text{O}/\text{H}_2\text{O} \).
2.3.8 Mass spectrometry

Mass spectra were also acquired for the compounds by ESI in positive mode. The system used was a Waters Alliance 2695 HPLC with a Thermo Navigator ESI. Diluted sample (50 μL) is injected into the mass spec with a flow rate of 0.2 mL/min 50:50 acetonitrile/water + 0.1%. The capillary voltage applied was 3.0 kV and the cone voltage applied was 25 V. Data was typically acquired over a range of 100 to 1000 m/z.

Mass spectra were acquired using an Applied Biosystems Voyager System DE PRO MALDI-TOF mass spectrometer using a nitrogen laser. The matrix was a saturated solution of α-cyano-4-hydroxycinnamic acid in a 50:50 mixture of acetonitrile and water containing 0.1% trifluoroacetic acid. Sample and matrix were mixed 1:1 and 1 μL of the solution was spotted onto a 100-well sample plate. All spectra were acquired in positive mode over the range of 600 - 2,500 Da under reflectron conditions (20 kV accelerating voltage, 350 ns extraction delay time) and 2 - 100 kDa under linear conditions (25 kV accelerating voltage, 750 ns extraction delay time).

2.3.9 Reversed-phase HPLC analysis

C$_{18}$ Phenomenex column (5 μm, 250 × 4.6 mm) with HPLC grade water containing 0.05 % TFA as solvent A and HPLC grade acetonitrile containing 0.05 % CF$_3$COOH (TFA) as solvent B. The gradient used for all the analyses was 30 % to 60 % solvent B in 30 min. The flow rate of 1 mL/min, column temperature of 25 °C and UV detection at 215 nm were used for the analysis.
2.3.10 Preparation of carboxylic acid Mannich salt 22 (Liberatore et al, 1990)

To a clean single neck 250 mL round bottom flask (RBF) containing a magnetic stirrer and absolute ethanol (30 mL), were added p-acetylbenzoic acid 21 (5.0 g, 30.45 mmol, 1 eq.), paraformaldehyde (2.74 g, 91.35 mmol, 3 eq.), piperidine hydrochloride (3.7 g, 30.45 mmol, 1 eq.) and concentrated hydrochloric acid (0.3 mL). A condenser was attached to the flask and the suspension was then heated to reflux (temperature of oil bath: 105 °C) with constant magnetic stirring. After 4 h of reaction, more paraformaldehyde (2.74 g, 91.35 mmol, 3 eq.) was added and the reaction was allowed to proceed for a further 6 h. After a total reaction time of 10 h, the reaction mixture was cooled to ambient temperature and then 40 mL of acetone was added to the suspension. A white crystalline solid product 22 was isolated by filtration in vacuo, dried (4.1 g, 45.2 %); IR spectrum (recorded neat using Nicolet Avatar 360 Omni-sampler FT-IR spectrometer) – 3203.9 cm⁻¹ (Carboxylic acid OH stretch), 1696.4 cm⁻¹ (Carboxylic acid C=O stretch); ¹H-NMR (dmsø-d₆, 400 MHz): 1.5 – 1.8 (2H CH₂ CH₂ CH₂ N and 4H CH₂ CH₂ CO), 3.23 (s, 4H CH₂ CH₂ CH₂ N), 3.38 (t, 2H, N CH₂ CH₂ CO, J = 7.6 Hz), 3.69 (t, 2H, NCH₂CH₂CO, J = 7.3 Hz), 8.10 (m, 4H, aromatic CH, J = 8.6 Hz), 10.3 (Br, 1H, COOH); ¹³C-NMR (dmsø-d₆, 400 MHz): 196.4, 166.0, 138.9, 134.9, 129.5, 128.1, 52.2, 50.8, 33.2, 30.6, 22.4, 21.2; mp = 198–200 °C, literature 200 °C.

2.3.11 Preparation of carboxylic acid bis-sulfide 23 (Liberatore et al, 1990)

To a clean 100 mL single-neck RBF containing a magnetic stirrer and a mixture of absolute ethanol (12 mL) and methanol (6 mL), were added 1-[3-(4-carboxy-phenyl)-3-oxo-propyl]-piperidinium HCl 22 (3.5 g, 11.7 mmol, 1 eq.), 4-methylbenzenethiol (2.91g, 23.48 mmol, 2
eq.), 37 % w/v formalin (3.5 mL) and piperidine (0.5 mL). A condenser was fitted to the flask and the reaction mixture was heated to reflux (temperature: 105 °C). After 1 h, additional formaldehyde (3.5 mL) was added and the refluxing continued for further 3 h. Analysis by thin layer chromatography (TLC) with the solvent mixture of methanol: acetic acid 9:1 indicated completion of the reaction. The reaction flask was then allowed to cool to ambient temperature and then subjected to rotary evaporation to obtain a crude creamy white residue. The pure product 23 was crystallised using methanol and isolated by filtration in vacuo, washed with methanol, dried to constant mass in vacuum (3.6 g, 75 %). IR spectrum (recorded neat using Nicolet Avatar 360 Omni-sampler FT-IR spectrometer) – 3013.5 cm⁻¹ (Carboxylic acid OH stretch), 1677.6 cm⁻¹ (Carboxylic acid C=O stretch); ¹H-NMR (CDCl₃, 400 MHz): 2.38 (s, 6H), 3.16-3.31 (m, 4H), 3.85 (q, 1H, J = 6.8 Hz), 7.15 (d, 4H, J = 8.1 Hz), 7.18 (d, 4H, J = 8.3 Hz), 7.64 (d, 2H, J = 8.6 Hz), 8.07 (d, 2H, J = 8.6 Hz); ¹³C-NMR (CDCl₃, 400 MHz): 200.5, 137.2, 131.5, 131.1, 130.2, 129.9, 128.3, 45.9, 36.4, 21.1; mp = 121 – 123 °C, literature 142 – 143 °C; TOF MS ES+ [M + H]+ : 437.1

2.3.12 Preparation of carboxylic acid bis-sulfone 13a (Liberatore et al, 1990)

![Figure 2.10. Preparation of carboxylic acid bis-sulfone 13a by Oxone oxidation.](image)

To a clean 500 mL RBF containing a magnetic stirrer and 1:1 methanol:water mixture, were added 4-(3-p-tolylsulfanyl-2-p-tolylsulfanylmethyl-propionyl)-benzoic acid 23 (3.0 g, 6.87 mmol, 1 eq.) and Oxone® (25.34 g, 41.22 mmol. 6 eq.). The white solid suspension formed was stirred at ambient temperature for 24 h. After the reaction period, 100 mL of chloroform was added to the flask to dissolve the bis-sulfone 13a. The mixture was transferred to an extraction funnel and the organic phase (bottom layer) was separated into a clean 250 mL RBF. To the remaining aqueous white solid suspension was added water (100 mL) to dissolve the remaining Oxone® and residual salts to give a homogenous aqueous phase. This was washed again with chloroform (100 mL) and the organic layer separated. The organic phases were then combined and transferred into a clean extraction funnel. This was washed with brine (100 mL) and the organic layer was separated into a clean 250 mL RBF. The organic phase was then dried using anhydrous magnesium sulfate (100 g) and filtered. The chloroform extraction steps were entirely carried out in a fume hood. The solvent was then subjected to rotary evaporation to give an off-white crude solid product. The product was re-crystallised using methanol to obtain a
white crystalline compound 13a (3.0 g, 84.2 %). IR spectrum (recorded neat using Nicolet Avatar 360 Omni-sampler FT-IR spectrometer) – 2974.6 cm⁻¹ (Carboxylic acid OH stretch), 1700.9 cm⁻¹ (Carboxylic acid C=O stretch); ¹H-NMR (CDCl₃, 400 MHz): 2.49 (s, 6H), 3.48 - 3.66 (m, 4H), 4.40 (q, 1H, J = 6.0 Hz), 7.37 (d, 2H, J = 8.1 Hz), 7.70-7.73 (m, 6H, J = 8.3 Hz), 8.10 (d, 2H, J = 8.6 Hz); ¹³C-NMR (CDCl₃, 400 MHz): 195.4, 170.1, 145.6, 138.2, 135.4, 133.7, 130.6, 130.2, 128.6, 128.4; mp = 164 - 165 °C, literature 174 - 175 °C; TOF MS ES⁺ [M + H]⁺: 501.1 Da.

2.3.13 Preparation of methyl ester bis-sulfide 39 by Fischer esterification (new compound)

To a clean 500 mL RBF containing a magnetic stirrer and methanol (10 mL), was added 4-(3-p-tolylsulfanyl-2-p-tolylsulfanylmethyl-propionyl)-benzoic acid 23 (0.8 g, 1.83mmol, 1 eq.). To this suspension was carefully added conc. sulfuric acid (2 mL). The flask was then fitted with a condenser and the solution was heated to reflux (temperature: 110 °C). After 1 h, the flask was removed and the clear solution formed was allowed to cool to ambient temperature. Concentrated ammonium hydroxide solution (5 mL) was added to the mixture and then the solvent subjected to rotary evaporation. The mixture was dissolved in acetone and then precipitated by addition of deionised water (1 mL). The precipitate 39 was filtered using a # 3 scintered funnel, dried in vacuum (0.7 g, 84.8 %). ¹H-NMR (CDCl₃, 400 MHz): 2.38 (s, 6H), 3.16-3.31 (m, 4H), 3.78 (q, 1H, J = 6.57 Hz), 3.95 (s, 3H), 7.15 (d, 4H, J = 8.1 Hz), 7.18 (d, 4H, J = 8.3 Hz), 7.64 (d, 2H, J = 8.6 Hz), 8.07 (d, 2H, J = 8.6 Hz), ¹³C-NMR (CDCl₃, 400 MHz): 200.4, 166.2, 139.7, 137.2, 133.9, 131.5, 129.8, 129.6, 128.2, 52.5, 45.7, 36.4, 29.7, 21.1.

2.3.14 Preparation of methyl ester bis-sulfone 40 (new compound)

Figure 2.12. Preparation of methyl ester bis-sulfone 40
To a 500 mL round bottom flask containing a magnetic stirrer and 1:1 methanol:water mixture (100 mL), were added 4-(3-p-tolylsulfanyl-2-p-tolylsulfanylmethyl-propionyl)-benzoic acid methyl ester 39 (0.425 g, 0.94 mmol, 1 eq) and Oxone® (25.34 g, 41.22 mmol, 44 equivalents). The white solid suspension obtained was stirred for 24 h. After the reaction period, chloroform (100 mL) was added to dissolve the bis-sulfone 40. This organic phase was separated by separating funnel to leave a white solid suspension in the aqueous phase. Deionised water (100 mL) was added to dissolve the residual salts to give a homogenous aqueous phase. This was washed again with chloroform (100 mL) and separated. The organic phases were combined and washed with brine (100 mL). The organic phase was dried with anhydrous magnesium sulfate and filtered. The solvent was removed by rotary evaporation to give an off-white crude solid product. The product was re-crystallised from methanol to give a white crystalline compound 40 (3.0 g, 84.2 %). IR spectrum (recorded neat using Nicolet Avatar 360 Omni-sampler FT-IR spectrometer) – 1720.3 cm⁻¹ (Ester C=O stretch), 1685.4 cm⁻¹ (Ketone C=O stretch); \(^1\)H-NMR (CDCl₃): 2.49 (s, 6H), 3.48-3.66 (m, 4H), 3.95 (s, 3H), 4.37 (q, 1H, \( J = 6.57 \) Hz), 7.37 (d, 4H, \( J = 8.1 \) Hz), 7.70 (d, 4H, \( J = 8.6 \) Hz), 7.73 (d, 2H, \( J = 8.3 \) Hz), 8.10 (d, 2H, \( J = 8.6 \) Hz); \(^13\)C-NMR (CDCl₃, 400 MHz): 195.2, 165.9, 145.5, 137.3, 135.2, 134.6, 130.2, 129.9, 128.4, 128.3, 55.5, 52.6, 35.7, 21.7; mp = 141 – 142 °C; TOF MS ES+: 515.00 Da.

2.3.15 Preparation of methyl ester bis-sulfone 40 using thionyl chloride (new compound)

![Figure 2.13. Preparation of methyl ester bis-sulfone 40 by thionyl chloride method](image)

To a clean dry 50 mL schlenk flask containing a magnetic stirrer, was added 4-[3-(toluene-4-sulfonyl)-2-(toluene-4-sulfonylmethyl)-propionyl]-benzoic acid 13a (0.05 g, 0.1 mmol) and sealed with a rubber septum. Thionyl chloride (1 mL) was taken using a glass syringe and injected into the flask. The solution was heated (temperature: 50 °C) for 2 h in an argon atmosphere. The excess thionyl chloride was removed by vacuum evaporation and the resultant mixture was dissolved in anhydrous dichloromethane. The solvent was again removed using vacuum and the process was repeated once more until white foam appeared. The crude product was dispersed in methanol (10 mL) and shaken for 10 min. A white precipitate formed upon addition of water (1 mL). The precipitate of compound 40 was filtered, dried in vacuum for 48 h (0.045 g, 90 %) IR spectrum (recorded neat using Nicolet Avatar 360 Omni-sampler FT-IR
Chapter 2. DB-PEG synthesis and reactivity

spectrometer) – 1720.3 cm⁻¹ (Ester C=O stretch), 1685.4 cm⁻¹ (Ketone C=O stretch); ¹H-NMR (CDCl₃): 2.49 (s, 6H), 3.48 -3.66 (m, 4H), 3.95 (s, 3H), 4.37 (q, 1H, J = 6.57 Hz), 7.37 (d, 4H, J = 8.1 Hz), 7.70 (d, 4H, J = 8.6 Hz), 7.73 (d, 2H, J = 8.3 Hz), 8.10 (d, 2H, J = 8.6 Hz); ¹³C-NMR (CDCl₃, 400 MHz): 195.2, 165.9, 145.5, 137.3, 135.2, 134.6, 130.2, 129.9, 128.4, 128.3, 55.5, 52.6, 35.7, 21.7; mp = 141 – 142 °C; TOF MS ES+: 515.00 Da.

2.3.16 Toluene distillation drying of mPEG 7 or mPEG amine 24

To a clean dry 50 mL schlenk flask fitted with a rubber septum and a magnetic stir bar, was added O-(2-aminoethyl)-O'-methylpolyethyleneglycol 24 or mPEG 1 (5 kDa, 10 kDa or 20 kDa) (0.5 to 1.0 g). Anhydrous toluene (5 mL) was injected into the flask using a clean glass syringe and needle. The flask was then gently warmed (temperature: ≈ 60 °C) to dissolve the PEG in toluene. The stoppered side arm of the schlenk flask was connected to vacuum oil pump fitted with an ice trap. Toluene was observed to slowly foam upon slow opening of the side arm stopper to vacuum. The flask was gently swirled to avoid spluttering of the mixture. Moisture formed on the outer walls of the flask was wiped until all the solvent was removed from the flask. The flask was then allowed to remain in vacuum for a further 30 min at ambient temperature. Anhydrous DCM (5 mL) was added to the flask under argon atmosphere to dissolve the PEG completely.

2.3.17 Preparation of PEG bis-sulfone 14a by thionyl chloride activation

To a clean dry 100 mL RBF containing a magnetic stirrer, was added 4-[3-(toluene-4-sulfonyl)-2-(toluene-4-sulfonylethyl)-propionyl]-benzoic acid 13a (100 mg, 0.2 mmol). Thionyl chloride (1.5 mL, 20.85 mmol) was carefully injected into the flask and a condenser was
attached to the flask. The heterogenous mixture was heated to reflux (temperature: 90 °C) with stirring for 1 h. After cooling to ambient temperature, the volatiles were removed by rotary evaporation to yield yellow foam. This was dissolved in anhydrous dichloromethane (5 mL) and the solvent removed by rotary evaporation. This solvent addition/removal process was repeated 1 – 2 more times to afford white foam. Anhydrous dichloromethane (5 mL) was again added to the product to afford a homogenous solution.

The activated compound 41 solution was injected into the toluene distillation dried O-(2-aminoethyl)-O'-methyl poly(ethylene glycol) 5,000 Da (400 mg, 0.08 mmol) solution drop wise immediately resulting in the evolution of a white gas. Anhydrous triethylamine (30 μL, 0.2 mmol) was charged into the PEG solution. The resulting solution was allowed to stir overnight at ambient temperature whereupon additional anhydrous triethylamine (30 μL, 0.2 mmol) was added. After a further 2 h, the resultant solution was added drop wise using a glass pipette into rapidly stirring chilled diethyl ether (~ 80 mL) placed in an ice bath. The precipitant obtained was isolated by centrifugation. The solid recovered is dried in a vacuum oven to afford an off-white product 14a (0.38 g, 86.4 %). 1H-NMR (CDCl3, 400 MHz): δ 2.49 (s, 6H, CH2Ar), 3.38 (s, 3H, CH3OPEG), 3.44 - 3.84 (m, PEG + 4H, CH2SO2), 4.34 CHCO (qn, IH, CHCO, J = 6.0 Hz), 7.36, 7.69 (AB q, SO2Ar, 4H, J = 8.2 & 8.4 Hz respectively), 7.64, 7.81 (AB q, COAr, 4H, J = 8.4 & 8.2 Hz respectively).

2.3.18 Preparation of PEG bis-sulfone 14a by DCC activation

Figure 2.15. Preparation of PEG bis-sulfone 14a by DCC method

To a clean dry schlenk flask containing a magnetic stirrer and toluene distillation dried O-(2-aminoethyl)-O'-methylpolyethylene glycol 24 5,000 g/mol (0.5 g, 100 μmol), was added 4-[3-(toluene-4-sulfonyl)-2-(toluene-4-sulfonylmethyl)-propionyl]-benzoic acid 13a (150 mg, 300 μmol) and N,N'-dicyclohexylcarbodiimide (62 mg, 300 μmol, Aldrich). The flask was sealed with a rubber septum and then connected to high vacuum for 30 min through the side arm. Argon was introduced into the flask with a argon filled rubber balloon fitted with a syringe and needle. The flask containing the stirred mixture was cooled on an ice bath. Anhydrous dichloromethane (10 mL) was added by syringe giving a homogenous solution. The stirred
solution was allowed to warm to ambient temperature slowly and then kept stirring for 12 h. The reaction mixture was filtered through a scintillated glass filter #3 to remove insoluble dicyclohexylurea (DCU). Dichloromethane was then removed by rotary evaporation and the viscous mixture was dissolved in acetone (20 mL) by gentle warming. The flask was placed on an ice bath for 5 min to precipitate the PEG bis-sulfone 14a which was isolated as a white solid by filtration (#3 scintillated glass funnel) and dried. (yield: 0.4 g, 72.7%). PEG bis-sulfone 14a was also prepared using mPEG amine 10 kDa 24 and mPEG amine 20 kDa 24 (0.5 g each) with similar stoichiometry. $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 2.49 (s, 6H, CH$_3$Ar), 3.38 (s, 3H, CH$_3$OPEG), 3.44 - 3.84 (m, PEG + 4H, CH$_2$SO$_2$), 4.34 CHCO (qn, 1H, CHCO, $J = 6.0$ Hz), 7.36, 7.69 (AB q, SO$_2$Ar, 4H, $J = 8.2$ & 8.4 Hz respectively), 7.64, 7.81 (AB q, COAr, 4H, $J = 8.4$ & 8.2 Hz respectively). $^{13}$C-NMR (CDCl$_3$, 500 MHz): $\delta$ 195.1, 166.1, 145.5, 139.4, 136.2, 135.4, 130.2, 128.6, 128.3, 127.7, 71.9, 70.6, 69.6, 59.0, 55.6, 40.0, 35.6, 21.7; MALDI-TOF-MS 5,378.1 Da.

2.3.19 Preparation of PEG bis-sulfide 42 by DCC activation

![Figure 2.16. Preparation of amide PEG bis-sulfide 42](image.png)

To a clean dry schlenk flask containing toluene distillation dried O-(2-aminoethyl)-O'-methyl poly (ethylene glycol) 5,000 g/mol (0.5 g, 100 μmol) and a magnetic stirrer, were added 4-(3-p-tolylsulfanyl-2-p-tolylsulfanylmethyl-propionyl)-benzoic acid 23 (150 mg, 343 μmol) and N,N'-dicyclohexylcarbodiimide (71 mg, 343 μmol, Sigma-Aldrich). The flask was sealed using a rubber septum and then subjected to high vacuum for 30 min through the side arm. Argon was introduced into the flask which was then placed on an ice bath. Anhydrous dichloromethane (10 mL) was injected into the flask and the contents stirred to form a homogenous solution. The flask containing the solution was brought to ambient temperature slowly and kept stirring for 12 h. The reaction solution was filtered through a scintillated glass filter #3 to remove insoluble dicyclohexylurea (DCU). Dichloromethane was then removed by rotary evaporation and the viscous mixture formed was dissolved in acetone (20 mL) by gentle warming. The flask was kept on an ice bath for 5 min to precipitate the PEG bis-sulfide 42. The precipitate was isolated using a #3 scintillated glass funnel and dried in vacuum to afford a white solid product (0.42 g, 77.2%). $^1$H-NMR (CDCl$_3$, 400 MHz) $\delta$ 2.33 (s, 6H, CH$_3$Ar), 3.38 (s, 3H, CH$_3$OPEG), 3.16 -
3.31 (m, 4H, CH$_2$SO$_2$), 3.44 – 3.84 (m, PEG), 4.34 CHCO (qn, 1H, CHCO), 7.05, 7.15 (AB q, SO$_2$Ar, 4H, J = 8.1 & 8.3 Hz respectively), 7.6, 7.75 (AB q, COAr, 4H, J = 8.3 & 8.1 Hz respectively).

2.3.20 Oxidation of PEG bis-sulfide 42 to PEG bis-sulfone 14a

To a 250 mL RBF containing 1:1 methanol: water (50 mL) and a magnetic stirrer, were added PEG bis-sulfide 42 (0.400 g) and Oxone® (10 g). The mixture formed was stirred for 24 h at ambient temperature. PEG bis-sulfone 14a was isolated by chloroform extraction as described earlier in section 2.3.14 (yield: 0.375 g, 93 %). $^1$H-NMR was consistent to PEG bis-sulfone 14a. $^1$H-NMR (CDCl$_3$, 400 MHz): δ 2.49 (s, 6H, CH$_3$Ar), 3.38 (s, 3H, CH$_3$OPEG), 3.44 - 3.84 (m, PEG + 4H, CH$_2$SO$_2$), 4.34 CHCO (qn, 1H, CHCO, J = 6.0 Hz), 7.36, 7.69 (AB q, SO$_2$Ar, 4H, J = 8.2 & 8.4 Hz respectively), 7.64, 7.81 (AB q, COAr, 4H, J = 8.4 & 8.2 Hz respectively).

2.3.21 Preparation of PEG mono-sulfone 15a

PEG bis-sulfone 14a ($\approx$ 5,500 g/mol, 0.25 g, 36 μmol) was dissolved in 50 mM sodium phosphate buffer, pH 7.8 (10 mL) and incubated overnight at ambient temperature. PEG mono-sulfone 15a was isolated from the solution by preparative reversed-phase flash chromatography (Section 2.3.22) (80 % pure by $^1$H-NMR with the remainder being the starting PEG bis-sulfone 14a) as a white solid (0.16 g, 71 %). $^1$H-NMR (CDCl$_3$, 400 MHz): δ 2.35 (s, 3H, CH$_3$Ar), 3.31 (s, 3H, CH$_3$OPEG), 3.39 - 3.76 (m, PEG), 4.28 (s, 2H, CH$_2$SO$_2$), 5.93, 6.22 (s, 2H, CH$_2$=C), 7.26, 7.64 (AB q, SO$_2$Ar, 4H, J = 8.3 & 8.6 Hz respectively), 7.72, 7.81 (AB q, COAr, 4H, J = 8.1 & 8.3 Hz respectively).
2.3.22 Preparative reversed phase flash chromatography

A #3 scinttered glass funnel (PYREX) packed with 10 g of reversed phase (Reversed phase silica gel 100 C18) silica was used for the purification of the PEG mono-sulfone 15a from the reaction solution. The preparative reversed-phase funnel reservoir was loaded with 25 mL (maximum reservoir capacity) of deionised water. Water was allowed to enter and flow through the C-18 packing bed by vacuum suction leaving the packing dry. The reaction mixture (LOAD - 25 mL) was then added to the reservoir and then vacuum applied to leave the funnel dry. The funnel was further washed with 50 mL (WASH - 25 mL x 2) of 25 % v/v aqueous acetonitrile to remove the buffer salts, the eliminated p-tolylsulfinic acid 25 and any underivatised mPEG amine 24. PEG mono-sulfone 15a was then eluted with 25 mL of 75 % v/v aqueous acetonitrile (eluent) and the filtrate diluted with more water and freeze dried to obtain the product as a white solid. The load and wash volumes were also collected separately and freeze dried to analyse the obtained products by 1H-NMR.

2.3.23 Preparation of ester PEG bis-sulfone 14b by DCC activation

![Figure 2.19. Preparation of ester PEG bis-sulfone 14b.](image)

To a 50 mL schlenk flask containing a magnetic stirrer and the toluene distillation dried O-(2-aminoethyl)-O'-methyl poly (ethylene glycol) 10,000 g/mol (0.5 g, 50 μmol), were added 4-[3-(toluene-4-sulfonyl)-2-(toluene-4-sulfonylmethyl)-propionyl]-benzoic acid 13a (150 mg, 300 μmol) and N,N'-dicyclohexylcarbodiimide (DCC, 62 mg, 300 μmol, Aldrich). The flask was sealed with a rubber septum and then subjected to high vacuum for 30 min through the side arm. Argon was introduced into it (with an argon filled rubber balloon) and the flask was then placed on an ice bath. Anhydrous dichloromethane (10 mL) was injected into the flask and the contents stirred to form a solution. The flask containing the solution was brought to ambient temperature slowly and kept stirring for 12 h.

The reaction solution was filtered through a #3 scinttered glass filter to remove insoluble DCU. DCM was then taken off by rotary evaporation and the viscous mixture was dissolved in acetone (20 mL) by gentle warming. The flask was kept on a dry ice bath for 5 min to
precipitate out the PEG bis-sulfide $^{14b}$. The precipitate was isolated with a #3 scinttered glass funnel and dried in vacuo to afford a white solid product (0.4 g, 76.2 %) $^1$H-NMR (CDCl$_3$, 400 MHz): $\delta$ 2.35 (s, 3H, CH$_3$Ar), 3.31 (s, 3H, CH$_3$OPEG), 3.39 - 3.76 (m, PEG), 4.28 (s, 2H, CH$_2$SO$_2$), 5.93, 6.22 (s, 2H, CH$_2$=C), 7.26, 7.64 (AB q, SO$_2$Ar, 4H, $J = 8.3$ & 8.6 MHz respectively), 7.72, 7.81 (AB q, COAr, 4H, $J = 8.1$ & 8.3 MHz respectively).

2.3.24 Preparation of urethane PEG bis-sulfide $^{34}$

![Figure 2.20. Preparation of urethane PEG bis-sulfide $^{34}$](image)

To a 50 mL schlenk flask containing a magnetic stirrer and toluene distillation dried mPEG 5,000 g/mol $^1$ (0.5 g, 100 µmol, 1 equivalent), were added paranitrobenzyl chloroformate activated amine bis-sulfide $^{33}$ (171.6 mg, 0.3 mmol, 3 eq) and DMAP (catalytic amount). The flask was sealed with a rubber septum and then subjected to high vacuum for 30 min through the side arm. Argon was introduced into the flask with an argon balloon and then anhydrous dichloromethane (10 mL) was injected into the flask. The contents were stirred to form a homogenous solution. The stirred solution was slowly brought to ambient temperature and kept stirring for 24 h. DCM was then removed by rotary evaporation and the viscous mixture was dissolved in acetone (20 mL) by gentle warming. The flask was kept on a dry ice bath for 5 min to precipitate out the PEG bis-sulfide $^{34}$. The precipitate was isolated using a #3 scinttered glass funnel and dried in vacuo to afford a white solid product $^{34}$ (0.46 g, 83.6 %). $^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 7.7 (d, 4H, $J = 8.3$ Hz), $\delta$ 7.45 (d, 2H, $J = 8.6$ Hz), $\delta$ 7.4 (d, 2H, $J = 8.6$), $\delta$ 7.35 (d, 4H, $J = 8.3$ Hz), $\delta$ 4.35 (m, 2H, br), $\delta$ 3.45-3.9 (PEG), $\delta$ 3.4 (s, 3H), $\delta$ 2.5 (s, 6H).

2.3.25 Preparation of urethane PEG bis-sulfone $^{14c}$

![Figure 2.21. Oxidation of urethane PEG bis-sulfide $^{34}$ to PEG bis-sulfone $^{14c}$](image)
To a 100 mL RBF containing a magnetic stirrer and a mixture of 1: 1 methanol and water (6 mL), were added urethane PEG bis-sulfide 34 (0.1 g, 9.6x10⁻³ mmol, 1 eq.) and Oxone® (41.38 mg, 0.067 mmol, 7 eq.). The white solid suspension formed was stirred for 21 h at ambient temperature. PEG bis-sulfone 14c was then extracted from the mixture with chloroform (7 x 3 mL). The collected organic layers were pooled and dried using anhydrous magnesium sulfate. The solution was filtered and then the solvent removed by rotary evaporation. The white solid product 14c obtained was dried \textit{in vacuo} (0.07 g, 63.6 %). \textsuperscript{1}H-NMR (400 MHz, CDCl₃): δ 2.4 (s, 6H, CH₃Ar), 3.3 (s, 3H, CH₃PEG), 3.35-3.8 (m, PEG), 7.3 (d, 4H, J = 8.3 Hz), 7.65 (d, 2H, J = 8.6 Hz), 7.35 (d, 4H, J = 8.3 Hz), 7.45 (d, 2H, J = 8.6 Hz).

2.3.26 Preparation of Paranitrobenzyl mPEG 38

To a 50 mL schlenk flask containing a magnetic stirrer and previously dried mPEG 1 (1 g, 200 μmol, 1 equivalent), were added paranitrobenzyl chloroformate (55 mg, 272 pmol, 1.36 equivalent) and DMAP (35 mg, 1.3 equivalent). The flask was fitted with a rubber septum. The side arm was connected to high vacuum and stoppered after 2-3 min. Argon was introduced in the flask with an argon filled rubber balloon fitted with a syringe and needle. Dichloromethane (10 mL) was then added to the flask and the solution was stirred for 16 h at ambient temperature. The solvent was removed by rotary evaporation and the viscous mixture in the flask was dissolved in acetone (20 mL) by gentle warming. The flask was then placed on an ice bath to precipitate the desired paranitrobenzyl PEG 38 as a white solid product (0.85 g, 77.3 %). \textsuperscript{1}H-NMR (CDCl₃, 400 MHz) δ 7.3 (d, 2H, J = 9.09), δ 7.2 (d, 2H, J = 9.09), δ 3.4-3.8 (PEG).

2.3.27 Preparation of carbonate PEG bis-sulfone 14d

To a clean dry 50 mL schlenk flask containing a magnetic stirrer, were added \textit{p}-nitrophenol activated PEG carbonate 38 (800 mg, 155 μmol, 1 eq), hydroxy bis-sulfone (216 mg, 457 μmol, 3 eq) and DMAP (catalytic). The flask was fitted with a rubber septum. The side arm was connected to high vacuum and stoppered after 2-3 min. Argon was introduced into the flask with an argon filled rubber balloon fitted with a syringe and needle. Anhydrous dichloromethane (10 mL) was added and the solution was stirred for 72 h at ambient temperature.
temperature. Dichloromethane was removed by rotary evaporation and the viscous mixture in the flask was dissolved in acetone (20 mL) by gentle warming. The flask was then placed on an ice bath to precipitate the desired carbonate PEG bis-sulfone 14d as a white solid which was filtered and dried in vacuo (650 mg, 76.6 %); $^1$H-NMR (CDCl$_3$, 400 MHz) $\delta$ 7.65 (d, 4H, $J = 8.3$), $\delta$ 7.55 (d, 2H, $J = 8.6$), $\delta$ 7.3 (d, 4H, $J = 8.3$), $\delta$ 7.1 (d, 2H, $J = 8.6$), $\delta$ 4.4 (m, 2H), $\delta$ 3.4-3.8 (PEG), $\delta$ 3.3 (s, 3H), $\delta$ 2.4 (s, 6H).

2.3.28 $^1$H-NMR characterisation of PEG bis-sulfone 14a elimination

To an eppendorf tube, 50 mM sodium phosphate buffer (1 mL), pH 7.2 was added and frozen. The frozen mixture was subjected to freeze drying. The residual phosphate salt was re-dissolved in 1 mL of D$_2$O to prepare the 50 mM sodium phosphate buffer (pH 7.2). PEG bis-sulfone 14a (5 mg, 5,500 Da, 0.91 pmol) was added. $^1$H-NMR spectrum was acquired every 1 h for 6 h.

2.3.29 RP-HPLC characterisation of PEG bis-sulfone 14a elimination

2.3.29.1 Effect of pH on the elimination reaction:

To an eppendorf tube, was added 5 kDa PEG bis-sulfone 14a (25 mg, 0.0045 mmol) and dissolved in different pH buffers (1 mL each, experiments done at separate times) to yield a PEG concentration of 25 mg/mL. The solution formed was immediately used for characterisation by reversed phase-HPLC. The sample volume used for each injection was 20 $\mu$L (0.5 mg, 0.091 pmol PEG bis-sulfone 14a). The solution after the initial injection (0 h - at the time of dissolution in buffer) was incubated at 25 °C and HPLC analysis carried out exactly every 1 h for 5 h. The buffers used for the study were 1) 50 mM potassium phosphate buffer, pH 4.8, 2) 50 mM sodium phosphate buffer, pH 6.0, 3) 50 mM sodium phosphate buffer, pH 7.0, 4) 50 mM sodium phosphate buffer, pH 7.8 and 5) 50 mM sodium phosphate buffer, pH 8.6

2.3.29.2 Effect of concentration on the elimination reaction

To three eppendorf tubes, were added 25 mg, 20 mg and 10 mg each of 5 kDa PEG bis-sulfone 14a and dissolved in 50 mM sodium phosphate buffer, pH 7.8 (1 mL) to yield the PEG bis-sulfone 14a concentrations of 25 mg/mL, 20 mg/mL and 10 mg/mL respectively (experiments done at separate times). The solutions formed were immediately used for characterisation by reversed phase-HPLC. The sample volume was adjusted to yield 0.5 mg of PEG bis-sulfone 14a in each injection (20 $\mu$L for 25 mg/mL experiment, 25 $\mu$L for 20 mg/mL experiment and 50 $\mu$L
for 10 mg/mL experiment). The solutions after initial injection at the time of dissolution (0 time) in buffer were incubated at 25 °C and the analysis carried out exactly every 1 h for 5 h.

2.3.29.3 Effect of temperature on the elimination reaction

To two eppendorf tubes (experiments done at separate times), were added 5 kDa PEG bis-sulfone 14a (10 mg, 0.0018 mmol) and dissolved in 50 mM sodium phosphate buffer, pH 8.6 (1 mL) to yield a PEG bis-sulfone 14a concentration of 10 mg/mL. The solutions formed were immediately used for characterisation by reversed phase-HPLC. The sample volume used for each injection was 50 μL (0.5 mg PEG bis-sulfone 14a). The solutions after initial injection at the time of dissolution in buffer (0 h time) were incubated at 4 °C and 25 °C respectively and the analysis carried out exactly every 1 h for 5 h.

2.3.30 1H-NMR characterisation of carbonate PEG bis-sulfone 14d elimination

To an eppendorf tube, was added carbonate PEG bis-sulfone 14d 5 kDa (25 mg) and dissolved in 50 mM sodium phosphate buffer, pH 7.8 (1 mL) to yield a PEG bis-sulfone 14d concentration of 25 mg/mL. The solution formed was incubated for 16 h at ambient temperature and solution buffer exchanged to D2O using a PD-10 column. The third 1 mL fraction collected from the PD-10 Desalting column was used for 1H-NMR analysis. The reaction was also repeated with 1 h of incubation at ambient temperature followed by buffer exchange with a PD-10 Desalting column to D2O and 1H-NMR analysis.

2.3.31 Molecular modeling studies of PEG bis-sulfones 14a, 14c & 14d

All computational studies were carried out using integrated molecular modeling package Maestro® v6.5 and Macromodel v9.1. The three bis-sulfones (14a, 14c and 14d) with truncated PEG chains (two repeat units) were subjected to Monte Carlo conformational search using Macromodel v9.1 (OPLS forcefield and implicitly defined solvent) and the lowest energy structures were further optimised using DFT calculations (B3LYP/6-31**) utilising Jaguar v6.5. The partial charges, the highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals, and the HOMO-LUMO gaps were calculated. Calculations were also conducted with 22 more repeat units to give a 1 kDa PEG moiety and a full Monte Carlo Multiple Minimum (MMMC) conformational search was done for the urethane 14c and carbonate 14d bis-sulfones.
2.3.32 Molecular modeling of amide PEG 10 kDa for proteins

Using Maestro®, PEG with linker subunit was constructed as a linear chain comprising 230 \( \text{CH}_2\text{CH}_2\text{O} \) monomer units (10 kDa). The OPLS-2005 force field was used with extended cut-off values for non-bonded interactions (Van der Waals – 8 Å, electrostatic – 20 Å, and hydrogen bond – 4 Å). Solvent effects were considered as an aqueous generalised Born/surface area (GB/SA) solvent model. The PEG structure was then subjected to the molecular dynamics simulation at 300 K; 1,500 steps of conjugated gradient minimisation, equilibration time of 10 ps, and 2,000 ps of simulation time and time step of 1 ps using shake algorithm for all bonds to hydrogen atoms. Snapshots of molecular structures were recorded at every 20 ps during simulations and energy minimised.

2.3.33 Preparation of GS-PEG-SG

To an eppendorf tube, were added 5 kDa PEG bis-sulfone \(14\text{a} \) (16 mg, 2.91 \( \mu \text{mol} \)) and GSH \( 26 \) (2 mg, 6.51 \( \mu \text{mol} \)), 50 mM sodium phosphate buffer, pH 7.8 containing 10 mM EDTA (900 \( \mu \text{L} \)). The tube was vortexed to dissolve the solids. The compounds were then allowed to react for 18 h at still and at ambient temperature. To the reaction solution, 100 \( \mu \text{L} \) of 1 N NaOH was added to ensure reaction completion. The solution (1 mL) was loaded onto a PD-10 desalting column pre-equilibrated with 9:1 \( \text{H}_2\text{O}:\text{D}_2\text{O} \). The third 1 mL fraction which contains the maximum amount of the reaction product was analysed by 500 MHz \( ^{1}\text{H}-\text{NMR} \) with water suppression.

2.3.34 Control incubation of PEG bis-sulfone \(14\text{a} \) (5 kDa) with GSSG

To an eppendorf tube, were added 5 kDa PEG bis-sulfone \(14\text{a} \) (10 mg, 1.82 \( \mu \text{mol} \)) and GSSG \(27 \) (10 mg, 16.3 \( \mu \text{mol} \)) and 50 mM sodium phosphate buffer, pH 7.8 containing 10 mM EDTA (1 mL). The tube was vortexed to dissolve the solids and the solution formed was allowed to react for 3 h at still and at ambient temperature. The reaction was then desalted to 9:1 \( \text{H}_2\text{O}:\text{D}_2\text{O} \) and the third 1 mL analysed by \( ^{1}\text{H}-\text{NMR} \) with water suppression.

Another reaction (same scale as above), after 3 h of incubation with GSH \( 26 \) was added with 25 \( \mu \text{L} \) of 1 N NaOH further kept still for reaction for 1 h. At this point, 1 N NaOH (25 \( \mu \text{L} \)) was added and the compounds allowed to react for further 30 min at still. This solution was then buffer exchanged to \( \text{D}_2\text{O} \) using a PD-10 column. The third 1 mL fraction from the column was analysed by \( ^{1}\text{H}-\text{NMR} \).
For comparison studies, reaction of PEG bis-sulfone 14a (10 mg, 1.82 µmol) with GSSG 27 (5 mg, 8.15 µmol) was also conducted with addition of 1 N NaOH. The solids were dissolved in 50 mM sodium phosphate buffer containing 10 mM EDTA (1 mL), pH 7.8 and then allowed to react for 3 h at still and ambient temperature. After 3 h, 25 µL of 1 N NaOH was added. The reaction solution was kept still for 1 h. At this point, 25 µL of 1 N NaOH was added. The solution was kept still and allowed to react for further 30 min. This solution was then buffer exchanged to D$_2$O using a PD-10 column. The third 1 mL fraction from the column was analysed by $^1$H-NMR.

### 2.3.35 Preparation of cys-PEG-cys 82

To an eppendorf tube, were added 5 kDa PEG bis-sulfone 14a (16 mg, 2.91 µmol) and cysteine HCl 81 (4 mg, 23.3 µmol), 50 mM sodium phosphate buffer, pH 7.8 containing 10 mM EDTA (900 µL). The tube was vortexed to dissolve the solids and then allowed to react for 12 h at still and at ambient temperature. After 3 h of reaction, 100 µL of 1 N NaOH was added to the solution and further incubated for 12 h at ambient temperature. The solution (1 mL) was loaded onto a PD-10 Desalting column pre-equilibrated with D$_2$O. The third fraction which contains the maximum amount of the reaction product was analysed by 400 MHz $^1$H-NMR.

### 2.3.36 Reaction of PEG mono-sulfone 15a with GSH 26 at pH 6.2

To an eppendorf tube charged with 50 % PEG mono-sulfone 15a (16 mg, 2.91 µmol) (50 % PEG mono-sulfone 15a and 50 % PEG bis-sulfone 14a mixture) and GSH 26 (2 mg, 6.51 µmol), 50 mM sodium phosphate buffer, pH 6.2 containing 2 mM EDTA (1 mL) was added. The tube was vortexed to dissolve the solids and then allowed to react for 2 h at still and at ambient temperature. After 2 h, the solution was then buffer exchanged to 9:1 H$_2$O:D$_2$O using a PD-10 column. The third 1 mL fraction from the column was analysed by $^1$H-NMR with water suppression.

### 2.3.37 Characterisation of urethane PEG bis-sulfone 14c reaction with GSH 26

To an eppendorf tube charged with urethane PEG bis-sulfone 14c (16 mg, 2.91 µmol) and GSH 26 (5 mg, 16.3 µmol), 50 mM sodium phosphate buffer, pH 7.8 containing 10 mM EDTA (900 µL) was added. The tube was vortexed to dissolve the solids and then allowed to react for 18 h at still and ambient temperature. After 18 h, the solution (1 mL) was loaded onto a PD-10 Desalting column pre-equilibrated with D$_2$O. The third fraction which contains the maximum amount of the reaction product was analysed by 400 MHz $^1$H-NMR.
2.3.38 Purification of mPEG 1 from paranitrophenol (Evaluation of PD-10 column)

To an eppendorf tube containing 1 N sodium hydroxide (1 mL) was added 25 mg of paranitrobenzyl PEG 38 (Section 2.3.26). A bright yellow colour solution formed immediately upon complete dissolution of the solids. This solution was then incubated for 3 h at ambient temperature. After 3 h, the solution was loaded onto a PD-10 column (pre-equilibrated with water) and the eluent obtained (1 mL) was discarded. Fractions of 1 mL (11 × 1 mL) were then added to the top of the column. The 3rd and 4th fractions (of elution) were collected in fresh eppendorf tubes and subjected to freeze drying procedure to obtain mPEG 1 (yield – 84 %).

2.4 RESULTS AND DISCUSSION

The main objective of the work described in this chapter was to prepare DB-PEG 14a and 15a and to characterise them for their reactivity and site-specificity. The amide PEG bis-sulfone 14a was derived from the carboxylic acid bis-sulfone 13a which was prepared by a sequence of Mannich and thiol addition reactions followed by thiol oxidation. The resulting carboxylic acid bis-sulfone 13a was then coupled to methoxy PEG amine 24. While the preparation of the carboxylic acid bis-sulfone 13a has been described (Liberatore et al. 1990; del Rosario et al. 1990), the synthetic procedure was improved while also demonstrating that this molecule can be effectively coupled through its carboxylic acid moiety. Prior work by Wilbur et al had been limited by not being able to effectively utilise the carboxylic acid moiety in 13a (Wilbur et al. 1994).

2.4.1 Preparation of carboxylic acid Mannich salt 22 (Mannich Reaction)

Substituted acetophenones are aryl methyl ketones which have an α-proton. Such molecules can undergo a Mannich reaction to form a new carbon-carbon bond in the form of a Mannich base (Figure 2.23). An amine nucleophile such as piperidine HCl is required to activate formaldehyde through its iminium salt 48 that is formed in situ. In these reactions formaldehyde was added in the solid trimer form, paraformaldehyde. Formaldehyde 44 was then formed in situ by the acid catalysed decompostion of paraformaldehyde. This in turn undergoes reaction with p-acetylbenczoic acid 21 to yield the Mannich base as a hydrochloride 22 (Figure 2.23) (Furniss et al. 1989a). The desired Mannich salt 22 was easily isolated as a solid by the addition of acetone to the reaction solution followed by filtration using a scinted glass funnel. The salt 22 could be used for further reactions without any additional purification.
2.4.2 Preparation of carboxylic acid bis-sulfide 23 (Thiol addition)

The Mannich salt 22 was then used for the preparation of 4-(3-p-tolylsulfanyl-2-p-tolylsulfanylmethyl-propionyl)-benzoic acid 23 (Figure 2.24). Mannich salt 22 undergoes elimination and Michael addition reactions with 4-methylbenzenethiol 52 in the presence of formaldehyde 44 and piperidine 45. Piperidine 45 causes elimination of the Mannich base. This is followed by Michael addition of 4-methylbenzenethiol 52. Piperidine 45 acts both as a base for elimination and to initiate a Mannich reaction in situ. Piperidine 45 also reacts with formaldehyde 44 to form an intermediate species 48 (Figures 2.23 and 2.24). This reactive species 48 is required to form the second arm via a second Mannich reaction. This is followed by a second elimination reaction initiated again by piperidine 45 and a subsequent Michael addition reaction. This sequence of reactions results in the bis-sulfide 23 which was purified by crystallisation using methanol. The compound 23 after drying in vacuum oven was analysed by $^1$H-NMR. Alternatively, the purity of the desired compound 23 could be improved by initial extraction with chloroform and subsequent crystallisation using hot methanol. Crystallisation could also be initiated by addition of one drop of deionised water to the cooled solution followed by overnight storage at 4 °C. Improvements were achieved by combining reactions. In prior literature, a mono-sulfone adduct was frequently isolated and then the second Mannich was done as a separate reaction.
2.4.3 Preparation of carboxylic acid bis-sulfone 13a (Oxidation)

Prior work (Liberatore et al, del Rosario et al) had shown that sulfone groups tend to be a good leaving group in elimination reactions. The oxidation of bis-sulfides to bis-sulfones is therefore an important step in the preparation of DB-PEG 14. The oxidation reaction was conducted in aqueous methanol using excess Oxone®. This reagent is a good substitute for meta-chloroperoxybenzoic acid and H₂O₂. It chemoselectively oxidises sulfides 62 to sulfones 64 (Trost and Curran, 1981; Davis et al. 1988). Oxone® is a triple salt with the chemical formula,
2KHSO₅:KHSO₄:K₂SO₄. Potassium peroxymonosulfate (KHSO₅) 60 is a potassium salt of Caro’s acid (H₂SO₅) and is the active chemical ingredient in Oxone®. Oxidation with Oxone® is a two-step process which usually requires at least 2 eq. moles of KHSO₅ per sulfur atom (Figure 2.25).

![Figure 2.25](image)

**Figure 2.25.** Steps involved in the mechanism of sulfide oxidation to sulfone by Oxone® (Figure adapted and modified from David and Lal, 1988).

For the efficient oxidation of the sulfide 62 to sulfone 64, it is required to stir the Oxone® and carboxylic acid bis-sulfide 23 mixture in 1:1 methanol: water mixture for 16-20 h time period at ambient temperature. Methanol facilitates the solubility of the bis-sulfide 23 and water is required to solubilise the KHSO₅ salt. The oxidation reaction is mild and can be conducted safely at ambient temperature inside a fume hood. The carboxylic acid bis-sulfone 13a formed after the reaction can be easily isolated by chloroform extraction. KHSO₅ and KHSO₄ salts remain in the aqueous phase during this extraction process. Typically, the isolated carboxylic acid bis-sulfone 13a is pure enough (as determined by 'H-NMR analysis) to be coupled directly to methoxy PEG amine 24 to give the desired PEG bis-sulfone 14a.

### 2.4.4 Thionyl chloride mediated coupling reaction

Initially, the use of SOCl₂ was planned for the coupling of mPEG amine 24 to carboxylic acid bis-sulfone 13a. However, it was necessary to ascertain if treatment of carboxylic acid bis-sulfone 13a with SOCl₂ cleanly gave the acid chloride 41 for reaction with mPEG amine 24. A simple method to do this was to prepare a methyl ester of bis-sulfone 40 via the thionyl chloride activation method and compare it with the Fischer esterified bis-sulfone product 40 (Figure 2.26). Carboxylic acids react with alcohol in the presence of concentrated HCl to form esters; the reaction is well-known as Fischer esterification (Furniss et al. 1989b). Initially, the Fischer esterification reaction was conducted on carboxylic acid bis-sulfide 23 to characterise the
product obtained by \(^1\)H-NMR. The Fischer esterification product was purified by precipitation with the addition of deionised water to the methanolic reaction solution. The product 39 indicated the presence of the three hydrogen atoms of the formed methyl ester in \(^1\)H-NMR analyses (Appendix 2). The product was subjected to Oxone\(^R\) oxidation to yield methyl ester bis-sulfone 40 as analysed by \(^1\)H-NMR (Appendix 2). The methyl ester bis-sulfone 40 was then prepared by thionyl chloride activation of the carboxylic group 41 followed by treatment with methanol. The isolated product was analysed by \(^1\)H-NMR to confirm the presence of methyl ester of bis-sulfone 40 (Appendix 2). This exercise showed that it is possible to activate the carboxylic acid present in bis-sulfone 13a completely with SOCl\(_2\), for reactions with nucleophilic groups.

\[
\text{HOOO} \quad \text{Fischer esterification} \quad \text{H}_{2}\text{COOO} \\
\begin{align*}
\text{23} \\
&\quad \text{Oxone} \ 1:1 \ \text{M ethanol:Water} \\
\text{39} \\
&\quad \text{Oxone} \ 1:1 \ \text{M ethanol:Water}
\end{align*}
\]

\[
\begin{align*}
\text{HOOO} \\
\text{SO}_2 \\
\text{SO}_2 \\
\text{ClOOC} \\
\text{SO}_2 \\
\text{SO}_2
\end{align*}
\]

\[
\begin{align*}
\text{13a} \\
&\quad \text{SOCl}_2 \ 50^\circ \text{C, 2 h} \\
\text{41} \\
&\quad \text{Methanol}
\end{align*}
\]

\[
\begin{align*}
\text{HOOO} \\
\text{H}_{2}\text{COOO}
\end{align*}
\]

\[
\begin{align*}
\text{Figure 2.26. Two different routes for the preparation of the methyl ester bis-sulfone 40}
\end{align*}
\]

2.4.5 Preparation of PEG bis-sulfone 14a

PEG bis-sulfone 14a was prepared by coupling the carboxylic acid bis-sulfone 13a to a molecule of methoxy PEG amine 24. For the carboxylic acid activation, it was necessary to maintain anhydrous conditions to help ensure that the coupling reaction could go to completion. For this, the mPEG amine 24 was dissolved in anhydrous toluene and the solvent then evaporated using high vacuum. The idea was to remove the trace amount of water (usually present along with PEG) by azeotropic distillation. Near complete dryness was then ensured by allowing the polymer to remain in high vacuum for a minimum time period of 30 min.

Thionyl chloride reacts with the carboxyl group to give the acid chloride bis-sulfone 41, HCl and \(\text{SO}_2\). The excess \(\text{SOCl}_2\) was then removed by high vacuum. Complete removal of \(\text{SOCl}_2\) was ensured by DCM dissolution of the reaction residue followed by solvent evaporation in vacuum. This process was repeated 2-3 times. This typically gave the acid chloride bis-sulfone
for reaction with methoxy PEG amine 24. The compound 41 upon reaction with mPEG amine 24 liberates HCl as a by-product. If HCl remains in the solution, it would ionise the amine group of methoxy PEG amine 24. This would retard the further coupling reactions of carboxylic acid bis-sulfone 13a with methoxy PEG amine 24. To avoid this, the HCl formed was neutralised using 1 equivalent of a tertiary amine base such as triethylamine.

Typically, 3 molar excess of the carboxylic acid bis-sulfone 13a was used for the SOCl₂ activation and subsequent reaction with mPEG amine 24. It was thought to be easier to remove impurities derived from 13a rather than PEG, during the purification to isolate the desired PEG bis-sulfone 14a. The coupling reaction typically required overnight stirring of the solution under argon at ambient temperature. After the reaction period, the solution was added drop-wise to ice chill and stirred diethyl ether. In ice chilled diethyl ether, only the PEG bis-sulfone 14a precipitates out leaving the excess unreacted bis-sulfone 41 in solution. This suspension was then filtered to obtain the pure bis-sulfone activated PEG 14a. Later, it was found that ice chilled acetone was efficient for the precipitation/purification procedure. Also, it was observed that precipitation of PEG in chilled diethyl ether or acetone was found to be better with higher molecular weight PEGs. Another observation was that better precipitation of PEG and therefore the yield was achieved when the acetone solution of PEG was placed on a dry ice bath for 3-5 min.

One disadvantage observed while using triethylamine as a proton scavenger was that it extracted the lone acidic α-hydrogen present in the carboxylic acid bis-sulfone 13a moiety. It is possible that the, β alkenyl group formed upon elimination of p-tolyl sulfinic acid, could react with the methoxy PEG amine 24 available in the solution. Another disadvantage is that the SOCl₂ method of carboxylic acid activation is a tedious process. This is because of need to remove excess SOCl₂.

DCC method of activation was later adopted for coupling mPEG amine 24 to carboxylic acid bis-sulfone 13a. This was because, in contrast to the SOCl₂ activation method, the carbodiimide method of activation was observed to be much easier. DCC activates the carboxylic acid bis-sulfone 13a in situ at a stoichiometrically equivalent amount for reaction with methoxy PEG amine 24. The by-product formed during this reaction was an inert compound, dicyclohexylurea (DCU) 72 (Figure 2.27). An important pre-requisite for an efficient DCC coupling reaction was the maintenance of anhydrous conditions. For this the mPEG amine 24 was dried by toluene distillation as described earlier. The dried mPEG amine 24 was typically allowed to react with 3 fold molar excess of carboxylic acid bis-sulfone 13a in the presence of DCC (1 equivalent to bis-sulfone). Typically, insoluble precipitates of the formed DCU could be observed in the
reaction solvent within 10 min of the reaction. However, to ensure complete reaction, the mixture was kept stirring for 16 h at ambient temperature. After the reaction period, the solution was filtered to remove the DCU formed. This was followed by rotary evaporation of the solvent and acetone (chilled) precipitation of the PEG compound 14a (determined by $^1$H-NMR analysis).

The DCC activation method resulted in the formation of insoluble DCU 72 as a side product. Although, it was convenient to remove the majority of the DCU formed by simple filtration, small amount of impurity was always found in the $^1$H-NMR spectrum of the purified PEG compound. DCU is an un-reactive molecule. Therefore, further purification was deemed not necessary for subsequent use of the prepared compound for PEGylation. However, for analytical purposes like $^1$H-NMR or MALDI-TOF, purification of the PEG compound 14a was achieved using a PD-10 Desalting column. For this, the PEG bis-sulfone 14a was dissolved in acidified deionised water (slightly acidified using HCl, pH 4.0) and loaded onto the PD-10 Desalting column pre-equilibrated with the acidified deionised water (25 mL). The pure PEG bis-sulfone 14a fractions were collected and freeze dried to obtain the pure compound 14a for analysis or even PEGylation purposes. Acidification of the deionised water was required to prevent the characteristic elimination reaction of PEG bis-sulfone 14a.

Figure 2.27. Mechanism of N,N'-dicyclohexylcarbodiimide activation of carboxylic bis-sulfone ($R^1$) carboxylic acid for the coupling of mPEG amine ($R^2$).
The DCC activation method typically yielded a product with good coupling efficiency (mPEG to \textit{bis}-sulfone). Since $^1$H-NMR could be used as a quantitative analytical method, the coupling efficiency was typically analysed by integration of the methyl peak (of \textit{bis}-sulfone) and methoxy peak (of mPEG) in $^1$H-NMR spectrum (Figure 2.28). Correlation of the integration values of PEG methoxy peak to methyl groups of the sulfonyl toluene signals gave the approximate efficiency of coupling reaction.

Colleagues in the lab also achieved the coupling of mPEG amine 24 to carboxylic acid \textit{bis}-sulfone 13a via isolation of NHS ester of \textit{bis}-sulfone and subsequent reaction with the methoxy PEG amine 24. This method was useful in the sense that the by-product formed after the coupling reaction of NHS ester of \textit{bis}-sulfone to methoxy PEG amine is soluble in acetone. This method therefore gave PEG \textit{bis}-sulfone 14a devoid of any impurity. However, for this thesis work, the DCC activation method of coupling was employed throughout for the preparation of different mw PEG \textit{bis}-sulfone 14a.

PEG \textit{bis}-sulfone 14a could also be prepared through an alternative route by first coupling PEG amine 24 to carboxylic acid \textit{bis}-sulfide 23 by SOCl$_2$ activation or DCC activation followed by Oxone® oxidation of PEG \textit{bis}-sulfide 42. This would avoid the problem of elimination occurring while using triethylamine in the SOCl$_2$ method of coupling reaction. This is because the sulfide groups are not as labile as the sulfone groups in undergoing elimination. Furthermore, PEG was observed to be stable to Oxone® oxidation and subsequent extraction processes. But, this route of synthesis did not yield any advantage for the preparation of amide PEG \textit{bis}-sulfone 14a via the DCC method. However, this route of synthesis was found to be useful in the preparation urethane PEG \textit{bis}-sulfone 14c.
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Figure 2.28. $^1$H-NMR shifts of PEG bis-sulfone 14a (top) and $^1$H-NMR spectrum of acetone precipitation purified PEG bis-sulfone 14a 5,500 Da in CDCl$_3$ (bottom). Important peaks are highlighted and shown within the spectrum. The diagnostic peaks for assessment of reaction efficiency are the singlet peak for methoxy group of PEG at $\delta$ 3.34 and the singlet bis-sulfone methyl peak at $\delta$ 2.49. Typically, in the $^1$H-NMR spectrum of PEG bis-sulfone 14a, the integrated peak for the methoxy group is calibrated to 3.00 (due to 3 hydrogen atoms of methoxy group). All other integrated peaks then show values relative to the calibrated methoxy group peak. In this case, the integrated bis-sulfone methyl groups peak (ideally 6.00 due to six hydrogen atoms) is observed to be 5.52, which indicates a coupling efficiency of 92% ($5.52/6.00 \times 100$).
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The PD-10 desalting step could also be employed for the initial purification of the PEG bis-sulfone 14a after the reaction with bis-sulfone 13a via DCC activation. The desalting method was found to be a very useful, convenient and highly efficient technique of purification when a small scale PEG reaction (for example, 100 mg PEG) was conducted. The reaction mixture after removal of DCM was dissolved in 5 mL of acidified deionised water, filtered using a Millex® 0.2 μm aqueous filter and then loaded (1 mL, 20 mg/mL) on to a PD-10 pre-equilibrated with acidified deionised water for purification. The elution volume (2.5 mL after the wash volume of 2 mL) was collected. This process was repeated 4 more times (for the remaining 4 mL solution) to collect the PEG fractions which are pooled and freeze dried to obtain the purified PEG bis-sulfone 14a with a yield of > 90%. Separation of a small molecule (< 1,000 Da) from a large molecule is efficient by PD-10 desalting as demonstrated with the separation of mPEG 1 from paranitrophenol (Figure 2.29).

2.4.6 Synthesis of ester, urethane and carbonate PEG bis-sulfone compounds 14

Ester, urethane and carbonate linkage PEG bis-sulfone compounds 14 were prepared from carboxylic acid bis-sulfone 13a, amine bis-sulfide 32 and bis-sulfone hydroxy 13c derivatives respectively. The synthesis of the compound 13c also involved the Mannich reaction, thiol addition, oxidation and coupling reactions similar to the preparation of carboxylic acid bis-sulfone 13a. Preparation of compound 32 involved Mannich reaction and thiol addition reactions. Ester 14b and urethane 14c PEG bis-sulfone are compounds of interest because mPEG 1 may be more economical than its amine derivative 24. Most amine reactive PEG reagents reported in the literature are prepared directly from mPEG 1 (Zalipsky, 1995). Ester PEG bis-sulfone 14b was prepared by reaction of carboxylic acid 13a with mPEG 1 by DCC activation in the presence of catalytic amounts of DMAP (Figure 2.4). The urethane PEG bis-sulfone 14c was prepared by activating the amine bis-sulfide using para-nitrobenzyl chloroformate. The chloroformate activated bis-sulfide 33 was typically used in 5 molar excess in its reaction with the mPEG 5,000 Da 1. Either DMAP or triethylamine was added as a catalyst and proton scavanger. The urethane PEG bis-sulfone 34 was purified by a similar process as was used for the amide PEG bis-sulfone 14a. The urethane PEG bis-sulfide 34 was then oxidised using excess of Oxone® in a 1:1 aqueous methanolic solution. The urethane PEG bis-sulfone 14c was then isolated by chloroform extraction followed by rotary evaporation of the solvent.

Since the carbonate bond can be more hydrolytically labile, it was thought that ultimately it might be important to have a DB-PEG in which PEG could be cleaved post protein conjugation. Carbonate PEG bis-sulfone 14d was prepared by first activating the mPEG 1 with para-
nitrobenzyl chloroformate followed by reaction for 48 h with hydroxy bis-sulfone 13c using catalytic amounts of DMAP. Carbonate PEG bis-sulfone 14d was then isolated by acetone precipitation for characterisation studies.

![Figure 2.29](image)

**Figure 2.29.** Separation of paranitrophenol from mPEG 5,000 Da using a PD-10 Desalting column. The column is loaded with 1 mL of 25 mg/mL PEG: paranitrophenol mixture (1:1) and additions of fresh deionised water (1 mL each) carried out until complete recovery of all compounds was achieved. The third and fourth fractions were found to have the maximum amount of PEG (as determined by the amount of PEG available after freeze drying of the collected fractions; ≈ 14 mg in the third fraction and ≈ 7 mg in the fourth fraction). These two fractions could be used for 1H-NMR analysis of PEG compounds or reactions. This experiment was carried out with prior experience of protein (RNase) desalting using PD-10 column (Appendix 3). Typically, collection of 2.5 mL after the wash volume of 2 mL was observed to yield 96 % recovery of the protein. (Symbols used - < = position of paranitrophenol in the PD-10 column, * = Collected fractions of interest).
2.4.7 Elimination studies of PEG bis-sulfone 14

Since PEGylation would be influenced by the relative amount of the PEG mono-sulfones 15 in solution we needed to evaluate the elimination reaction of the PEG bis-sulfones 14. Also it seemed that it would be more efficient synthetically to use the PEG bis-sulfones 14 rather than the more difficult to prepare PEG mono-sulfones 15. The $^1$H-NMR analysis of the elimination of amide PEG bis-sulfone 14a was carried out in 50 mM sodium phosphate buffer, pH 7.2 prepared using D$_2$O (Figure 2.30).

![Figure 2.30](image)

**Figure 2.30.** Elimination reaction of PEG bis-sulfone 14a: **A)** Mechanism of elimination of toluene sulfinic acid 25 from PEG bis-sulfone 14a to generate PEG mono-sulfone 15a which is required for the first thiol addition reaction to occur. **B)** Elimination reaction was monitored by $^1$H-NMR in 50 mM phosphate buffer in D$_2$O (pH 7.2) with 51 % conversion to PEG mono-sulfone 15a observed after 4 h as calculated by integration of the geminal vinylic protons. Typically, overnight incubation at ambient temperature was required for maximum conversion of the compound.
Figure 2.31. Reverse phase HPLC chromatograms at t = 15 min (broken line) and t = 5 h (solid line) for the elimination of toluene sulfonic acid from PEG bis-sulfone 14a to give PEG mono-sulfone 15a in 50 mM sodium phosphate buffer, pH 7.8 at ambient temperature. The peaks were confirmed by $^1$H-NMR.

The manner in which the PEG bis-sulfone 14a undergoes elimination was monitored by RP-HPLC (Figure 2.31). Factors which affect the elimination rate were studied by this analytical method (Figure 2.32). Also, the peaks found in the chromatograms were collected manually, freeze dried and subjected to $^1$H-NMR analysis to confirm the identity of the compounds. The first peak observed in the chromatogram was confirmed by $^1$H-NMR as p-tolylsulfinic acid 25 (Appendix 2). As observed in the reversed phase (C-18) chromatogram, this compound was found to be hydrophilic and was soluble only in D$_2$O for $^1$H-NMR analysis. The other main peaks seen in the chromatogram at approximately 13 min and 17 min were that of PEG mono-sulfone 15a and PEG bis-sulfone 14a respectively as confirmed by $^1$H-NMR analysis (Appendix 2). Both these compounds were soluble in D$_2$O and CDCl$_3$. As analytical controls, mPEG amine 24 5,000 Da and carboxylic acid bis-sulfone 13a were analysed by the RP-HPLC using the same analytical conditions used for PEG bis-sulfone 14a. Methoxy PEG amine 24 was not detectable due to its non-absorbance even at the UV wavelength of 215 nm. The carboxylic acid bis-sulfone 13a was observed to elute at approximately 19 min. From these observations, it is evident that the hydrophobicity of PEG bis-sulfone 14a and PEG mono-sulfone 15a is mainly due to the presence of sulfonyl toluene group present in the structure. However, the p-tolylsulfinic acid 25 is hydrophilic in nature.
Figure 2.32. Effect of A. pH; B. Concentration and C. Temperature on the elimination reaction of PEG bis-sulfone 14a as monitored by reversed phase-HPLC using a C-18 Phenomenex column (5 μm, 250 x 4.6 mm); Solvent (A): water containing 0.05% TFA, Solvent (B): acetonitrile containing 0.05% CF₃COOH (TFA); gradient used – 30-60% B in 30 min with flow rate of 1 mL/min at ambient temperature and UV detection at 215 nm. Sample injection volumes were adjusted to give 0.0045 mmol/mL concentration of the starting PEG bis-sulfone 14a for temperature and concentration experiments at pH 7.8.
Chapter 2. DB-PEG synthesis and reactivity

The elimination reaction of PEG bis-sulfone 14a is dependent on pH, concentration and temperature (Figure 2.32). Typically, preparation of PEG mono-sulfone 15a was accomplished by overnight incubation of the PEG bis-sulfone 14a at a concentration of 25 mg/mL in 50 mM sodium phosphate buffer, pH 7.8 followed by reversed-phase flash chromatography purification. However, the product usually has low amounts of the starting PEG bis-sulfone 14a (typically, 10 – 20% as calculated by 'H-NMR analysis). When pure PEG mono-sulfone 15a was required for characterisation and reactions, it was isolated by RP-HPLC (Figure 2.33).

It was observed from the RP-HPLC studies that the PEG mono-sulfone 15a could also undergo alkylation reactions with hydroxyl groups in aqueous buffer solution. The α, β alkenyl group of the PEG mono-sulfone 15a undergoes Michael addition with a hydroxyl group to form PEG mono-hydroxy (mono-sulfone) 74 product (Figure 2.35). The peak for this compound eluted with maximum time at 12.16 min in the RP-HPLC chromatogram (Figure 2.34) of the overnight incubation of PEG bis-sulfone 14a in pH 7.0 buffer. The peak eluent was collected and the

Figure 2.33. 'H-NMR spectra (D₂O) of PEG bis-sulfone 14a (top) and reversed-phase HPLC purified PEG mono-sulfone 15a (bottom).
structure of the compound 74 was confirmed by $^1$H-NMR (Appendix 2, compound 74). Further, it is hypothesised that the compound 74 undergoes the second elimination to form product 75 with the $\alpha$, $\beta'$ alkenyl group which can ultimately form a PEG bis-hydroxy compound 76. The chromatogram peak eluting at 9.717 min (Figure 2.34) could be compound 75 or 76. This hypothesis could not be tested since the amount of PEG compound in the peak at 9.717 min was too low for $^1$H-NMR analysis.

The formation of compounds 74 and 75 are disadvantageous for efficient conjugation. Compound 74 (with one hydroxy arm) can undergo second elimination quickly to form the $\alpha$, $\beta'$ alkenyl product which can potentially react with a nucleophile including cysteine thiol in a protein. But this thiol conjugated product might not undergo the 3-carbon bridging reaction with another thiol of the reduced disulfide since it lacks the sulfonyl leaving group on the other arm. The products are therefore not desired during the preparation of PEG mono-sulfone 15a. The bis-hydroxy compound 76 would be completely non-reactive since it would not typically undergo elimination. It was possible to avoid the formation of these hydrolysed products by using the PEG bis-sulfone during PEGylation. Addition of thiols is much faster at pH 7.8 than water.

Figure 2.34. Reverse phase HPLC chromatogram of the PEG bis-sulfone 14a incubation in sodium phosphate buffer, pH 7.0 for 17 h. Elimination of p-tolylsulfinic acid 25 to give PEG mono-sulfone 15a. The peak at 12.162 min was identified by $^1$H-NMR as 74 (Figure 2.35).
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2.4.8 Elimination of urethane 14c and carbonate 14d PEG bis-sulfones

The elimination reactions for the urethane 14c and carbonate 14d PEG bis-sulfones were monitored by their formation of the corresponding PEG mono-sulfones 15c and 15d in phosphate buffer, pH 7.8 by $^1$H-NMR. This was typically accomplished by desalting the PEG elimination product 15c or 15d into D$_2$O using a PD-10 Desalting column (pre-equilibrated with D$_2$O). Efficient desalting of the product can be achieved since the mass exclusion limit for the PD-10 column is 5,000 Da (for proteins). In the case of PEG 5,000 Da, it behaves in aqueous solution like a protein of molecular mass > 20,000 Da (Figure 3.11, SEC-HPLC of 20 kDa PEG bis-sulfone 14a, Chapter 3). This is because the PEG has a large hydrodynamic volume in aqueous solutions due to its water binding property (Roberts et al. 2002). When the solution containing the elimination reaction mixture of PEG bis-sulfone 14 was subjected to PD-10 desalting, compounds such as $p$-tolylsulfinic acid 25, phosphate buffer salts and water (H$_2$O) were separated from the reaction product, PEG mono-sulfone 15.
Urethane PEG bis-sulfone 14c underwent relatively less elimination in 50 mM sodium phosphate buffer, pH 7.8. Slight elimination was found to occur immediately after dissolution in the buffer, but no further elimination occurred even after overnight incubation at ambient temperature. This observation was in contrast to the amide PEG bis-sulfone 14a elimination reaction. Liberatore et al and del Rosario et al have also reported low reactivity with the amine bis-sulfone and sulfonamide bis-sulfone compounds (Liberatore et al. 1990; del Rosario et al. 1990). They have attributed this effect to the poor electrophilic nature of the carbonyl group of acetophenone in their bis-sulfone compounds. However, the elimination reaction of urethane PEG bis-sulfone 14c was found to occur in organic solvents such as DCM in the presence of catalytic amounts of DMAP (during the urethane PEG bis-sulfone 14c preparation step) (Appendix 2). Since this elimination was observed in an organic solvent, the effect of mechanistic electronic interference within the bis-sulfone linker is not well understood.

In contrast, the elimination was observed to be rapid for the carbonate PEG bis-sulfone 14d (Figure 2.37). Elimination up to 40% was observed after 1 h of reaction and 100 % elimination after 16 h of reaction. However, 35 % of carbonate linkage was found to cleave during the reaction period making the compound ideal for reversible PEGylation studies. This is because the carbonate bond typically is relatively hydrolytically labile. This difference in elimination behaviour of the prepared 14c and 14d was not clear. Both the linkage functionalities are electron donating and therefore the active carbonyl group (*) (Figure 2.36) in both the compounds should react similarly. Therefore, urethane PEG mono-sulfone 14c should also have undergone elimination reaction.

**Figure 2.36.** Chemical structures of urethane PEG bis-sulfone 14c and carbonate PEG bis-sulfone 14d. Due to the amine functionality in 14c, elimination did not occur in 50 mM sodium phosphate buffer, pH 7.8. However, in the case of carbonate PEG bis-sulfone 14d, elimination was found to occur rapidly in similar conditions (*) = active carbonyl group.)
Chapter 2. DB-PEG synthesis and reactivity

Figure 2.37. 'H-NMR spectra (D$_2$O) of carbonate PEG bis-sulfone 14d reactions purified and buffer exchanged to D$_2$O using a PD-10 Desalting column; 40 % activation was observed in 1 h and complete activation in 16 h. The integrations (16 h reaction) also show approximately 35 % cleavage of the carbonate bond occurred during the reaction period.

The initial assumption was that the elimination of p-tolylsulfinic acid was prevented due to the 'hardness value' \( h = \frac{(E_{\text{LUMO}} - E_{\text{HOMO}})}{2} \) of the urethane PEG bis-sulfone 14c being higher than the hardness value of carbonate 14d PEG bis-sulfone. This value is often considered as a measure of reactivity (or stability) of a molecule (Zhou and Parr, 1990). The lower the hardness value, the higher will be the reactivity (or lower stability) of the molecule and vice versa.

In collaboration with Dr Mire Zloh, the three bis-sulfones (amide 14a, urethane 14c and carbonate 14d) with truncated PEG chains (two repeat units) were therefore subjected to Monte Carlo conformational search using Macromodel v9.1 and the lowest energy structures were further optimised using density functional theory (DFT) calculations (B3LYP/6-31**) utilising Jaguar v6.5 (Figure 2.38). The partial charges [the highest occupied (HOMO) and lowest unoccupied (LUMO) molecular orbitals] and the HOMO-LUMO gap suggested that there was no significant difference in the induction effects caused by the PEG linking structures (Table 2.1). Therefore, the difference in reactivity could not be correlated to hardness values. Additionally, DFT calculations of the inductive effects caused by the urethane and carbonate
linkages to PEG did not indicate differences in the electron density or charges on the atoms involved in the elimination. In short, a rationale based on differences of partial charges could not explain why the urethane PEG bis-sulfone did not undergo elimination in mild aqueous buffered conditions.

![Urethane 14c and Carbonate 14d](image)

**Figure 2.38.** HOMO (top) and LUMO (bottom) orbitals of 14c (left) and 14d (right).

<table>
<thead>
<tr>
<th>Values (Hartree)</th>
<th>Amide 14a</th>
<th>Urethane 14c</th>
<th>Carbonate 14d</th>
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<td>Energy</td>
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<td>-2,684.51534</td>
<td>-2,664.82065</td>
</tr>
<tr>
<td>HOMO</td>
<td>-0.36558</td>
<td>-0.34885</td>
<td>-0.33700</td>
</tr>
<tr>
<td>LUMO</td>
<td>0.05301</td>
<td>0.06648</td>
<td>0.07022</td>
</tr>
<tr>
<td>Hardness value</td>
<td>0.20929</td>
<td>0.20766</td>
<td>0.20361</td>
</tr>
</tbody>
</table>

**Table 2.1.** DFT calculated values of amide PEG bis-sulfone 14a, urethane PEG bis-sulfone 14c and carbonate PEG bis-sulfone 14d; the hardness value is calculated using the formula \(\frac{E_{\text{LUMO}} - E_{\text{HOMO}}}{2}\).

During the study it was noted that when only 2 ethylene glycol repeat units were included in the preliminary calculations, there were differences in the 3D arrangement of the PEG chain in the urethane PEG bis-sulfone 14c compared to the carbonate PEG bis-sulfone 14d. As a result,
calculations were also conducted with 22 more repeat units to give a 1 kDa PEG moiety and a full Monte Carlo Multiple Minimum (MMMC) conformational search was conducted for the urethane 14c and carbonate bis-sulfones 14d (Figure 2.39). Considering the lowest energy structures, the electronegative oxygen atoms of the carbonate bond in PEG bis-sulfone 14d helped to create an extended PEG structure that exposed the bis-sulfone moiety. In contrast, the urethane PEG linking moiety in bis-sulfone displayed a hydrogen bond donor site that was close to the aromatic ring which attracted PEG oxygen. PEG was conformationally constrained to localise close to the bis-sulfone aromatic rings through a network of favourable hydrogen bonding interactions. These interactions in water may have the potential to inhibit the elimination reaction for urethane PEG bis-sulfone in buffer at pH 7.8. Therefore, the non-reactivity of urethane PEG bis-sulfone 14c might be because it adopts a conformation which prevents the base attack on the bis-sulfone α-hydrogen atom which initiates the elimination. Elimination of urethane PEG bis-sulfone 14c to give the PEG mono-sulfone 15c was observed in CHCl3/water with DMAP as base (1 mL of 20 mg/mL PEG bis-sulfone 14c in CHCl3, 10 eq. DMAP and 2 drops of water). PEG bis-sulfone 14a would not have hydrogen bonding interactions in solvents such as CHCl3 and therefore could undergo elimination. This might also explain why slight elimination was seen in DCM during the preparation of urethane PEG bis-sulfone 14c.

![Urethane PEG bis-sulfone 14c and Carbonate PEG bis-sulfone 14d](image)

**Figure 2.39** Lowest energy structures of urethane PEG bis-sulfone 14c (left) and carbonate PEG bis-sulfone 14d (right). Conformational search was carried out using Macromodel, MMFF force field and implicit representation of solvent (H2O) using Generalised Born Surface Area (GBSA) method. Urethane PEG bis-sulfone 14c has a constricted conformation in water.

### 2.4.9 Characterisation of GS-PEG-SG 28 and cys-PEG-cys 82

The sequential elimination-addition reactions of PEG bis-sulfone 14a with GSH 26 gave an adduct 28 (GS-PEG-SG) of two glutathione molecules connected via a 3-carbon bridge PEG molecule (Figure 2.40). GSH 26 has a primary amine nucleophile apart from the cysteine thiol.
Therefore, it was possible to analyse the reaction specificity of PEG bis-sulfone 14a to thiols by $^1$H-NMR and two dimensional $^1$H-$^1$H TOCSY and $^1$H-$^1$H NOESY analyses. This experiment is basically a sulfhydryl exchange reaction of PEG bis-sulfone 14a with GSH 26 which showed the presence of 3-carbon bridge between the cysteine thiols of two GSH 26 molecules.

Figure 2.40. PEG bis-sulfone 14a reaction with GSH 26 in 50 mM sodium phosphate buffer, pH 7.8 to form PEG(SG)$_2$ 28.

Figure 2.41. Chemical structures of cysteine 80, cystine 81 and cys-PEG-cys 82.

Alternatively, cysteine HCl 80 and cystine 81 and the reaction product cys-PEG-cys 82 (Figure 2.41) could be used for similar characterisation studies. However GSH 26 and GSSG 27 were used mostly since they were readily soluble in water when compared to cysteine. GSH 26 and GSSG 27 showed several distinct peaks during $^1$H-NMR analysis (Figure 2.42) which were helpful in the $^1$H-NMR characterisation of the GS-PEG-SG 28. Furthermore, GSH 26 and GSSG 27 have peptide bonds similar to proteins which are observed in the $^1$H-NMR spectrum (Figure 2.42).
Chapter 2. DB-PEG synthesis and reactivity

Figure 2.42. Chemical structures and peak assignments in the $^1$H-NMR spectra of GSH 26 and GSSG 27 in 9:1 H$_2$O: D$_2$O

The one dimensional and two dimensional $^1$H-NMR spectra of GSH 26 and GSSG 27 were acquired and assigned using standard procedure for proteins (Muller et al. 1986). The hydrogen atoms of amide bonds (solvent exchangeable hydrogen atoms) which are usually not visible in the spectrum acquired in D$_2$O solvent can be observed while using 9:1 H$_2$O: D$_2$O solvent. This is a routine method used to observe the amide hydrogen atoms in proteins. In the case of GSH 26 and GSSG 27, the amide region of the one dimensional $^1$H-NMR spectrum had only two peaks (the doublet for NH of cysteine residue and the triplet for the NH of glycine residue (Figure 2.43). The only difference which can be observed in the GSH 26 and GSSG 27 spectra is the hydrogen atom peak for the $\beta$-methylene groups of cysteine residues. The two beta hydrogen atoms of the GSSG cysteine residues are magnetically non equivalent (2.8 – 3.25 ppm) showing two distinct chemical shifts. Beta hydrogen atoms of the cysteine residue in GSH 26 (the reduced form of the glutathione) shows only a single peak in the spectrum. This is because the $\beta$-methylene hydrogen atoms are magnetically equivalent due to free rotation (in absence of the disulfide bond).
The peak assignment in the NMR spectra for PEGylated glutathione 28 (GS-PEG-SG) (Figure 2.43A) was achieved by observing the spin system patterns in $^1$H-$^1$H TOCSY spectrum (Figure 2.43B) for peptide cysteine (NH doublet at 8.3 ppm) and glycine (NH triplet at 8.05 ppm). The $^1$H-$^1$H-NOESY crosspeak at 8.39 and 4.45 ppm (Figure 2.43C) confirmed the sequential connectivity between the alpha hydrogen of cysteine (*) and NH of glycine. The triplet for the NH of the amide bond of GS-PEG-SG 28 was at 8.7 ppm. This peak signal had a crosspeak in the $^1$H-$^1$H TOCSY spectrum to the methylene hydrogen atoms of PEG at 3.7 ppm. This confirmed that the two GS tripeptides were connected to the PEG. The 1:2 ratio for the integration of amide hydrogen atoms of the PEG compound and the amide hydrogen atoms of the peptides was consistent with that expected for conjugation. The 1:2 ratio is also observed while comparing the aromatic peaks at 7.9 ppm and 8.1 ppm to the peptide methylene peaks at ~2.4 ppm and 2.0 ppm (Figure 2.43B). The keto α-hydrogen atom can be seen at 4.2 ppm. The β-methylene hydrogen atoms of cysteine residues in the GS-PEG-SG 28 were non-equivalent indicating the loss of free rotation observed in GSH 26 (Figure 2.42) and that the sulfur atoms were no longer in the form of free thiols. These β-methylene hydrogen atoms from the two cysteine residues are diastereotropic and without free rotation. Therefore, they are magnetically non-equivalent and display slightly different chemical shifts. This type of chemical shift is also observed in the $^1$H-NMR spectrum of GSSG 27 (Figure 2.42). Also observed along with the two cysteine β-methylene groups are the β-methylene groups of the two arms of the PEG connecting to the two peptides. The integration value for both these β-methylene groups amount to ~8 which further confirms the 3-carbon bridging reaction. The possibility of PEG bis-sulfone 14a conjugation to the NH$_2$ group of the N-terminal γ-glutamic acid can be ruled out since no new peak for the hydrogen atom due to amine alkylation was observed. This way the site-specificity of the PEG bis-sulfone 14a to thiols is also confirmed.
Chapter 2. DB-PEG synthesis and reactivity

Figure 2.43. Thiol selectivity of PEGylation was demonstrated with GSH 26 to give GS-PEG-SG 28. A. Chemical structure of GS-PEG-SG 28; bond connectivity to the cysteine thiols was confirmed by $^1$H-$^1$H TOCSY and $^1$H-$^1$H NOESY (B and C respectively). The important peaks which show connectivity are 2 (peak of amide bond of PEG at 8.7 ppm), 9 (amide bond of cysteine at 8.3 ppm), 8 (amide of glycine at 8.05 ppm), 3 & 4 (aromatic peaks at 8.1 ppm and 7.9 ppm respectively) and 6 & 7 (cysteine $\beta$ methylene peaks at $\sim$3 ppm).

The reaction of PEG bis-sulfone 14a with reduced glutathione on termination after 5 h followed by purification by PD-10 desalting and $^1$H-NMR analysis showed the presence of GS-PEG-SG 28 and PEG-bis-sulfone 14a with no PEG mono-sulfone 15a or PEG mono-glutathione/mono-sulfone 78 (Figure 2.44). This suggested that the second elimination-addition reaction of
Chapter 2. DB-PEG synthesis and reactivity

glutathione–PEG mono-sulfone 78 was thermodynamically driven and was more favoured to the first elimination process of a new PEG bis-sulfone 14a. This is an important observation since it shows that the initial alkylation reaction of PEG bis-sulfone 14a with a protein would lead to immediate elimination of the second sulfonyl group to undergo the second alkylation which completes the 3-carbon bridging reaction.

Figure 2.44. $^1$H-NMR spectra of PEG bis-sulfone 14a reaction with GSH 26 for 5 h followed by desalting to D$_2$O; The only two compounds seen are PEG bis-sulfone 14a and GS-PEG-SG 28. No PEG mono-sulfone 15a was observed which indicated that the second thiol addition reaction was thermodynamically driven. The unreacted GSH was eliminated during the desalting step.

Control reactions of PEG bis-sulfone 14a with GSSG 27 was also carried out to confirm the non-reactivity of PEG bis-sulfone 14a to amine groups present in GSSG 27. For this, the PEG bis-sulfone 14a was initially incubated for 3 h with GSSG 27 in 50 mM sodium phosphate buffer, pH 7.8, then desalted to D$_2$O and analysed by $^1$H-NMR. The spectrum showed the presence of only PEG mono-sulfone 15a which indicated no reaction with amine groups. When the reaction solution after 3 h incubation was supplemented with 50 $\mu$L of 1 N NaOH, conjugation to amine groups present in GSSG 27 was observed as expected in the $^1$H-HMR spectrum (after the desalting procedure) (Appendix 2). However, the reaction to amines was not efficient since the presence of $\alpha$, $\beta'$ alkenyl group was also observed in the spectrum.

PEG mono-sulfone 15a when incubated with GSH 26 for 2 h at pH 6.2 followed by PD-10 desalting and $^1$H-NMR analysis showed the PEG mono-glutathione (mono-sulfone) 78 adduct (Appendix 2). This meant that the second elimination reaction required a basic pH. However, this experiment showed that the initial Michael thiol addition with one molecule of GSH 26 occurs efficiently at pH 6.2. This is because all of the PEG mono-sulfone 15a underwent conjugation to GSH 26. The second elimination might occur slowly at this pH to undergo the subsequent second thiol addition. However, this was not confirmed with GSH 26.
PEG bis-sulfone 14a was also incubated with cysteine HCl in 50 mM sodium phosphate buffer, pH 7.8. The reaction solution after 3 h was supplemented with 100 µL of 1 N NaOH to ensure completed reaction. The solution was then loaded onto a PD-10 column for buffer exchange to D₂O. ¹H-NMR analysis of the reaction product showed 85 % reaction with thiols and remaining with the amine groups based on the integration values of the hydrogen atom peaks. This was because the chemical shifts for thiol reacted aromatic peaks of PEG were different from amine reacted aromatic peaks of PEG. The reaction to amine groups in cysteine occurred mainly because the solution pH raised to 10 due to addition of 100 µL 1 N NaOH. Conjugation of PEG bis-sulfone 14a to amine groups is expected at this pH since the amine groups are nucleophilic enough to undergo reaction.

2.4.10 Urethane PEG bis-sulfone 14c reaction with GSH

Urethane PEG bis-sulfone 14c was incubated overnight with GSH in 50 mM sodium phosphate buffer, pH 7.8 and purified by PD-10 desalting to D₂O. ¹H-NMR analysis showed the presence of 75 % PEG bis-sulfone 14c and 25 % PEG-bis-glutathione based on the integration values of the hydrogen atom peaks. This further supports the observation that once the elimination occurs, the 3-carbon bridging reaction proceeds to completion. The initial elimination is therefore the limiting step in the PEG bis-sulfone 14 reaction with thiols.

2.5 CONCLUSIONS

PEG bis-sulfone compounds with amide 14a, ester 14b, urethane 14c and carbonate 14d linkage were prepared by conventional coupling reactions on their corresponding prepared bis-sulfide or bis-sulfone compounds. These compounds were purified and characterised mainly by ¹H-NMR analysis. The amide PEG bis-sulfone 14a was considered appropriate for further characterisation and PEGylation reactions due to its relatively stable amide linkage. Urethane PEG bis-sulfone 14c was prepared in pursuit of a cost-effective compound for protein PEGylation. Ester 14b and carbonate 14d PEG bis-sulfone compounds were prepared for PEG detachable PEGylation studies.

Mechanistically, the α, β alkenyl group of the PEG mono-sulfone 15 is required to initiate the sequential thiol Michael addition/elimination reactions. PEG bis-sulfone 14a undergoes an initial elimination reaction to form the PEG mono-sulfone 15a compound. The prepared PEG bis-sulfones 14a, 14c and 14d were therefore studied for their elimination reactivity in basic aqueous buffers. The formation of PEG mono-sulfone 15a as determined by ¹H-NMR and RP-HPLC was found to be dependent on the solution pH, temperature and compound concentration.
Interestingly, the linkage between the bis-sulfone 13 and the mPEG 1 or its amine derivative 24 was found to have an effect on the elimination.

GSH 26 was used to characterise the thiol site-specificity and reactivity of the PEG bis-sulfone 14a. Bis-alkylation to GSH 26 thiols was confirmed using 1D 1H-NMR and 2D 1H-1H TOCSY NMR analyses. Based on the results, DB-PEG was found to be specific to the GSH 26 thiols. The formation of 3-carbon disulfide bridge was confirmed with these studies. Another important observation was that the second sequential elimination/thiol Michael addition reactions were thermodynamically driven. The observed efficiency in reactivity and site-specificity is ideal for the PEGylation of proteins with accessible disulfide bonds.
Chapter 3

Assessing the DB-PEGylation process on proteins –

DB-PEGylation of L-asparaginase
3.1 INTRODUCTION

L-asparaginase is a therapeutic enzyme used in the treatment of lymphoblastic leukaemia. Its antitumour effect was discovered by Kidd in 1953 (Vaan et al. 1971). For therapeutic purposes, L-asparaginase is mainly produced in E.coli (Ho et al. 1970). The enzyme has various other sources such as Erwinia chrysanthemi, Erwinia carotovora and Acinetobacter calcoaceticus (Joner, 1976; Kotzia and Labrou, 2005; Kotzia and Labrou, 2006). L-asparaginase, being a non-human protein causes initiation of immunological reactions when administered parenterally (Schein et al. 1969). The main limitation of its therapeutic use is the associated hypersensitivity reactions (Killaender et al. 1976). PEGylation is a well known approach to reduce the immunogenicity of proteins (Abuchowski et al. 1977a; Abuchowski et al. 1977b). Other advantages of PEG conjugation to L-asparaginase include longer systemic half-life and better stability (Soares et al. 2002). PEG-L-asparaginase was first prepared in the 1970s and clinical trials were carried out in the 1980s. Currently, PEG-L-asparaginase is marketed by Enzon as PEGASPARGASE® for clinical use. Conjugation of N-acetylneuraminyl lactose (Marsh et al. 1977), polysialic acid (Fernandes and Gregoriadis, 2001), poly (N-vinylpyrrolidone-co-maleic anhydride) (Qian et al. 1997) to L-asparaginase have also been examined. However, these preparations have not been developed into clinical products.

Various PEGylation studies reported indicate that L-asparaginase was PEGylated non-specifically with mPEG 5,000 Da (Zalipsky, 1995). The number of attachment sites ranged from 30 to 73. PEGylated L-asparaginase displayed reduced enzymatic activity ranging from 7 % to 54 % (Zalipsky, 1995). However, the effect of site-specific attachment of relatively larger PEG molecules on L-asparaginase has not been reported so far. The disulfides present in L-asparaginase are involved with the stability of the protein rather than its biological function (Toddokoro et al. 1975). Therefore, L-asparaginase was an ideal model both to study the reactivity of DB-PEG and also to evaluate the in vitro biological effects of PEG attachment to disulfides of L-asparaginase. The hypothesis is that since the disulfides are distant from the catalytic site of L-asparaginase (Swain et al. 1993), the DB-PEGylated L-asparaginase should retain most of its enzymatic activity while possibly displaying reduced antigenicity due to the attached larger molecular weight mPEGs.

3.1.1 Structural features of E.coli L-asparaginase

E.coli L-asparaginase (L-asparagine amidohydrolase, E.C 3.5.1.1) has a molecular weight range of 133 – 141 kDa and is a tetramer composed of four identical subunits, each with an active catalytic site (Figure 3.1) (Frank, 1970). Each monomer subunit is made up of 326 amino acids
with the molecular weight of approximately 34 kDa. The disulfide bond is located between the amino acid residues 77 and 105 in each monomer subunit. The quaternary tetrameric structure is maintained by non-covalent interactions. The tetrameric form of L-asparaginase is required for its biological activity (Frank, 1970). The specific activity of the enzyme is between 200 and 400 μmol of substrate (L-asparagine 83) per milligram of protein (Wriston and Yellin, 1973). The isoelectric point (pl) is pH 4.9 and the $K_m$ for L-asparagine 83 is $1.25 \times 10^{-5}$ mol/L (Wriston, 1970).

![Figure 3.1. Modeled structure of L-asparaginase tetramer (pdb id 4eca) drawn with Maestro®; substrates (L-aspartic acid 84) are shown as ball and stick model. Monomer subunits in different colours.](image)

**3.1.2 Mechanism of action of L-asparaginase**

L-asparaginase catalyses the hydrolysis of the amino acid L-asparagine 83 to L-aspartic acid 84 and ammonia 85 (Figure 3.2). Acute lymphoblastic leukaemia (ALL) cells have low levels of the enzyme asparagine synthetase and progression of the disease relies on L-asparagine from the surrounding tissues. L-asparaginase depletes the endogenous supply of L-asparagine leading to the death of acute lymphoblastic leukaemia cells (Graham, 2003). Since the healthy cells can produce L-asparagine for metabolism, the depletion caused by L-asparaginase normally does not affect them.
Chapter 3. DB-PEGylation of L-asparaginase

Figure 3.2. L-asparaginase catalysed hydrolysis of L-asparagine \text{83} to L-aspartate \text{84} and ammonia \text{85}.

The rationale for choosing L-asparaginase as a model protein for the DB-PEG conjugation studies are:

1. L-asparaginase has a single and readily accessible disulfide bond in each of its subunits. The disulfide bond exists between the cysteine residues 77 and 105 and is far from the active site (Palm et al. 1996).
2. L-asparaginase retains 85 % of its specific activity after disulfide reduction and alkylation of the thiols derived (Todokoro et al. 1975). This indicates that the disulfides are not crucial for the enzyme’s biological activity.
3. Non-specific amine PEGylated L-asparaginase is already marketed as PEGASPARGASE® (Enzon) for antitumour therapy. This would help in the comparison studies of DB-PEG-L-asparaginase with the marketed product. The commercially available product from Sigma-Aldrich has approximately 40 molecules of PEG (5 kDa) attached to a single protein molecule via secondary amine linkage (Sigma-Aldrich product literature). The clinical product is not a human recombinant protein. Therefore, it is highly immunogenic and many PEG molecules are required to mask its antigenicity. The effect of less number of larger PEG molecules (20 kDa) on the protein could therefore be evaluated.
4. The biological activity of L-asparaginase could be easily determined (assay protocol provided by Sigma-Aldrich).
5. \textit{E.coli} L-asparaginase is affordable and easily available. Therefore, it would provide an ideal opportunity to study and understand the DB-PEGylation process in detail for further studies on expensive therapeutic proteins.

3.2 MATERIALS

1. L-asparaginase from \textit{E.coli} (Sigma-Aldrich, cat. no. A4887)
2. PEG-L-asparaginase, lyophilised powder (Sigma-Aldrich, cat. no. A5336)
3. Dithiothreitol (Sigma-Aldrich, cat. no. 43819, m.w – 154)
4. EDTA (DTNB, Sigma-Aldrich-Fluka, cat. no. 03682)
5. Ellman’s reagent (Sigma-Aldrich, cat. no. D8130)
6. Bovine pancreatic RNAse (Sigma-Aldrich, cat. no. R4875)
7. Sephadex G-25 (PD-10) column (GE Healthcare, cat. no. 17-0851-01)
8. HiTrap Q FF (Amersham Biosciences, cat. no. 17-5053-01)
9. SDS-PAGE system (BDH)
10. Novex Bis-tris 4 – 12 % gels (Invitrogen, cat. no. NP0321BOX)
11. Colloidal blue staining kit (Invitrogen, cat. no. LC6025)
12. 0.1 M Perchloric acid
13. 5 % Barium chloride solution
14. Trizma base (Sigma-Aldrich, cat. no. T1503)
15. L-asparagine (Sigma-Aldrich, cat. no. A0884)
16. 6 M Trichloroacetic acid (Sigma-Aldrich, cat. no. T0699)
17. Nessler’s reagent (Sigma-Aldrich, cat. no. 34,518-8)
18. Ammonium sulfate (Sigma-Aldrich, cat. no. A5312)
19. Nunc 96-well ELISA plate
20. Bovine Serum Albumin (Sigma-Aldrich, cat. no. 05477)
21. Tween 20 (Sigma-Aldrich, cat. no. P7949)
22. Rabbit anti-asparaginase polyclonal antibody (Chemicon Int., USA)
23. Anti-rabbit IgG-horseradish peroxidase conjugate (Sigma-Aldrich, cat. no. )
24. Tetramethylbenzidine (Sigma-Aldrich, cat. no. 860336)
25. MicroBCA assay kit (Pierce, cat. no. 23235)
26. Hydrogen peroxide
27. Glacial acetic acid (BDH)
28. Sulfuric acid, concentrated
29. Eppendorf tubes, 1.5 mL and 2 mL
30. HPLC
31. UV spectrophotometer

3.3 METHODS

The main steps involved in the DB-PEGylation process are 1) disulfide reduction, 2) DB-PEGylation and 3) purification of the reaction mixture. The disulfide bonds in L-asparaginase were reduced with DTT 16. Reduced L-asparaginase was then isolated from DTT 16 with a PD-10 Desalting column. Reduction was ascertained by Ellman’s assay and sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The reduced L-asparaginase was then allowed to react with PEG mono-sulfone 15a. The reaction mixture was analysed initially by SDS-PAGE, then purified by ion exchange chromatography and characterised by SEC-
Chapter 3. DB-PEGylation of L-asparaginase

HPLC and MALDI-TOF MS. Biological characterisation was conducted by enzymatic and ELISA assays.

An important objective of the study was to characterise the prepared PEG mono-sulfone 15a in terms of its reactivity and selectivity to thiols. Characterisation of the reactions was mostly conducted by SDS-PAGE analyses.

3.3.1 Disulfide reduction of L-asparaginase with DTT 16

_E. coli_ L-asparaginase was procured from Sigma-Aldrich as 5 mg in ~1 mL of a 50% aqueous buffered glycerol solution, pH 6.5. Typically, L-asparaginase solution was prepared in an eppendorf tube at a concentration of 0.2 mg/mL (1 mL) using 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8. This was achieved by diluting the volume containing 0.2 mg of L-asparaginase from the 5 mg stock solution, pH 6.5 (typically 50 μL) to 1 mL with 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8. DTT 16 (15.4 mg) was then added as a solid to the solution to achieve a concentration of 100 mM. The solution was then incubated without agitation for 30 min at ambient temperature and in the dark. The solution mixture (1 mL) was then loaded onto a PD-10 Desalting column pre-equilibrated with 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 (25 mL) and the eluent (1 mL) discarded. Fresh buffer (2 mL, wash volume) was then added to the PD-10 Desalting column and the eluent (2 mL) discarded. Another fresh buffer (2 mL) was added to the PD-10 Desalting column and the eluent collected and assayed for protein concentration by UV absorbance (relative to buffer). Disulfide reduction was then evaluated by Ellman’s assay and SDS-PAGE.

3.3.2 Ellman’s assay

Disposable plastic 2 mL cuvettes (3 in number) were added with deionised water (1 mL each). To each of the cuvette was added freshly prepared Ellman’s reagent solution (100 μL of 4 mg/mL prepared in 50 mM sodium phosphate buffer, pH 7.8). To one of the cuvette was added, reduced L-asparaginase solution in 50 mM sodium phosphate buffer, pH 7.8 (200 μL of the 2 mL isolated from the PD-10 column and UV absorbance assay concentration determined). To the other two cuvettes (controls), was added 50 mM sodium phosphate buffer, pH 7.8 (200 μL). The solutions were mixed and incubated for 10 min at ambient temperature. The absorbance of the sample solution was then measured at 412 nm relative to the blank (controls). The value obtained was used for the calculation of number of thiols using the molar extinction coefficient (MEC) of TNB⁻ (14,150 M⁻¹ cm⁻¹). Initially the concentration of thiols in 0.2 mL of the sample
solution was calculated. The value obtained was divided by the concentration of L-asparaginase in the sample solution (Appendix 3) to obtain the number of thiols in the solution.

3.3.3 SDS-PAGE analysis

SDS-PAGE analyses of L-asparaginase reduction and DB-PEGylation were conducted with a Novex® bis-tris 4 - 12 % precast gel (Invitrogen) in an electrophoresis tank (BDH). Samples were prepared with NuPAGE® LDS sample buffer (4 X). The sample volumes loaded were typically 10 µL. The running buffer was NuPAGE® MOPS SDS running buffer (20 X). The voltage applied for electrophoresis was 200 V and the run time was 1 h. The prestained protein molecular weight standards used were SeeBlue® Prestained standard or SeeBlue® Plus2 Prestained standard (Invitrogen).

3.3.4 Conjugation of L-asparaginase with PEG mono-sulfone 15a

To a solution of L-asparaginase (0.2 mg/mL or 0.5 mg/mL, 1 mL) in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8, was added DTT 16 (100 mM, 15.4 mg). The solution was then incubated for 30 min at ambient temperature and in dark. The mixture was then loaded onto a PD-10 Desalting column pre-equilibrated with 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 and the eluent (1 mL) discarded. Fresh buffer (2 mL, wash volume) was then added to the PD-10 Desalting column and the eluent discarded again. More fresh 2.5 mL buffer was added while collecting the eluent for PEGylation. To the reduced protein solution (~ 0.19 mg/mL, 2.5 mL; yield was typically 96% of the starting amount of protein), was added PEG mono-sulfone 15a (1.3 eq. to each reduced disulfide) as a freshly prepared solution (20 mg/mL) in 20 mM sodium acetate buffer, pH 4.0 (approx. 5 µL, 10 µL and 20 µL PEG mono-sulfone 15a solution for 5 kDa, 10 kDa and 20 kDa PEGylation reactions respectively) and allowed to react for 16 h at 4 °C without agitation. Purification was accomplished by ion exchange to remove any PEG species. SEC-HPLC was conducted to characterise (not to isolate) the asparaginase-PEG conjugate.

3.3.5 Ion exchange purification of L-asparaginase conjugates

The L-asparaginase and PEG mono-sulfone 15a reaction solution (0.19 mg/mL, 2.5 mL) was buffer exchanged to 50 mM tris buffer, pH 8.6 using a PD-10 Desalting column (giving ~ 0.13 mg/mL, 3.5 mL, yield ~ 91 % of the starting amount of protein). The solution was then manually injected into a HiTrap Q FF 1 mL column (pre-equilibrated with 10 mL of 50 mM tris buffer, pH 8.6) using a 5 mL disposable syringe at a flow rate of 1 mL/min. The column was
washed manually with 5 mL of fresh 50 mM tris HCl buffer, pH 8.6 taken in a fresh syringe. The pure conjugate was recovered upon injecting the column with 1 M sodium chloride in 50 mM tris buffer (2.0 mL) while collecting the eluent. The collected fraction containing the conjugate was characterised by SEC-HPLC.

3.3.6 Mass spectrometry

Mass spectra were acquired using an Applied Biosystems Voyager System DE PRO MALDI-TOF mass spectrometer using a nitrogen laser. The matrix was a saturated solution of α-cyano-4-hydroxycinnamic acid in a 50:50 mixture of acetonitrile and water containing 0.1% trifluoroacetic acid. Sample and matrix were mixed 1:1 and 1 µL of the solution was spotted onto a 100-well sample plate. All spectra were acquired in positive mode over the range of 2 – 150 kDa under linear conditions (25 kV accelerating voltage, 750 ns extraction delay time).

3.3.7 SEC-HPLC of PEG bis-sulfone 14a and L-asparaginase conjugates

The ion exchange purified conjugates and different molecular weight PEG bis-sulfones 14a were characterised by SEC-HPLC mainly with HiLoad™ Superdex 200 prep grade column. The eluent buffer used for the analyses of PEG bis-sulfones 14a was 20 mM sodium acetate buffer, pH 4.0 containing 150 mM NaCl. The solvent buffer used for the characterisation of L-asparaginase conjugates was 50 mM tris buffer, pH 8.0. The flow rate for both analyses was 1 mL/min and the run time was 120 min. The UV detection wavelengths used for the PEG bis-sulfones 14a and L-asparaginase conjugates were 215 nm and 280 nm respectively. Superose 12 column was also used with a flow rate of 0.25 mg/mL and UV detection at 215 nm.

3.3.8 Molecular modeling studies of L-asparaginase

Initially, L-asparaginase (pdb id – 4eca) with all disulfide bonds reduced was attached with the modelled DB-PEG 10 kDa (Chapter 2, section 2.3.32). The structure was then subjected to stochastic simulation protocol with the same parameters used for the molecular dynamics simulation of PEG. Due to the size of conjugate, the number of atoms was exceeding the software limit. Therefore, only the attached PEG was subjected to simulation. L-asparaginase structure was kept rigid but its environment was considered during simulation.
3.3.9 **MicroBCA assay for determination of protein concentration**

L-asparaginase solution (0.5 mL) (0.1 mg/mL) was prepared in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8. For obtaining the standard curve, concentrations of 0 (blank), 10, 20, 30 and 40 µg/mL were prepared by diluting 0 (blank), 50 µL, 100 µL, 150 µL and 200 µL respectively of 0.1 mg/mL L-asparaginase solution to 500 µL with 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 in a 3 mL glass test tube. Samples were also prepared by diluting 50 µL each of the protein/conjugate solutions to 500 µL with 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8. MicroBCA solution (500 µL) (Solutions A:B:C ratio of 25:24:1) was added to all the test tubes. The tubes were mixed well and then incubated at 60 °C for 30 min. After the incubation period, 300 µL (x 3) of all the solutions were added in to a Nunc 96 well-plate and the plate read at 570 nm. The concentration of the protein in the sample solution was calculated based on the obtained standard curve.

3.3.10 **Biological activity assay for L-asparaginase and its conjugates**

The protein concentration in PEG-L-asparaginase was determined by microBCA protein assay using L-asparaginase as the standard curve. The enzymatic activities of L-asparaginase, DB-PEGylated L-asparaginase and the commercially available PEGylated L-asparaginase (15 µg/mL each) were determined by measuring ammonia formation (i.e., hydrolysis of L-asparagine to L-aspartic acid and ammonia) (Mashburn and Wriston, Jr., 1964) (Sigma-Aldrich assay protocol). The samples were prepared at a concentration of 15 µg/mL and then (100 µL each) were incubated with approximately 20 mM L-asparagine in 50 mM tris-HCl buffer (pH 8.6) for 30 min at 37 °C. The reaction was then stopped by the addition of 1.5 M trichloroacetic acid. Nessler’s reagent was then added to the solution which formed a yellow coloured solution if ammonia had been generated. The absorbances of these solutions were measured at 436 nm. The ammonia produced in the samples was determined using an ammonium sulfate standard curve.

3.3.11 **Enzyme-linked immunosorbent assay (ELISA) of L-asparaginase conjugates**

Nunc 96-well ELISA plate wells were coated with 10 µg/mL each of native L-asparaginase and 20 kDa PEG-L-asparaginase conjugate solution in 0.2 M carbonate coating buffer solution, pH 9.6 by incubating overnight at 4 °C. The coated wells were washed four times with 0.5 % (v/v) Tween 20 in PBS solution (PBS-T). BSA (0.5 % w/v) in PBS (200 µL) was added to the wells and incubated at 37 °C for 1 h. The wells were washed again with PBS-T. Rabbit anti-asparaginase polyclonal antibody (100 µL of 1:1000 dilution) was added and incubated at 37 °C.
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for 1 h. To the wells after washing with PBS-T, was added anti-rabbit IgG-horseradish peroxidase conjugate diluted 1500-fold in PBS-T and incubated at 37 °C for 1 h. After washing the wells with PBS-T, 100 μL of tetramethylbenzidine and 0.03 % (v/v) hydrogen peroxide solution in 0.1 M sodium acetate-acetic acid, pH 6.0 were added and the plate incubated for 5 min at ambient temperature. The reaction was then stopped using 100 μL of 2.5 M sulfuric acid. The absorbance was measured at 405 nm using a 96-well plate reader.

3.4 RESULTS AND DISCUSSION

E.coli L-asparaginase was the first protein model chosen for studying the DB-PEGylation process. Reduction of the L-asparaginase disulfides was required prior to the DB-PEGylation with PEG mono-sulfone 15a. DTT 16 was used as the disulfide reductant for L-asparaginase. Many studies suggest the use of DTT 16 at a concentration of 10 – 100 mM for reduction purposes (Hermanson, 1996). For this study, reduction of L-asparaginase was conducted with 100 mM DTT 16 in 50 mM sodium phosphate buffer, pH 7.8. Removal of DTT 16 from the reduced L-asparaginase was required prior to conjugation reactions. PD-10 Desalting column procured from GE Healthcare was employed for this purpose.

PD-10 Desalting column basically consists of a Sephadex™ G-25 medium (GE Healthcare PD-10 Desalting product literature). It works on the principle of gel filtration (size exclusion) and efficiently separates a high molecular weight compound (> 5,000 Da) from a low molecular weight compound (< 1,000 Da). PD-10 Desalting column is chemically stable to most aqueous buffers (pH range 2 – 13); it is often used for protein desalting (from compounds like NaCl, DTT 16 or buffer salts) and buffer exchange (to exchange the protein to new buffer) with 95 % protein recovery (GE Healthcare PD-10 Desalting product literature). The column is supplied filled with an aqueous solution of antimicrobial agent by the manufacturer. Therefore, equilibration (washing) of the column with required buffer (25 mL) has to be conducted prior to use.

3.4.1 Disulfide reduction of L-asparaginase, Ellman’s assay and SDS-PAGE analysis

Reduction of the disulfides in L-asparaginase typically occurred within 30 min at ambient temperature without agitation. The reduced protein was isolated from the DTT 16 using a PD-10 Desalting column and the protein concentration assayed by UV absorbance at 280 nm. Since disulfides also contribute to the extinction coefficient of a protein (Pace et al. 1995), microBCA was also used to quantify the reduced protein. However, no significant difference in the reduced L-asparaginase concentration was observed with the UV_{280 nm} and MicroBCA assays. This could
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be due to the fact that the disulfides (4 in number) in L-asparaginase contribute only about 0.5% to the protein's absorbance at 280 nm (Pace et al. 1995).

The number of thiols formed (disulfides reduced) was measured by Ellman's assay. Ellman's assay is a reliable method to quantify the amount of thiols present in a solution (ELLMAN, 1959; Hermanson, 1996). Ellman's reagent or DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)) is a water-soluble compound which works on the principle of thiol/disulfide exchange reaction. DTNB reacts with a thiol (R-SH) present in a protein to form a mixed disulfide, thereby releasing the 2-nitro-5-thio benzoic acid (TNB). The thiolate anion (TNB\(^-\)) absorbs strongly at 412 nm. This absorbance is measured and used for the calculation of thiols formed in the solution. Based on the calculation (Appendix 3), the Ellman's assay indicated nearly complete reduction of the disulfides in L-asparaginase (7.06 thiols out of the maximum possible 8 thiols for 4 disulfides).

Disulfide reduction was also evaluated by SDS-PAGE analysis. SDS-PAGE is a widely used analytical and quantitative method for the detection and characterisation of proteins (Bollag et al. 1996a). Proteins that are run on the gel separate based on their molecular weights (Laemmli, 1970). Staining and visualising of the separated proteins is typically conducted with colloidal blue or silver staining methods. During the electrophoresis, SDS present in the SDS-MOPS running buffer disrupts the hydrophobic non-covalent interactions within the protein. L-asparaginase was found to appear at \( \approx 39 \) kDa as monomers (not as a tetramer of \( \approx 140 \) kDa) in the gel. This is due to the dissociation of tetrameric structure induced by SDS. The reduced
protein being more open migrated to a lesser extent in comparison to the native (unreduced) protein (Figure 3.4, lanes 2 and 3). This is because, once the disulfides in a protein are reduced, the protein completely unfolds (in the presence of SDS) and will have a larger apparent structure while running in the gel. Some studies also show that SDS-PAGE analysis can also be used to determine the extent of protein disulfide reduction (Aitken and Learmonth, 2002b).

Native PAGE analysis where no SDS is used does not disrupt or denature the protein (Bollag et al. 1996b). This method of analysis is therefore known as non-denaturing gel electrophoresis. Non-native PAGE with SDS in the buffer is a denaturing method where typically, the sample is incubated at 60 °C for 30 min before analysis. Also, disulfide reduction is conducted with a thiol reductant like 2-ME or DTT. However, during this study, a non-native (denaturing), but non-reducing (no reductant used) SDS-PAGE method was used for all the analyses.

3.4.2 Effect of EDTA on disulfide reformation

For efficient DB-PEGylation to occur, the reduced disulfide must remain open so that the two thiols are free. When the protein solution was incubated in a buffer prepared with no EDTA, the reduced protein was found to slowly re-oxidise over a time period of 4 h (Figure 3.5, left, lanes 3–7). This reoxidising property was not observed when the reaction buffer was supplemented with 10 mM EDTA (Figure 3.5, right, lanes 3–7). The re-oxidation observed in the non-EDTA system could be due to the presence of metals such as Cu²⁺ in the solution. Therefore, a chelating agent like EDTA was necessary to prevent metal-catalysed re-oxidation of the reduced disulfides.
3.4.3 Control incubation of PEG mono-sulfone 15a with native L-asparaginase

The reactions of PEG mono-sulfone 15a with L-asparaginase were conducted even before understanding its specificity to thiols (as studied with GSH 26, chapter 2). Therefore, a series of control experiments was conducted with 5 kDa PEG mono-sulfone 15a by incubating it with the native (unreduced) L-asparaginase at different pH buffers and at ambient temperature for 24 h.

This study was conducted to determine if the PEG mono-sulfone 15a could react to the amine groups, which would be nucleophilic at basic pH. L-asparaginase (0.25 mg/mL) was prepared in different buffers (50 mM sodium acetate buffer, pH 4.8; 50 mM bis-tris buffer, pH 6.0; 50 mM sodium phosphate buffer, pH 7.3 and 50 mM tris buffer, pH 8.6) for reaction with 10 eq. of 5 kDa PEG mono-sulfone 15a. Conjugation of PEG mono-sulfone 15a was assessed by SDS-PAGE. A high excess of the PEG mono-sulfone 15a was employed for this study since L-asparaginase has many nucleophilic residues (136 nucleophilic residues in the structure) (Swain et al. 1993). No conjugation was noted in any of the reactions as analysed by SDS-PAGE, (Figure 3.6, lanes 4 - 7). This suggested that the PEG mono-sulfone 15a is non-reactive to the amine groups in L-asparaginase. The possibility of the amine groups present in L-asparaginase being non-nucleophilic at pH 7.0 – 8.6 can be ruled out since the protein is PEGylated in this pH range in many of the studies reported (Soares et al. 2002; Veronese et al. 1996). However, when the protein (0.2 mg/mL) was disulfide reduced and treated with 5 kDa PEG mono-sulfone 15a, conjugation was observed (Figure 3.6, lane 10). This experiment suggested that the PEG mono-sulfone 15a was reactive only to thiols.
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3.4.4 Conjugation of PEG-MAL 7 and PEG mono-sulfone 15a to L-asparaginase

Comparison reactions were also conducted on the reduced L-asparaginase with 5 kDa PEG-MAL 7 and 5 kDa PEG mono-sulfone 15a (50 equivalents to one reduced disulfide of L-asparaginase). Reaction of reduced L-asparaginase with 5 kDa PEG mono-sulfone 15a showed a band corresponding to the ≈ 51 kDa prestained marker in the SDS-PAGE gel. Reaction with 5 kDa MAL-PEG 7 showed a band corresponding to ≈ 64 kDa prestained marker. This suggested that, monoPEGylation occurred with the 5 kDa PEG mono-sulfone 15a reaction (Figure 3.7, lane 5) and diPEGylation occurred with the 5 kDa PEG-MAL 7 (Figure 3.7, lane 4). The monoPEGylation with 5 kDa PEG mono-sulfone 15a was expected since it is designed to utilise two thiols during conjugation. DiPEGylation with 5 kDa MAL-PEG 7 occurred because the reagent could conjugate to both the thiols separately. The reaction of L-asparaginase with 5 kDa MAL-PEG 7 confirmed that both the thiols of the reduced disulfide were available for reaction. DiPEGylation was not observed even with high equivalents of the PEG mono-sulfone 15a. Therefore, the monoPEGylation observed with 5 kDa PEG mono-sulfone 15a reaction was only due to the 3-carbon bridge formed between the two thiols of the reduced disulfide. 
Also, observed with these studies was that the L-asparaginase monomer once PEGylated had a bigger apparent size in the gel compared to the protein standard of the same molecular mass (Figure 3.7). This property of the conjugate is due to the attached mPEG molecule which is uncharged in nature. Therefore, the conjugate migrates to a lesser extent in the gel compared to a protein of same molecular mass.

**Figure 3.7.** SDS-PAGE analysis of reduced L-asparaginase reactions with 5 kDa PEG-MAL 7 and 5 kDa PEG mono-sulfone 15a: Novex bis-tris 12% gel stained with colloidal blue, lane 1: molecular weight standards, lane 2: native L-asparaginase monomer, lane 3: reduced L-asparaginase monomer, lane 4: 5 kDa MAL-PEG 7 (50 eq.), lane 5: 5 kDa PEG mono-sulfone 15a (50 eq.).

### 3.4.5 Conjugation of PEG mono-sulfone 5/10/20 kDa 15a to L-asparaginase

DB-PEGylation was then accomplished on reduced L-asparaginase using 5 kDa, 10 kDa and 20 kDa PEG mono-sulfones 15a. Efficient reaction was observed with the stoichiometry of PEG mono-sulfone 15a being 1.3 equivalents to each reduced disulfide in L-asparaginase (total of 5.2 equivalents to the protein calculated as a tetramer). Control reactions with the native L-asparaginase were also conducted using these compounds. No conjugation with native L-asparaginase was observed with any of the different molecular weight compounds 15a. This result was consistent with the initial control reaction studies (Section 3.4.3). SDS-PAGE analysis was used mainly for the characterisation of each reaction. The gel was initially stained with colloidal blue for protein staining followed by barium iodide staining which is specific for PEG (Protocols 6.3.1 and 6.3.2 respectively). The PEG conjugate displayed a distinct brown stain due to the PEG's iodine binding property (Kurfurst, 1992).

Conjugation with 1.3 equivalents of the PEG mono-sulfone 15a to disulfide, typically gave near quantitative conversion of mono-PEGylated L-asparaginase monomers (Figure 3.8, lanes 4-6). Trace amount of monomers (approximately 16 % as calculated by densitometry) were found unreduced and hence unPEGylated even after reduction with 100 mM DTT 16 and reaction with high equivalents of PEG mono-sulfone 15a to each reduced disulfide. This suggested that the disulfides of some monomers either refolded during the PEGylation process or were inaccessible for disulfide reduction and DB-PEGylation. Again, no conjugation was observed.
with any of the different PEG mono-sulfones 15a (Figure 3.8, lanes 7 – 9), which suggested that the compound reacts only with thiols and not with amine nucleophiles (Section 3.4.3). Since alkylation of the amine nucleophiles was observed with bis-sulfone compounds 13 by Liberatore et al while cross-linking studies on reduced antibody, no conjugation observed with PEG mono-sulfone 15a even at pH 8.6 was a surprising result.

Figure 3.8. SDS-PAGE analysis of reduced L-asparaginase reaction with 5 kDa, 10 kDa and 20 kDa PEG mono-sulfone 15a; Novex Bis-Tris 12 % gel stained with colloidal blue followed by barium iodide lane 1: SeeBlue standards, lane 2: native L-asparaginase monomer, lane 3: reduced L-asparaginase monomer, lane 4: reaction with PEG mono-sulfone 5 kDa 15a (1.3 eq.), lane 5: reaction with PEG mono-sulfone 10 kDa 15a (1.3 eq.), lane 6: reaction with PEG mono-sulfone 20 kDa 15a (1.3 eq.), Lane7-9: Control incubations of PEG mono-sulfone 5/10/20 kDa 15a (1.3 eq.) respectively with native L-asparaginase.

3.4.6 Purification and characterisation of conjugates

Anion exchange chromatography using HiTrap Q FF column was used as the main step in the purification of the reaction products. Ion exchange chromatography utilises the amphoteric property of the protein (Bollag et al. 1996c). The protein in a solution above or below its isoelectric pH is charged. Therefore, it non-covalently interacts with the oppositely charged column matrix (quaternary ammonium compound in the case of HiTrap Q FF). This helps in removing the excess of any unreacted PEG (which is an uncharged molecule) from the unreacted protein and conjugate (Figure 3.9, lanes 5 and 6). The ion exchange column (HiTrap Q FF) was chosen based on L-asparaginase’s isoelectric point (pI) which is 4.9. For ion exchange chromatography, the reaction solution (50 mM sodium phosphate buffer, pH 7.8) (2.5 mL) was shifted to 50 mM tris HCl buffer, pH 8.0 – 8.6 (3.5 mL) by PD-10 desalting. Tris buffer, pH 8.0 was chosen as the eluent based on the HiTrap Q FF’s recommended buffer for the pH range 8 - 9 (GE Healthcare product literature). The buffer exchanged solution was then
manually loaded onto the 50 mM tris HCl buffer pre-equilibrated ion exchange column. Typically, washing the column with fresh 2 mL of 50 mM tris HCl buffer removed any excess PEG (Figure 3.9, lanes 5 and 6). The unreacted protein and the conjugate eluted upon washing the column with 1 M NaCl in 50 mM tris HCl buffer, pH 8.0. The ion exchange purified fraction obtained was then characterised by SDS-PAGE and SEC-HPLC. Unlike observed in the SDS-PAGE, the protein and the conjugates eluted as tetramers during SEC-HPLC (Figure 3.10). This indicated that the protein remained as a tetramer in solutions.

![Image of SDS-PAGE analysis](image)

**Figure 3.9.** SDS-PAGE analysis of the ion exchange purification of DB-PEGylated L-asparaginase conjugate; Novex bis-tris 4 - 12% gel stained with colloidal blue; lane 1: SeeBlue Standards, lane 2: native L-asparaginase monomer, lane 3: reduced L-asparaginase monomer, lane 4: 20 kDa PEG mono-sulfone 15a (2 eq.), lane 5: reaction of reduced L-asparaginase with 20 kDa PEG mono-sulfone 15a (2 eq.), lane 6: conjugate isolated from the ion exchange column.

It was noted during many SDS-PAGE analyses that the 20 kDa PEG mono-sulfone 15a, when used in high concentrations (2 or more equivalents) to the protein, stained also with colloidal blue (Figure 3.9, lane 4). However, this staining property was not observed when 1.3 eq. of 20 kDa PEG mono-sulfone 15a was used for the reaction (Figure 3.8, lane 9). This is because most of the 20 kDa PEG mono-sulfone 15a was consumed during DB-PEGylation.

It was also noted that the 10 kDa PEG mono-sulfone 15a in the control lane, stained with colloidal blue or barium iodide when used in high concentrations only. PEG staining was not observed with 5 kDa PEG mono-sulfone 15a with either colloidal blue or barium iodide staining. This might be because the lower molecular weight PEGs do not stain well with colloidal blue or barium iodide staining. L-asparaginase concentration used for the reactions were typically low (0.1 mg/mL or 0.2 mg/mL). Also, PEG mono-sulfone 15a stoichiometry employed in the reaction with the reduced protein is low (1.3 eq.). Therefore, the SDS-PAGE analyses do not typically show the stained PEG well in the control or reaction lanes. From these observations, it was understood that only the higher molecular weight PEGs (20 kDa or more) exhibit good staining property with colloidal blue or barium iodide staining.
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Figure 3.10. SEC-HPLC of L-asparaginase and the three-carbon disulfide bridged PEG-L-asparaginase on a Superdex 200 pg column. The protein elutes in a tetrameric form; (a) native L-asparaginase at 70.82 min; (b) 5 kDa DB-PEG-L-asparaginase at 56.50 min; (c) 10 kDa DB-PEG-L-asparaginase at 51.47 min; (d) 20 kDa DB-PEG-L-asparaginase at 48.82 min.

3.4.7 SEC-HPLC studies of PEG bis-sulfones 14a

SEC-HPLC was used to characterise the 5 kDa, 10 kDa and 20 kDa PEG bis-sulfones 14a (Figure 3.11). The chromatograms obtained indicated that the PEGs had a large hydrodynamic size in aqueous solution due to its water binding property. The 20 kDa PEG bis-sulfone 14a eluted even earlier than the 140 kDa L-asparaginase (Figures 3.10, 3.11C and 3.12A & B). Also, a clear separation between the conjugate and the 20 kDa PEG mono-sulfone 15a was observed in SEC-HPLC (Figure 3.12C). This avoided the need of ion exchange chromatographic purification to remove any trace unreacted PEG mono-sulfone 15a. The presence of the protein conjugate was confirmed by analysing the ion exchange purified conjugate (characterised by SDS-PAGE) in the SEC-HPLC (Figure 3.12D). No 20 kDa PEG mono-sulfone 15a was noted in the chromatogram after ion exchange.
Figure 3.11. SEC-HPLC analyses of different molecular weight PEG bis-sulfones 14a. Superdex 200 prep grade column was employed for the analyses with the eluent (20 mM sodium acetate buffer containing 150 mM NaCl) flow rate of 1 mL/min and UV detection at 280 nm. It can be noted that the 20 kDa PEG bis-sulfone 14a has a large apparent hydrodynamic size in solutions and elute even earlier (64.02 min) than a 140 kDa protein, L-asparaginase (70.82 min) (Figure 3.10).
Figure 3.12. SEC-HPLC chromatograms of L-asparaginase and 20 kDa PEG mono-sulfone 15a. Elution with a Superdex 12 analytical column (GE Healthcare) of A) L-asparaginase, B) 20 kDa PEG mono-sulfone 15a, C) L-asparaginase reaction with 10 eq. of 20 kDa PEG mono-sulfone 15a and D) 20 kDa PEG-L-asparaginase conjugate.
3.4.8 MALDI-TOF analysis

MALDI-TOF MS is a routine method employed for the detection of protein mass. Mass of PEGylated protein can also be analysed by this method. Therefore, after purification by ion exchange, the conjugates were buffer exchanged by PD-10 Desalting column to deionised water. The solution was then analysed by MALDI-TOF-MS. Typically 50 µL of each of the native L-asparaginase and the conjugate were used for the analyses. The MALDI-TOF spectra of the native L-asparaginase showed peaks corresponding to the molecular masses of the L-asparaginase monomer and dimer (Figure 3.13A).

![MALDI-TOF spectrum](image)

**Figure 3.13.** MALDI-TOF spectrum of A) L-asparaginase; peaks represent the monomer, dimer, trimer and the tetramer and B) 20 kDa L-asparaginase-PEG.

The peaks of native L-asparaginase showed two different molecular masses, which confirmed the observation of separate L-asparaginase bands in the SDS-PAGE (Figure 3.8, lane 2). The native protein showed a molecular mass range of 127,300 – 137,600 Da. The MALDI-TOF spectrum of the conjugate showed a monomer molecular mass peak of 34,409 Da and 55,977 Da (Figure 3.13B). The 34,409 Da peak represents the unreacted refolded monomer whereas the 55,977 Da peak represents the 20 kDa monoPEG conjugated monomer. Other peaks were not observed which along with the SDS-PAGE results suggested that there was no diPEGylated product.

3.4.9 MicroBCA assay for determination of protein concentration

Bicinchoninic acid (BCA) assay is a well known method of protein quantitation which can be conducted in alkaline conditions (Smith et al. 1985; Walker, 2002). The peptide bonds present in a protein converts Cu^{2+} to Cu^{+} under alkaline conditions, a reaction widely known as Biuret reaction. Cu^{+} forms a complex with the bicinchoninic acid to develop an intense purple colour.
solution. The complex has an absorbance maximum at 562 nm. The standard BCA assay has a protein concentration sensitivity of 0.1 – 1.0 mg/mL and the microBCA assay has a sensitivity of 0.5 – 10 μg/mL. MicroBCA was used throughout this study since the isolated conjugates typically had a concentration range of 10 – 100 μg/mL.

3.4.10 In vitro activity assays

L-asparaginase cleaves L-asparagine 83 to ammonia 85 and L-aspartic acid 84 at pH 8.6. The reaction can be stopped by the addition of TCA to lower the pH to acidic conditions. The ammonia formed during the incubation can be measured with Nessler’s reagent. Nessler’s reagent is a solution of 0.09 M potassium tetraiodomercurate (II) in 2.5 M potassium hydroxide. The reaction forms a compound which has absorbance at 436 nm.

A known concentration (15 μg/mL) of L-asparaginase and partially reduced L-asparaginase were assayed for their enzymatic activity. The native enzyme was found to liberate 242.7 units of ammonia per mg of L-asparaginase. This complied with the specifications of Sigma-Aldrich for that batch of L-asparaginase stock solution. L-asparaginase from the same stock solution after reduction for 2 h with DTT 16 and separation using PD-10 column liberated 238.6 units of ammonia per mg of L-asparaginase. Reduction was assessed by Ellman’s assay (5.2 thiols) and also by SDS-PAGE. This confirmed that the disulfide bonds are not crucial for the activity of L-asparaginase enzyme. L-asparaginase from a different stock was assayed for its enzymatic activity and was found to liberate 233 units of ammonia per mg of protein. DB-PEG 5 kDa, 10 kDa and 20 kDa conjugates prepared from these batches showed enzymatic activity of 230 units/mg of protein (96 % activity) (Figure 3.14). The amount of protein in the conjugate was assayed using microBCA assay. The Sigma-Aldrich PEG-L-asparaginase was found to liberate 70 units/mg of protein, equivalent to 30 % of the native enzyme specific activity. This showed that the presence of large number of polymers on the surface of the protein (commercial PEG-L-asparaginase) interfered with the binding and interaction of the substrate with the active site. In the case of DB-PEGylated L-asparaginase, the PEG is attached distal to the catalytic site. Therefore, DB-PEG-L-asparaginase retained its activity completely (Figure 3.14).
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3.4.11 In vitro immunogenicity assays

Enzyme linked immuno-sorbent assay (ELISA) is a well-known method to identify an antigen protein with the help of a polyclonal/monoclonal antibody. L-asparaginase has many antigenic sites. This is evident from the hypersensitivity reactions observed upon its parenteral administration. Blocking these antigenic sites is one of the objectives of PEGylation. ELISA is a method of *in vitro* analysis of the efficiency in blocking the antigenic sites of the protein (antigen).

In ELISA, initially the protein (antigen) is allowed to bind to the bottom surface of the 96-well plate by overnight incubation at 4 °C. Strong binding between the protein and the well surface occurs which cannot be disrupted with PBS-T. Washing of the wells is conducted with PBS-T to remove any contaminant present. As a control, BSA is also coated to the well surface along with the samples in serial half dilutions. The primary polyclonal antibody (rabbit anti-asparaginase antibody) when added and incubated in the wells, binds to the antigenic sites present on L-asparaginase or the conjugate. Any excess antibody which does not bind is removed easily by washing with PBS-T. A secondary antibody (anti-rabbit IgG-horseradish peroxidase conjugate) when added to the wells, binds to the previously bound primary antibody.
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Again, the excess secondary antibody is washed away with PBS-T. The complex when treated with TMB gives a coloured compound which can be measured at 405 nm.

ELISA revealed high degree of antibody binding to native L-asparaginase and the DB-PEG 20 kDa L-asparaginase (Figure 3.15). In contrast, the sigma PEG conjugated L-asparaginase was found to be free of any antibody binding. This indicated the efficiency of large number of PEG molecules on the protein in evading antibody recognition. This could be due to either the amine based site of PEGylation of the commercially available PEG-L-asparaginase and/or to it having ≈ 40 molecules of 5 kDa PEGs per protein molecule (Sigma-Aldrich literature). The DB-PEG 20 kDa conjugated L-asparaginase having four molecules of PEG (Figure 3.16) does not prevent antibody recognition.

Figure 3.15. Antigenicity of DB-PEG 20 kDa conjugated L-asparaginase (87 ± 7.5) and Sigma-Aldrich L-asparaginase-PEG conjugate (4.6 ± 3.7) as determined by ELISA; values shown as mean ± S.E.M.
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Figure 3.16. Stochastic simulation modeled 10 kDa DB-PEG L-asparaginase (initial structure pdb id 4eca); the substrates (L-aspartic acid) are shown as balls in pink colour. The theoretically modeled structure shows that the presence of PEG 10 kDa on all the disulfides does not impede substrate approach to the substrate binding site. Also, a large protein surface is available for intermolecular interactions. This could lead to immune recognition and initiation of hypersensitivity reactions.

L-asparaginase was found to retain both its enzymatic activity and antigenicity upon DB-PEGylation. These observations strongly suggest that the four 20 kDa PEGs attached to the protein do not interfere with the protein’s substrate binding ability or mask the antibody recognition sites. This inference was further supported with the molecular modeling studies where it was observed that the attached 10 kDa PEGs were distal to the substrate binding site and also a large protein surface with possible antigenic sites was available for antibody recognition (Figure 3.16).

3.5 CONCLUSIONS

Due to the presence of the single biologically non-functional disulfide bond in L-asparaginase monomer, it was an ideal model for the DB-PEGylation studies. From the many conjugation experiments conducted with L-asparaginase, it was evident that DB-PEGylation is specific to the reduced disulfides in a protein. The reduced L-asparaginase and L-asparaginase DB-PEG conjugates that were prepared displayed similar enzymatic activity compared to the native
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protein, irrespective of the size of PEG attached. This indicated that the protein structure and function were not disrupted after the DB-PEGylation process. Also, DB-PEGylation showed that it was possible to attach four PEG molecules to sites on a protein that are distant to its biologically active site without disrupting its biological activity. However, the DB-PEGylated L-asparaginase retained all of its antigenic properties, which therefore did not serve the purpose of PEGylation. In contrast, the commercially available amine PEGylated L-asparaginase was less antigenic, although it displayed much less enzymatic activity. The DB-PEGylated *E. coli* L-asparaginase would not be a clinically useful molecule unless more sites on the protein were PEGylated to hide its antigenic sites. The important fact is that no conjugation was observed with the native unreduced protein even at pH 8.6. This study suggested that DB-PEGylation would be useful to improve the pharmacokinetics of recombinant human proteins, thus potentially reducing the risk of immunogenicity that would be due to frequent dosing.
Chapter 4

DB-PEGylated interferon α-2b
4.1 INTRODUCTION

Interferons (IFNs) are a family of secretory cytokines having immunomodulatory functions in eukaryotic cells (Isaacs and Lindenmann, 1957). IFNs are expressed by cells in response to a viral infection. The secreted IFNs regulate directly or indirectly the immune activities in the body (Brassard et al. 2002). IFNs have been categorised based on their expression cells mainly as Type I which includes IFN α, β & ω and Type II which includes only IFN-γ (Bekisz et al. 2004). Type I IFNs which are expressed by lymphoid cells bind to the IFNAR receptor complex which comprises of IFNAR1 and IFNAR2 subunits (Mogensen et al. 1999). Type II IFN (IFN-γ) which is expressed by non-lymphoid cells binds to IFNGR receptor complex which comprises of IFNGR1 and IFNGR2 subunits (Aguet et al. 1988). The variants of IFN-α (Type I IFN), IFN α-2a and IFN α-2b are widely used in conjunction with ribavirin (an antiviral agent) for the treatment of chronic hepatitis C infection (Luxon et al. 2002). The most popular recombinant α-IFN products available for clinical use are Roferon® (IFN α-2a, Roche Inc) and Intron® (IFN α-2b, Schering-Plough Corp.).

The main limitation of the parenterally administered α-IFNs is that they have a very short systemic half-life and therefore a short pharmacodynamic activity (Luxon et al. 2002). PEGylated α-IFNs have a sustained in vivo activity profile which significantly reduces the number of required administrated doses (Luxon et al. 2002; Caliceti, 2004). Currently available marketed PEGylated α-IFN products are PEGASYS® (Roche Inc) and PEGINtron® (Schering-Plough Corp.). PEGASYS® is IFN α-2a with a conjugated 40 kDa “branched” PEG and PEGINtron® is IFN α-2b attached with a 12 kDa linear PEG. The “branched” 40 kDa PEG used in PEGASYS® is actually two 20 kDa PEGs that have been conjugated to the two amines of lysine. IFN α-2a is then conjugated via the carboxylic acid of the lysine. However, these two products are heterogenous mixtures of monoPEGylated positional isomers with activities that vary at which amino acid the PEG is conjugated (Bailon et al. 2001; Grace et al. 2001; Luxon et al. 2002). For example, the marketed product of PEGylated IFN α-2b (PEGIntron®) is a mixture of 14 PEG positional isomers with their in vitro antiviral activity ranging from 6 % for the Lys^{164}–PEG isomer to 37 % for the His^{34}–PEG isomer relative to the native IFN (Wang et al. 2002). The conjugate mixture as such is a clinically useful product with an average in vitro antiviral activity of 28 % in the particular assay that was used.

The DB-PEGylation approach requires the presence of an accessible disulfide bond which can be reduced while maintaining protein tertiary structure. IFN α-2b is a good example protein to examine the limitations of DB PEGylation because this protein has two accessible disulfides and there has been a considerable amount of information published on PEGylated IFN α-2. The
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goal of this work with IFN α-2b is that DB-PEGylation after reduction of its two disulfide bonds would give a maximum of two PEG positional isomers. This would be a vast improvement for the PEGylation of native IFN α-2b. Since thiol selective conjugation is usually chemically more efficient, it is anticipated that DB-PEGylation of IFN α-2b would also be much more efficient than is possible with other reagents. It is hoped these two advantages of site-specificity and increased conjugation efficiency can be accomplished while maintaining the biological properties of IFN α-2b.

4.1.1 Structural features of IFN α-2b

IFN α-2b is a monomeric protein (Figure 4.1) comprised of 165 amino acids with the molecular mass of 19,269 Da. It is a five helical barrel structure with two accessible disulfide bonds between the residues, Cys1 – Cys98 & Cys29 – Cys138. IFN α-2b differs from IFN α-2a by just one replaced amino acid at position 23 (lysine for α-2a and arginine for α-2b) (Bekisz et al. 2004).

![Figure 4.1 Homology modelled 3-D structure of IFN α-2b showing the position of its two disulfide bonds (initial structure pdb id – IITF; ribbon structure drawn with Maestro®).](image)

4.1.2 Mechanism of IFN antiviral activity

IFN-α is secreted in the body by fibroblasts, T-cells, natural killer (NK) cells, macrophages, monocytes and dendritic cells (Brassard et al. 2002). IFN α-2a and IFN α-2b acts extracellularly by binding to the receptor subunits, IFNAR-1 and IFNAR-2 and act via the janus kinase (JAK) – transcription factors (STAT) pathway (Bekisz et al. 2004). Initially, the binding of IFN to the IFNAR complex induces the phosphorylation of JAK and Tyrosine kinase (TAK) with subsequent release of STAT. The STAT in complex with p48 (a DNA binding protein) forms IFN-stimulated gene factor-3 responsible for binding to IFN-stimulated response
elements (ISRE) and initiating the transcription and immunomodulatory functions. IFN-α regulates the innate immunity (for example, to increase the proliferation and cytolytic activity of natural killer cells) and the adaptive immunity (for example, increased MHC Class I and MHC Class II expression, increased activities of CD8+ T-cell, B-cell and macrophages) in the body (Brassard et al. 2002).

The detailed rationales for choosing IFN as a model protein for the DB-PEGylation studies are:

1. PEGylated IFN is already marketed as PEGINtron® (Schering-Plough) and PEGASYS® (Roche) for the treatment of Hepatitis C infection. However, the product is a mixture of 14 PEG-positional isomers. DB-PEGylation of IFN would drastically reduce the number of PEG positional isomers.

2. IFN has two accessible disulfide bonds that are ideal for DB-PEGylation. The two disulfide bonds exist between the residues Cys1 – Cys98 and Cys29 – Cys138.

3. The sulfurs of the Cys1 – Cys98 disulfide can be alkylated while maintaining majority of the protein’s biological activity (Brassard et al. 2002; Morehead et al. 1984). However both disulfides in the oxidised form are needed to maintain protein structure. Hence maintenance of structure in vivo would be necessary to display activity.

4. The comparative biological activity of PEGylated IFN can be determined by established in vitro assays (Grace et al. 2005b).

5. The two major positional isomers reported for PEGINtron® are the His\textsuperscript{34} isomer (47.8 %) and Cys\textsuperscript{1} isomer (13.2 %) displaying 37 % and 11 % antiviral activities respectively relative to the native IFN α-2b (Wang et al. 2002; Grace et al. 2005b). However, there are 12 other positional isomers in the mixtures with lower activities. His\textsuperscript{34} and Cys\textsuperscript{1} residues are near to the position of disulfide bonds in IFN α-2b. One hypothesis of this study is that the DB-PEGylated IFN α-2b isomers would derive similar structural conformations to the major PEGINtron® isomers and display similar biological activities.

6. Since IFN has two disulfide bonds, it would also provide an opportunity to prepare diPEGylated IFN by DB-PEGylation of both the reduced disulfides. The biological activity profile of diPEGylated IFNs is not discussed in any detail in the literature.

7. It would be possible to evaluate the applicability of DB-PEGylation to prepare other therapeutically relevant α-helical PEGylated proteins.

4.2 MATERIALS

8. Recombinant human IFN α-2b (Shanferon®, Shantha Biotechnics, India)

9. Dithiothreitol (Sigma-Aldrich, cat. no. 43819, m.w – 154)
10. Sodium dihydrogen orthophosphate dihydrate (Na$_2$H$_2$PO$_4$.2H$_2$O, Fisher Scientific UK cat. no. S/3760/53)
11. Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich-Fluka, cat. no. 03682)
12. Reduced L-glutathione (GSH, Sigma-Aldrich, cat. no. G4251)
13. Oxidised L-glutathione (GSSG, Sigma-Aldrich, cat. no. G4376)
14. PD-10$^\text{®}$ column (GE Healthcare, cat. no. 17-0851-01)
15. Corning$^\text{®}$ tubes, 15 mL
16. 1 mL HiTrap SP FF (GE Healthcare, cat no. 17-5054-01)
17. HiLoad 16/60 Superdex 200 prep grade column (GE Healthcare, cat. no. 17-1069-01)
18. Human lung fibroblast cell line A549 cells
19. DMEM media
20. Foetal calf serum (FCS)
21. Encephalomyocarditis virus (EMCV)
22. Formaldehyde
23. Methyl violet 2B
24. Peristaltic pump
25. HPLC system with UV detector
26. SDS-PAGE system (BDH)
27. Novex Bis-tris 12 % gels (Invitrogen, cat. no. NP001 BOX)
28. Colloidal blue staining kit (Invitrogen, cat. no. LC6025)
29. 0.1 M Perchloric acid
30. 5 % Barium chloride solution
31. MicroBCA assay kit (Pierce, cat. no. 23235)
32. Glacial acetic acid (BDH)
33. Sulfuric acid, concentrated
34. Eppendorf tubes, 1.5 mL and 2 mL
35. UV spectrophotometer
36. Schrödinger suite installed Linux® computer system

4.3 METHODS

Recombinant human IFN α-2b (IFN, 8 mg, 1.3 mg/mL, 6.15 mL) was provided by Shantha Biotechnics, India in 20 mM sodium acetate buffer, pH 4.0 as a frozen lot in an 8 mL cryogenic vial. For reaction purposes, the frozen stock was thawed and an aliquot containing 2 mg of IFN was transferred each time to a new 8 mL cryogenic vial and stored at 4 °C. The remaining IFN was frozen and stored at -20 °C for future use.
Similar to the DB-PEGylation of L-asparaginase, the steps involved in the preparation of monoPEGylated IFN were 1) disulfide reduction, 2) DB-PEGylation and 3) conjugate purification. Disulfide reduction of IFN was conducted with 100 mM DTT in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 which was followed by PD-10 desalting of the solution to separate the reduced IFN from DTT. The reduced IFN was then used for DB-PEGylation reactions with PEG mono-sulfone and PEG bis-sulfone. Glutathione refolding solution (GRS) comprising of 1 mM GSH: 1 mM GSSG was used for the re-oxidation of the unreacted reduced disulfide. The main objective of the study was to prepare monoPEG-IFN and evaluate its biological activity. However, since IFN has two disulfides, it was also possible to prepare diPEG-IFN and evaluate its biological activity. Purification of the conjugates was then achieved by ion exchange chromatography and SEC-HPLC. The 3-carbon bridging (bis-alkylation) between the cysteines of the reduced disulfides in IFN with carboxylic acid bis-sulfone was also examined.

Unlike L-asparaginase, the supply of native IFN for the study was limited. Two reaction scales of 0.2 mg/mL IFN and 0.5 mg/mL IFN were typically used during the study. The 0.2 mg/mL IFN reaction scale was used mainly to characterise the PEG mono-sulfone reactions with reduced IFN by SDS-PAGE. A higher reaction scale of 0.5 mg/mL IFN was used when preparation of the monoPEG-IFN conjugate or the diPEG-IFN conjugate was required for biological activity studies. The higher scale reaction typically gave bigger peak intensities during SEC-HPLC analysis.

4.3.1 Disulfide reduction of IFN and protein quantitation

To a solution of IFN (0.2 mg/mL, 1 mL) in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 was added DTT (100 mM, 15.4 mg). The solution formed was incubated at ambient temperature (at still and dark conditions) for 30 min and the DTT then removed with a PD-10 Desalting column. For this, the IFN-DTT solution (1 mL) was loaded onto the PD-10 Desalting column (pre-equilibrated with 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8) while discarding the eluent (1 mL) from the bottom. After this, fresh buffer (2 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 – wash volume) was added and the eluent (2 mL) from bottom discarded again. Fresh buffer (2 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8) was added to the column and the eluent (2 mL) from bottom collected in a 15 mL Corning centrifuge tube carefully letting the droplets trickle through sides of the tube. The collected solution was analysed for its UV absorbance at 280 nm (MEC used – 17,803 M^(-1)cm^(-1)). A small amount of solution (50 µL) was drawn and used for microBCA assay.
To a solution of IFN (0.5 mg/mL, 1 mL) (× 2) in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 was added DTT 16 (100 mM, 15.4 mg). The solution formed was incubated at ambient temperature (at still and dark conditions) for 30 min and the DTT 16 then removed with a PD-10 Desalting column. For this, the IFN-DTT 16 solution (1 mL) was loaded onto the PD-10 column (pre-equilibrated with 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8) and discarding the eluent (1 mL) from the bottom. After this, fresh buffer (2 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 – wash volume) was added and the eluent (2 mL) discarded again. Fresh buffer (2.5 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8) was added to the column and the eluent (2.5 mL) collected in a 15 mL Corning® centrifuge tube carefully letting the droplets trickle through sides of the tube. The collected solution was analysed for its UV absorbance at 280 nm. A small amount of solution (50 µL) was drawn and used for microBCA assay.

The concentrations of reduced IFN obtained from the UV\textsubscript{280 nm} absorbance assay and microBCA assay were also matched with the elution profile of native (unreduced) bovine pancreatic ribonuclease (RNase) on a PD-10 column. RNase concentrations of 0.217 mg/mL and 0.510 mg/mL were prepared for the experiment. Three different PD-10 Desalting columns were equilibrated with 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 (25 mL). RNase solution (1 mL, load volume, either 0.217 mg/mL RNase or 0.510 mg/mL RNase) was added to the PD-10 Desalting columns and the eluent discarded. Fresh buffer (2 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 – wash volume) was then added to the PD-10 Desalting column and the eluent discarded again. Fresh buffer (50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8) (2 × 1 mL for 0.217 mg/mL experiment and 2.5 mL for 0.5 mg/mL experiment) was added to the PD-10 Desalting column while collecting the eluent in a 15 mL Corning® tube. The third and fourth 1 mL fractions were collected separately for the experiment with 0.217 mg/mL RNase and UV\textsubscript{280 nm} assayed (MEC− 9,590 M\textsuperscript{−}cm\textsuperscript{−1}).

4.3.2 PEGylation of IFN with PEG mono-sulfone 15a

To a solution of IFN (0.2 mg/mL, 1 mL) in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 was added DTT 16 (100 mM, 15.4 mg). The solution formed was incubated at ambient temperature (at still and dark conditions) for 30 min and the DTT 16 then removed with a PD-10 Desalting column. For this, the IFN-DTT 16 solution (1 mL) was loaded onto the PD-10 Desalting column discarding the eluent (1 mL) from the bottom. After this, fresh buffer (2 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 – wash volume) was added and the eluent (2 mL) discarded again. Fresh buffer (2 mL) was added to the column and
Chapter 4. DB-PEGylated interferon α-2b

the eluent (2 mL) collected carefully in a 15 mL Corning® centrifuge tube letting the droplets trickle through sides of the tube. To the reduced protein solution (≈ 0.090 mg/mL, 2 mL), was added PEG mono-sulfone 15a (1 equivalent, ≈ 8 µL from a 13 mg/mL of 10 kDa PEG mono-sulfone 15a solution in sodium acetate buffer) and gently swirled to mix. The reaction mixture was kept still at 4 °C for 2 h. After the reaction period, 20 mM sodium acetate buffer, pH 4.0 (0.5 mL) was added to the solution. The solution (2.5 mL) was buffer exchanged to 20 mM sodium acetate buffer, pH 4.0 (3.5 mL) with a PD-10 Desalting column (equilibrated with 20 mM sodium acetate buffer, pH 4.0) and then subjected to ion exchange chromatography purification (section 4.3.6).

4.3.3 PEGylation of IFN with PEG mono-sulfone 15a followed by GRS re-oxidation

To a solution of IFN (0.2 mg/mL, 1 mL) in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 was added DTT 16 (100 mM, 15.4 mg). The solution formed was incubated at ambient temperature (at still and dark conditions) for 30 min and the DTT 16 then removed with a PD-10 Desalting column. For this, the IFN-DTT 16 solution (1 mL) was loaded onto the PD-10 Desalting column discarding the eluent (1 mL) from the bottom. After this, fresh buffer (2 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 – wash volume) was added and the eluent (2 mL) discarded. Fresh buffer (2 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8) was added to the column and the eluent (2 mL) collected in a 15 mL Corning® centrifuge tube carefully letting the droplets trickle through the sides of the tube. To the reduced protein solution (≈ 0.090 mg/mL, 2 mL), was added PEG mono-sulfone 15a (1 equivalent, ≈ 8 µL from a 13 mg/mL of 10 kDa PEG mono-sulfone 15a solution in 20 mM sodium acetate buffer, pH 4.0) and gently swirled to mix. The reaction was kept still at 4 °C for 2 h. GRS (1 mM) (40 µL of 50 mM GSH 26: 50 mM GSSG 27 in 20 mM sodium acetate buffer, pH 4.0) was added to the reaction mixture and allowed to react for a further 16 h time period at 4 °C. After the reaction period, the solution (2 mL) was added with 20 mM sodium acetate buffer, pH 4.0 (0.5 mL). This solution (2.5 mL) was then buffer exchanged to 20 mM sodium acetate buffer, pH 4.0 (3.5 mL) with a PD-10 Desalting column (equilibrated with 20 mM sodium acetate buffer, pH 4.0) and subjected to ion exchange chromatography purification (section 4.3.6).

4.3.4 PEGylation of higher conc. IFN with PEG mono-sulfone 15a followed by GRS re-oxidation

To a solution of IFN (0.5 mg/mL, 1 mL) in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 was added DTT 16 (100 mM, 15.4 mg). The solution was incubated for 30 min

100
at ambient temperature and the DTT 16 then removed with a PD-10 Desalting column. For this, the protein solution (1 mL) was loaded onto the PD-10 column discarding the eluent (1 mL) from the bottom. After this, fresh buffer (2 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 – wash volume) was added and the eluent (2 mL) discarded again. Fresh buffer (2.5 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8) was added to the column and the eluent (2.5 mL) collected carefully in a 15 mL Corning® centrifuge tube letting the droplets trickle through sides of the tube. To the reduced protein solution (≈ 0.2 mg/mL, 2.5 mL), was added PEG mono-sulfone 15a (1 eq., ~ 20 µL from a 13 mg/mL of 10 kDa PEG mono-sulfone 15a solution in sodium acetate buffer, pH 4.0) and gently swirled to mix. The reaction was kept still at 4 °C for 4 h. GRS (1 mM) (50 µL of 50 mM GSH 26: 50 mM GSSG 27 in 20 mM sodium acetate buffer, pH 4.0) was added to the reaction mixture and allowed to react for a further 16 h time period at 4 °C. After the reaction period, the solution (2.5 mL) was buffer exchanged to 20 mM sodium acetate buffer, pH 4.0 (3.5 mL) with a PD-10 Desalting column (equilibrated with 20 mM sodium acetate buffer, pH 4.0) and then subjected to ion exchange purification (section 4.3.6).

4.3.5 PEGylation of higher conc. IFN with PEG bis-sulfone 14a followed by GRS re-oxidation

To a solution of IFN (0.5 mg/mL, 1 mL) (× 2) in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 was added DTT 16 (100 mM, 15.4 mg). The solution was incubated for 30 min at ambient temperature and the DTT 16 then removed with a PD-10 Desalting column. For this, the IFN-DTT 16 solution (1 mL) was loaded onto the PD-10 column discarding the eluent (1 mL) from the bottom. After this, fresh buffer (2 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 – wash volume) was added and the eluent (2 mL) discarded. Fresh buffer (2.5 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8) was added to the column and the eluent (2.5 mL) collected in a Corning® centrifuge tube carefully letting the droplets trickle through the sides of a 15 mL Corning® centrifuge tube. To the reduced protein solution (≈ 0.2 mg/mL, 2.5 mL), was added PEG bis-sulfone 14a (1 equivalent, ~ 20 µL from a 13.4 mg/mL solution of 10 kDa PEG bis-sulfone 14a in 20 mM sodium acetate buffer, pH 4.0) and gently swirled to mix. The reaction was kept still at 4 °C for a period of 8 h and then 1 mM GRS (50 µL of 50 mM GSH 26: 50 mM GSSG 27 in 20 mM sodium acetate buffer, pH 4.0) was added to the reaction mixture and allowed to react for a further 16 h at 4 °C. After the reaction period, the solution (2.5 mL) was buffer exchanged to 20 mM sodium acetate buffer, pH 4.0 (3.5 mL) with a PD-10 Desalting column (equilibrated with 20 mM sodium acetate buffer, pH 4.0) and then subjected to ion exchange purification (section 4.3.6).
4.3.6 SDS-PAGE analysis

SDS-PAGE analysis of IFN reduction and DB-PEGylation were characterised with a Novex bis-tris 12 % precast gel (Invitrogen) in a electrophoresis tank (BDH). Samples were prepared with NuPAGE LDS sample buffer (4 X). The sample volume was typically 10 μL. The running buffer was NuPAGE MOPS SDS running buffer (1 X). The voltage applied for analysis was 200 V and the run time was typically 1 h. The prestained protein molecular weight standards used were SeeBlue Prestained standard.

4.3.7 Ion exchange purification of interferon conjugates

The reaction solution (2.5 mL) after the PEGylation period, was loaded onto a PD-10 Desalting column (pre-equilibrated with 25 mL of 20 mM sodium acetate buffer, pH 4.0) while discarding the eluent (2.5 mL). Fresh buffer (3.5 mL of 20 mM sodium acetate buffer, pH 4.0) was added to the PD-10 Desalting column while collecting the eluent (3.5 mL) in the same Corning® tube that was used for the DB-PEGylation. The solution collected was then drawn into a 5 mL disposable syringe with the help of a needle. The Corning® tube was washed again with fresh buffer (1.5 mL of 20 mM sodium acetate buffer, pH 4.0) and pooled to the 3.5 mL solution previously drawn. The solution (total 5 mL) was then loaded manually into the ion exchange column (HiTrap SP FF, 1 mL column, GE Healthcare) with the help of a syringe at a flow rate of 1 mL/min. The ion exchange column was washed with fresh buffer (2 mL of 20 mM sodium acetate buffer, pH 4.0) at a flow rate of 2 mL/min with the help of a peristaltic pump. A fresh syringe was loaded with fresh buffer (5 mL of 20 mM sodium acetate buffer, pH 4.0 containing 1 M NaCl) and attached to the ion exchange column. The buffer in the syringe was then injected manually at a flow rate of 1 mL/min. The initial eluent (1 mL) was discarded. The next eluent volume (2 mL) (which contains the protein and the conjugation mixture) was then collected in a 2 mL eppendorf tube. The ion exchange purified conjugates solution was then injected to SEC-HPLC (typically, 1.9 mL) for purification of the protein and the conjugation mixture.

4.3.8 SEC-HPLC of interferon conjugates

The ion exchange purified conjugates were characterised by SEC-HPLC using Superdex 200 prep grade column. The eluent buffer used for the characterisation was 20 mM sodium acetate buffer, pH 4.0 containing 150 mM NaCl at the flow rate of 1 mL/min and run time of 120 min. The UV detection wavelength used for the analysis was 280 nm. These conditions were same for all the analyses unless specified otherwise.
4.3.9 Preparation of 3-carbon disulfide double bridged IFN

To a solution of IFN (0.2 mg/mL, 1 mL) in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 was added DTT 16 (100 mM, 15.4 mg). The solution was incubated for 30 min at ambient temperature (at still and dark conditions) and then loaded onto a PD-10 Desalting column pre-equilibrated with 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8. For this, the IFN-DTT 16 solution (1 mL) was loaded onto the PD-10 column discarding the eluent (1 mL) from the bottom. After this, fresh buffer (2 mL of 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 – wash volume) was added and the eluent (2 mL) discarded. Fresh buffer (2 mL) was added to the column and the eluent (2 mL) collected carefully in a 2 mL eppendorf tube letting the droplets trickle through sides of the tube. To the reduced IFN (~ 90 μg/mL, 2 mL) was then added 1 – 8 eq. of the carboxylic acid bis-sulfone 13a as a solution in acetonitrile (0.625 mg/mL). The mixture was gently shaken and incubated for 2 h at 4 °C. The reaction solution was made up to 2.5 mL and then buffer exchanged to 20 mM sodium acetate buffer containing 150 mM NaCl, pH 4.0 using a PD-10 Desalting column to isolate the three-carbon double bridged IFN (3.5 mL, ≈ 50 μg/mL).

4.3.10 MALDI-TOF Mass spectrometry

Mass spectra were acquired using an Applied Biosystems Voyager System DE PRO MALDI-TOF mass spectrometer using a nitrogen laser. The matrix was a saturated solution of α-cyano-4-hydroxycinnamic acid in a 50:50 mixture of acetonitrile and water containing 0.1% trifluoroacetic acid. Sample and matrix were mixed 1:1 and 1 μL was spotted onto a 100-well sample plate. All spectra were acquired in positive mode over the range of 600 – 2,500 Da under reflectron conditions (20 kV accelerating voltage, 350 ns extraction delay time) and 2 – 100 kDa under linear conditions (25 kV accelerating voltage, 750 ns extraction delay time).

4.3.11 Concentration of sample solutions

The protein solutions were concentrated using Vivaspin® 6 mL or Vivaspin® 20 mL spin concentrators. The spin conditions used for Vivaspin® 6 mL concentrator with sample volume of 5 mL were 8,000 rpm (6,010 rcf) at 4 °C for 40 min. The spin conditions used for Vivaspin® 20 mL concentrator with sample volume of 14 mL were 8,000 rpm (6,010 rcf) at 4 °C for 3 h.
4.3.12 Trypsin digestion for MALDI-TOF analysis

IFN, reduced IFN, DB-PEGylated IFN and 3-carbon bridged IFN solutions (1 mL each of 0.2 mg/mL in 10 mM sodium phosphate buffer, pH 8.0) were concentrated to 200 μL using Vivaspin® 6 mL concentrators (MWCO 5,000 Da, Sartorius). Sequencing grade modified trypsin (50 μL of 0.2 mg/mL, Promega) was added to each of the samples followed by incubation at 37 °C for 14 h. To each solution, was added acetonitrile (30 μL) and the incubation continued at 37 °C for another 4 h. Finally, each digest was diluted with 220 μL of acetonitrile and the resulting samples analysed with a Voyager MALDI-TOF-MS. High resolution reflectron mode was used to observe low molecular masses. Low resolution linear mode was used to observe high molecular masses.

4.3.13 Molecular modelling studies (Godwin et al, 2006)

The homology modelled 3-D structure of native IFN α-2b (based on the NMR determined 3-D structures of interferon α-2a (1itr) and the X-ray crystallography determined structures of ovine interferon Tau (1b51) and human interferon-β-1au1) was obtained using the Automated Protein Modelling Server (SWISS-MODEL) (Peitsch, 1996; Peitsch et al. 1996). The resultant modelled IFN α-2b was subjected to further dynamics studies. The first step was to import IFN α-2b into Maestro® and to add hydrogen atoms to all the heavy atoms; this was further subjected to 1,500 steps of conjugate gradient minimisation using the AMBER force field and Macromodel v.8.5. Water was defined as an aqueous generalised Born/surface area (GB/SA) solvent model to consider the solvent effect.

Using Maestro®, two separate IFN α-2bs were built which had one unmodified disulfide bond and the other with a 3-carbon methylene bridge between the sulfur atoms. The first model had the 3-carbon methylene bridge between Cys1 and Cys98 and the second model had the 3-carbon methylene bridge between Cys29 and Cys138. The modified models were then subjected to the modified stochastic dynamic simulation. Initially, 100 steps of conjugate gradient minimisation using the software AMBER force field and GB/SA solvent models were used to remove clashes between added atoms and the rest of the protein. Models were then subjected to equilibration at 300 K for 1 ps followed by stochastic simulation for 200 ps in 1.5 fs steps at 300 K. The SHAKE algorithm was applied to the bonds containing hydrogen. Snapshots of molecular structures were recorded at every 2 ps (to obtain 100 structures) during simulations and their energy minimised. The double-bridged IFN α-2b model was also built with the 3-carbon bridges (4-[2,2-bis-methylene-acetyl] benzoic acid) on both of the native disulfides of IFN (CYS1-CYS98 and CYS29-CYS138). The modified models were then subjected to the modified
stochastic dynamic simulation as described above but with an extended stochastic simulation of 2,000 ps in 1.0 fs steps at 300 K. Snapshots of molecular structures were recorded at every 20 ps during simulations and energy minimised.

PEG with linker subunit was constructed as a linear chain comprising 230 monomers (10 kDa) and the simulation performed using the parameters defined above - except that molecular dynamics was used instead of stochastic dynamics (Chapter 2). Initially, the molecular model of IFN with both disulfide bonds reduced was subjected to the molecular simulation protocol and the final snapshot structure was used to build the IFN models with a PEG (10 kDa) attached at Cys1-Cys98 or Cys29-Cys138 and at both disulfides. These three different models obtained were subjected to the stochastic dynamics protocol after removal of atomic clashes.

All the modelled structures were then compared to native conformations of interferon α-2a by calculating the root mean square displacement (RMSD) values. They were determined using NMRClust 1.2 software and the McLachlan algorithm as implemented in the program ProFit v.1.8.

4.3.14 CD spectroscopy studies of DB-PEGylated IFN

IFN (≈ 0.5 mg/mL) and DB-PEG-IFN (≈ 0.275 mg/mL) in acidified water, pH 2.85 were prepared for CD analysis. Each sample was diluted to an appropriate concentration and the required volume for 1 cm cell path length (Table 4.1).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration (mg/mL)</th>
<th>Dil. conc. (mg/mL)</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN</td>
<td>0.5</td>
<td>0.175</td>
<td>Acidified water, pH 2.85</td>
</tr>
<tr>
<td>PEG-IFN</td>
<td>0.275</td>
<td>0.11</td>
<td>Acidified water, pH 2.85</td>
</tr>
</tbody>
</table>

Table 4.1. IFN samples prepared for circular dichroism spectroscopy studies

The UV & CD spectra of both the solutions were obtained from the Applied Photophysics Ltd Chirascan spectropolarimeter. Cell path lengths of 1 cm and 0.05 cm were employed in the regions 400 – 220 nm and 260 – 190 nm respectively. All spectra were buffer baseline subtracted. This experiment was conducted at the King’s College in Prof. Alex Drake’s lab.
Chapter 4. DB-PEGylated interferon α-2b

4.3.15 In vitro antiviral biological assay for IFN

The human lung fibroblast cell line A549 cells were maintained in DMEM media supplemented with 10 % foetal calf serum (FCS), 50 units/mL penicillin and 50 μg/ml streptomycin (assay media). For the antiviral assay, A549 cells were resuspended at 0.3 × 10⁶ cells in assay media and were aliquoted at 50 μL/well in 96-well microtitre plates. On the following day (after 12 h), 50 μL of protein samples prepared in assay media were added to test wells in serial 2-fold dilutions. Protein samples were tested in quadruplicate wells. Control wells contained only cells (negative control) or cells with the virus (as positive control). The plates were incubated overnight at 37 °C. Media was then removed and the cells were infected with encephalomyocarditis virus (EMCV) for 1 h in DMEM supplemented with 2 % FCS and penicillin/streptomycin at 50 μL/well. Assay media containing the EMCV was then removed and fresh assay media was added to the plates at 100 μL/well. On the following day, the extent of cell death were monitored every 2 h until an even spread of cell death was observed (80 %) across the dilution range. Wells were then washed twice with PBS and 50 μL 4 % formaldehyde/0.1 % methyl violet 2B solution was added per well for 30 min. Plates were washed twice with PBS and dried. Dye was solubilised by agitation in 50 μL/well 10 % SDS and the OD was measured at 570 nm.
4.4 RESULTS AND DISCUSSION

4.4.1 Disulfide reduction of IFN and protein quantitation

IFN α-2b has two disulfide bonds, Cys1 – Cys98 and Cys29 – Cys138 which are accessible for disulfide reduction (Morehead et al. 1984). Similar to L-asparaginase studies, the disulfide reduction of IFN α-2b was conducted with 100 mM DTT in 50 mM sodium phosphate buffer, pH 7.8 containing 10 mM EDTA. During the IFN reduction studies, it was observed that vigorous agitation by vortexing or exposure to heat and sunlight caused precipitation of the protein. This might be because the reduced protein unfolds upon agitation and the hydrophobic residues usually present in the protein core are exposed to water. Precipitation was not observed when the protein-reductant solution was kept still for 30 min in dark and at ambient temperature conditions. The disulfide reduction was assessed during each DB-PEGylation process by SDS-PAGE analysis. For this, the reduced protein sample (30 μL) was collected after the PD-10 desalting from the reductant, frozen instantly using a dry ice bath and stored at -20 °C until PAGE analysis.

![Figure 4.2. SDS-PAGE analysis of IFN and reduced IFN; Novex bis-tris 12% lane 1 - protein molecular weight standards, lane 2 - native (unreduced) IFN, lane 3 - completely reduced IFN.](image)

The completely disulfide reduced protein (Figure 4.2, lane 3) migrated less on the PAGE gel compared to the native protein (Figure 4.2, lane 2). This was due to the more open structure of the reduced protein. Disulfide reduction could typically be achieved without any denaturants such as guanidine HCl or urea.

It was noted during DB-PEGylation studies of L-asparaginase that in the presence of metals like Cu²⁺ in the solution, the reduced protein could re-oxidise rapidly before conjugation reactions could occur as observed by SDS-PAGE analysis. Therefore, Type I deionised water was used for the preparation of 50 mM sodium phosphate buffer, pH 7.8 which was also supplemented with 10 mM EDTA.
4.4.2 Protein concentration after disulfide reduction and PD-10 desalting

One important observation during the DB-PEGylation studies on L-asparaginase was that the disulfide reduced L-asparaginase did not show much difference in UV$_{280}$ nm absorbance from the native L-asparaginase. Therefore, the amount of protein available for DB-PEGylation could be easily determined by UV$_{280}$ nm absorbance analysis. Compared to L-asparaginase, IFN is a much smaller protein with a low extinction coefficient value (MEC - 17,803 M$^{-1}$cm$^{-1}$). Disulfide bonds can contribute to the absorbance of a protein at UV$_{280}$ nm (Pace et al. 1995). An example is demonstrated using GSH 26 and GSSG 27 (Figure 4.3). GSSG 27 has a single disulfide bond which entirely contributes to its absorbance at 280 nm, whereas the GSH 26 showed no absorbance at UV$_{280}$ nm.

![Figure 4.3. SEC-HPLC analyses of GSH 26 and GSSG 27 with UV detection at 280 nm. The disulfide bond present in the GSSG 27 entirely contributes to its UV absorption at 280 nm. Therefore, reduction of disulfide bonds in a protein could slightly affect the protein's absorbance at UV 280 nm.](image)

Knowledge of IFN concentration after disulfide reduction and PD-10 desalting was important to calculate the amount of PEG mono-sulfone 15a required to add 1 equivalent for reaction with reduced IFN. The reduction of two disulfide bonds in IFN was expected to have a small effect on its UV absorbance at 280 nm. Based on Pace et al.'s equation for the calculation of the extinction coefficient of proteins, the disulfide bonds in IFN contribute 1.33 % of its total UV$_{280}$nm absorbance of IFN (Pace et al. 1995). Therefore the difference in the UV$_{280}$ nm absorbance of native and reduced IFNs should only be negligible. However, the concentration of the reduced protein was also assayed by microBCA (using the standard curve obtained with the native protein) to compare with the UV$_{280}$ nm absorbance assay.
Typically for DB-PEGylation studies, 0.2 mg/mL or 0.5 mg/mL (1 mL) concentrations were used for disulfide reduction, PD-10 desalting and DB-PEGylation. For the desalting process of 1 mL of 0.2 mg/mL IFN and DTT 16 solution, typically 90% of the IFN was recovered in the pooled third and fourth 1 mL fractions from the PD-10 column (UV$_{280}$ nm assay). The IFN concentration of 0.2 mg/mL was used for many reactions since the stock supply of native IFN was limited. The third and fourth 1 mL fractions were collected from the PD-10 and used for DB-PEGylation reactions. This is because these fractions typically had the maximum amount of reduced IFN for DB-PEGylation and hence were useful for SDS-PAGE studies with colloidal blue staining method. Unlike silver staining method, the colloidal blue staining method was less sensitive and required a larger quantity of protein in the gel.

With the desalting process of 0.5 mg/mL IFN and DTT 16 solution, it was observed that 95% (UV$_{280}$ nm assay) of reduced IFN was recovered in 2.5 mL (third and fourth 1 mL fractions and fifth 0.5 mL fraction). Further desalting of this 2.5 mL IFN solution gave approximately 90% of the starting amount of reduced IFN in a resulting 3.5 mL final solution. These results were also consistent with the microBCA assay results.

The UV$_{280}$ nm absorbance and the microBCA calculated amount of reduced IFN available after PD-10 desalting was also compared to a typical protein (molecular weight more than 5,000 Da) elution profile on a PD-10 column. For this experiment, bovine pancreatic ribonuclease (RNAse, mw – 13,700 Da) was chosen since it is an inexpensive protein which would represent a protein above the molecular weight of 5,000 Da (PD-10 exclusion limit). Concentrations of approximately 0.5 mg/mL (Figure 4.4A & B) and 0.2 mg/mL (Appendix 3) were used for the separation studies. The results were similar by UV$_{280}$ nm assay and microBCA assay. Based on these results, it was inferred that the UV$_{280}$ nm assay (easiest method to determine protein concentration) of reduced IFN could be used for quantitation after PD-10 desalting.
4.4.3 Control studies

Before conducting reaction studies on reduced IFN with PEG mono-sulfone 15a at pH 7.8, it was necessary to learn if the compound 15a would react or undergo conjugation with the non-reduced protein at pH 7.8. Control studies were routinely conducted by incubating the PEG mono-sulfone 15a with native IFN to determine if there was any conjugation with other nucleophilic sites. Incubation of 5-fold molar excess of the PEG mono-sulfone 15a with the native protein at pH 7.8 at ambient temperature for 24 h did not show any conjugation as observed by SDS-PAGE analysis (Figure 4.5, lanes 3 & 4). This suggested that the PEG mono-sulfone 15a does not undergo reaction with non-reduced IFN. These control studies were consistent with those that had been conducted with GSSG 27 and L-asparaginase.

Figure 4.4. (A) – PD-10 desalting of bovine pancreatic RNase using three different PD-10 desalting columns (n = 3); the yield obtained from the columns were consistent. (B) – An average of 96.10 % protein was recovered after desalting 1 mL of 0.5 mg/mL protein to 2.5 mL solution; an average of 90 % (of starting concentration) was recovered in 3.5 mL after the desalting of the obtained 2.5 mL.

Figure 4.5. SDS-PAGE analysis of IFN incubation with 10 kDa PEG mono-sulfone 15a; Novex bis-tris 12% gel stained with colloidal blue (left) and barium iodide (right); lane 1 – molecular weight standards, lane 2 – native IFN, lane 3 – 10 kDa PEG mono-sulfone 15a, lane 4 – IFN incubated with 5 eq. of 10 kDa PEG mono-sulfone15a for 24 h at ambient temperature.
4.4.4 Initial DB-PEGylation studies using PEG mono-sulfone 15a

Preliminary studies on DB-PEGylation of IFN using PEG mono-sulfone 15a were conducted in collaboration with other colleagues in the lab. These studies had focused on the partial reduction of one of the IFN disulfides. This was normally accomplished in 20 mM sodium phosphate buffer containing 150 mM NaCl and 5 mM EDTA, pH 6.0 (argon purged) using 6 eq. of TCEP HCl in the presence of 2 eq. of selenol (as a catalyst) at ambient temperature and for 6 min. Without prior removal of the excess TCEP HCl, 20 kDa PEG mono-sulfone 15a was added and the solution formed was incubated at 4 °C for 2 h.

Tris (2-carboxyethyl) phosphine HCl (TCEP HCl) 90 is a phosphine derivative capable of rapid reduction of protein disulfides (Burns et al. 1991). TCEP HCl 90 cleaves the disulfide bond to generate free thiols and thereby undergo irreversible oxidation of the phosphine group (Figure 4.6). TCEP HCl 90 is a more stable reductant which can reduce at a wide pH range of 1.5 – 8.5 (Getz et al. 1999). Typically, reduction can be accomplished with stoichiometric equivalents of TCEP HCl 90 to the protein which avoids the need to remove the oxidised reductant 93 prior to conjugation with maleimide reagents (Getz et al. 1999).

![Diagram](image)

**Figure 4.6.** TCEP 90 reduction of disulfides (adapted and modified from Hermanson, 1996)

After cation exchange purification, the monoPEGylated IFN was successfully isolated by SEC-HPLC and identified by SDS-PAGE, western blot (Figures 4.7, lane 8 and 4.8, lanes 6, 7 respectively) and by MALDI-TOF (data not shown). A control study conducted with 2 eq. of the PEG mono-sulfone 15a incubated with native (unreduced) IFN for 2 h at 4 °C showed no conjugation in the SDS-PAGE gel. This suggested no reaction of PEG mono-sulfone 15a to amine groups (Figure 4.7, lane 5).
Figure 4.7. SDS-PAGE analysis of DB-PEGylation of reduced IFN with 20 kDa PEG mono-sulfone 15a at acidic pH 6.0 without removal of TCEP HCl (Courtesy: Dr. Elisa Pedone, Dr. Antony Godwin, Dr. Ji-won Chot); Novex bis-tris 12 % gel stained using colloidal blue (left) followed by barium iodide (right); lane 1 – molecular weight standards, lane 2 – native IFN, lane 3 – IFN partially reduced with selenol (2 eq.) and TCEP (6 eq.) to protein for 6 min at 20 °C, lane 4 – DB-PEGylation with 8 eq. of 20 kDa PEG mono-sulfone 15a after 2 h at 8 °C, lane 5 - control incubation mixture of native (unreduced) IFN with 8 eq. of 20 kDa PEG mono-sulfone 15a after 2 h, lane 6 – 20 kDa PEG mono-sulfone 15a, lane 7 – reaction mixture after cation exchange chromatography, lane 8 – 20 kDa PEG-IFN and lane 9 – completely reduced IFN using 50 mM DTT at 70 °C and for 10 min.

DB-PEGylation occurred quickly (within 2 h) at pH 6.0 in the presence of TCEP and selenol. The 20 kDa monoPEG-IFN conjugate prepared thus showed ≈ 5 % antiviral activity compared to the native IFN (in vitro antiviral assay conducted by infection of A549 cells with EMCV; data not shown). However, the use of 8 eq. of PEG mono-sulfone 15a was inefficient compared to the DTT 16/DB-PEGylation method with L-asparaginase (1.3 eq. of PEG mono-sulfone 15a to 1 reduced disulfide). These preliminary studies however provided a frame of reference for the purification and characterisation of monoPEG-IFN.

Both DTT 16 and TCEP (non-oxidised) are nucleophilic at neutral pH and can react to the PEG mono-sulfone 15a, thus making it non-reactive to the reduced protein. Presence of these reducing agents in the reaction solution (like the in situ TCEP/DB-PEGylation method) would therefore interfere with the PEG mono-sulfone 15a conjugation to reduced IFN. The aim of the work on IFN described in this thesis was to adopt and optimise the DTT 16/DB-PEGylation method for the preparation of monoPEG-IFN and diPEG-IFN conjugates. The hypothesis is that the DTT 16/DB-PEGylation would be more efficient than the in situ TCEP/DB-PEGylation method in terms of reactant stoichiometry and product yields. This is because, in the DTT
16/DB-PEGylation method, conjugation is conducted only after separating the reduced protein from the DTT 16.

Another objective of the study was to determine the effect of PEG chain length on the in vitro antiviral activity of the prepared PEG-IFN conjugates. PEG (compounds 14a and 15a) with molecular weights of ≈ 10 kDa and ≈ 20 kDa were employed throughout this study to prepare the monoPEG-IFN and diPEG-IFN conjugates.

4.4.5 DB-PEGylation with PEG mono-sulfone 15a after reduction with DTT 16

DB-PEGylation of the completely disulfide DTT 16 reduced IFN was then conducted with 10 kDa PEG mono-sulfone 15a initially in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 for 2 h at 4 °C. PEGylation with 1 eq. of 10 kDa PEG mono-sulfone 15a gave monoPEG-IFN, diPEG-IFN and unreacted IFN mixture (of completely reduced, partially reduced and native IFN) as observed by SDS-PAGE (Figure 4.8, lane 6). The amount of monoPEG-IFN was high (~ 57 %) as observed in the SEC-HPLC (Figure 4.9) and the SDS-PAGE (Figure 4.8, lane 6). No conjugation was observed in the control incubation of PEG mono-sulfone 15a with native (unreduced) IFN (Figure 4.8, lane 5).

As observed earlier with DB-PEGylation of L-asparaginase, the 10 kDa PEG mono-sulfone 15a typically did not appear clearly in the controls (Figure 4.8, lanes 4 and 5) even with barium iodide staining. This was probably due to the low concentration of the PEG mono-sulfone 15a employed in the reaction. The 10 kDa PEG mono-sulfone 15a appears clearly in the gel with both colloidal blue staining and barium iodide staining when a higher concentration is used as control (Figure 4.5, lanes 3 & 4).
Figure 4.8. SDS-PAGE analysis of 10 kDa PEG *mono*-sulfone 15a reaction with IFN; 12 % Novex bis-tris gel stained with colloidal blue, lane 1 – protein molecular weight standards, lane 2 – native IFN, lane 3 – reduced IFN, lane 4 – 10 kDa PEG *mono*-sulfone 15a, lane 5 – IFN incubated with 1 eq. of 10 kDa PEG mono-sulfone 15a for 24 h at ambient temperature, lane 6 – reduced IFN reaction with 1 eq. of 10 kDa PEG mono-sulfone 15a. A mixture of completely reduced, partially reduced and native IFN can be seen in lane 6.

After the reaction incubation period, the solution containing the reaction mixture (in pH 7.8 buffer) had to be buffer exchanged to 20 mM sodium acetate buffer, pH 4.0. This was done to aid the ion exchange chromatography procedure. Unlike the DB-PEGylation process of L-asparaginase where anion exchange chromatography was used to purify the conjugates, cation exchange chromatography was used for the DB-PEGylation process of IFN. The pI of IFN is ~ 6.0 (Wetzel et al. 1981). The pH selected for cation exchange chromatography was 4.0 since IFN would be positively charged at this pH. The buffer recommended for cation exchange chromatography with HiTrap sulphopropyl fast flow (SP FF 1 mL column) at pH 4.0 is sodium acetate buffer, pH 4.0 (GE Healthcare HiTrap SP FF product literature). The reaction mixture after cation exchange purification was injected into the SEC-HPLC for the isolation of the PEGylated conjugates and the unreacted protein. Superdex 200 preparative grade column (GE Healthcare) was chosen for all the SEC-HPLC analyses. A preparative scale column was chosen since it would allow a large volume (up to 2 mL) for load injection. In the chromatogram that was obtained, the major peak maxima were at 47.32 min, 59.67 min, 69.80 min and 102.38 min (Figure 4.9).
The peak at \( \approx 100 \) min was for the IFN as known from the SEC-HPLC analysis of native IFN (Appendix 3) prior to the analysis of the conjugation reaction mixture. Based on the SDS-PAGE analysis (Figure 4.8), the other expected peaks were for the monoPEG-IFN and diPEG-IFN. Since the peak with maximum at 69.80 min was large, it was thought to be the PEG-IFN. The peak with its maximum at 59.67 min could therefore be the diPEG-IFN (In SEC, larger molecular masses elute earlier).

The peak with its maximum at 47.32 min was thought to be soluble protein aggregates. This peak eluent was collected manually from the SEC-HPLC for analysis by SDS-PAGE. This peak as observed by SDS-PAGE analysis (silver staining) was soluble aggregates composed of mainly the reduced IFN and the PEG-IFN conjugate (Figure 4.10B, silver staining, lane 3). Aggregates break up to their individual components during SDS-PAGE analysis. Aggregation is

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Figure 4.9. SEC-HPLC of ion exchange purified reaction mixture of reduced IFN and 1 eq. of 10 kDa PEG mono-sulfone 15a; Peaks were collected manually and characterised by SDS-PAGE and MALDI-TOF analyses.
an unfavourable event in protein reactions and the soluble aggregate formed is usually an unwanted product.

**Figure 4.10.** SDS-PAGE analysis of the SEC-HPLC conjugate fractions; A) SEC-HPLC chromatogram showing the conjugate fractions B) Novex bis-tris 12 % gel stained with colloidal blue (left) and silver staining (right); Lane 1 – molecular weight standards, Lane 2 – ion exchange purified mixture, Lane 3 – SEC fraction 47.32 min, Lane 4 – SEC fraction 60 min, Lane 5 – SEC fraction 70 min.

All the other eluting peaks had to be collected manually and concentrated for SDS-PAGE and MALDI-TOF analyses. The eluents (typically 5-8 mL) were concentrated by freeze drying. Freeze drying was typically conducted only for the chemical characterisation of the conjugates. For the biological characterisation (antiviral activity studies), the concentration procedure is not required as the eluent could be used directly for analysis. The freeze dried solid obtained from the peak with its maximum at 59.67 min was re-dissolved in deionised water (1 mL). The solution was then desalted using a PD-10 Desalting column to deionised water (2.5 mL). This procedure was conducted to remove the buffer salts from the conjugate prior to MALDI-TOF analysis. Typically, for MALDI-TOF analysis, the amount of salts in the sample should be as low as possible. This solution was then concentrated using the Vivaspin® 6 mL concentrator with 5,000 MWCO to 50 µL and analysed by SDS-PAGE and MALDI-TOF MS. The MALDI-TOF spectrum showed the compound to have a peak molecular weight of ≈40,000 Da (Figure 4.11) suggesting that the conjugate obtained was the diPEG-IFN (IFN – 19,269 Da + 2 PEGs – 20,000 Da). Due to the PEG present in the conjugate, the spectrum peak showed polydispersity. This eluent concentrate was found to appear at ≈ 64 kDa of the standard molecular weight marker in the SDS-PAGE gel (Figure 4.10B, lane 4).
The solid isolated from the peak with its maximum at 69.80 min was dissolved in deionised water (1 mL). This solution was then buffer exchanged with a PD-10 Desalting column to deionised water (2.5 mL). The diluted solution was then concentrated using Vivaspin® 6 mL concentrator with 5,000 Da MWCO to 150 μL and analysed by SDS-PAGE and MALDI-TOF-MS. The MALDI-TOF spectrum showed the compound to have a peak molecular weight of 30,000 Da (Figure 4.12) suggesting that the conjugate obtained was monoPEG-IFN (IFN: 19,269 Da + PEG -10,000 Da). This eluent concentrate was found to appear at ≈ 39 kDa of the standard molecular weight marker in the SDS-PAGE gel (Figure 4.10B, lane 5). Based on these results, a distinct separation of protein and its 10 kDa PEG conjugates could be achieved with Superdex 200 column.
As observed in both SDS-PAGE (Figure 4.10B, lane 3) and SEC-HPLC (Figures 4.9 and 4.10A), the reaction mixture had a tendency to form soluble aggregates. The aggregate formed is not a desirable product. Aggregation observed could be due to the instability of the reduced protein in the reaction buffer pH 7.8. If reduced protein is still present during purification, aggregation could also occur. No aggregation was observed on SEC-HPLC analysis of the incubated solution of either the native or the reduced protein in 50 mM sodium phosphate buffer, pH 7.8 containing 10 mM EDTA even after incubation for 24 h at 4 °C. This suggested that the aggregation observed was not a consequence of the protein instability in the reaction buffer (pH 7.8). The protein aggregation could be induced either by the acidic pH of the 20 mM sodium acetate buffer or the ionic strength of the ion exchange purified mixture buffer. Since no aggregation was observed for the native protein in either 20 mM sodium acetate buffer, pH 4.0 or 20 mM sodium acetate buffer, pH 4.0 containing 1 M NaCl, the aggregation observed in the conjugation mixture could have been due to the instability of the reduced protein in the acidic pH.

Reduction with 100 mM DTT yields an IFN molecule with both the disulfides reduced. When monoPEGylation of IFN is desired, one of the reduced disulfides remains open after the DB-PEGylation reaction. Also, both the disulfides in the unreacted IFN remain open after the reaction. The disulfide reduced IFN displayed no antiviral activity (Appendix 3) and also could induce aggregation during the purification process. Therefore, re-oxidation of the cysteines to their corresponding disulfides in IFN and monoPEG-IFN was necessary.
4.4.6 Re-oxidation of the reduced IFN

Re-oxidation of any free cysteines of the unreacted reduced IFN and PEG-IFN is an important step required to prevent aggregation in the DB-PEGylation process. It was therefore necessary to conduct a literature search about how reduced proteins can be re-oxidised during refolding. Fahey et al. have shown the effect of a mixture of GSH 26 and GSSG 27 on the re-oxidation of the reduced disulfides and recovery of activity of serine protease domain of urokinase plasminogen activator (Fahey et al. 2000). They found that the optimum ratio of GSH 26: GSSG 27 for efficient refolding was 0.5: 0.5 mM or 1:1 mM.

The formation of a disulfide bond 91 in a protein occurs via an ‘oxido-shuffling’ (thiol/disulfide exchange reaction) process (Figure 4.13) (Bradshaw et al. 1967; Creighton, 1997; Bulaj, 2005). In the presence of a small molecular weight disulfide containing compound 94 such as cystine 81 or GSSG 27, the oxidation of thiols in a high molecular weight reduced protein 92 is initiated. Initially, a mixed disulfide 95 (i.e., reduced protein with low molecular weight compound) forms. This however is an unstable disulfide bond 95. Reduction of this intermolecular disulfide bond 95, which is initiated by a thiol in the high molecular weight protein, results in the formation of a correct and stable intramolecular disulfide bond 91. GSSG 27 along with small molecular weight thiol compounds such as GSH 26, cysteine and mercaptoethanol have been commonly used for protein refolding purposes (Ahmed et al. 1975). The thiol group is required to initiate the redox process and to correct misfolded (wrong disulfide) proteins.

![Figure 4.13. Process of protein disulfide bond formation via thiol disulfide exchange process initiated by small molecular weight (R) disulfide compounds.](image)

Re-oxidation studies was initially attempted with 0.5 mM GSH 26: 0.5 mM GSSG 27 on reduced IFN (separately and not in the conjugation mixture) to assess the applicability of Fahey et al.'s observation to the DB-PEGylation process of IFN. The reduced IFN was incubated with and without agitation at 4 °C or at ambient temperature in 50 mM sodium phosphate buffer, pH 7.8 for 16 h with 0.5 mM GSH 26: 0.5 mM GSSG 27. These solutions were analysed by SDS-PAGE (Figure 4.14, lanes 5-8). Two controls were used for this study: (1) immediately after
disulfide reduction, an aliquot of reduced IFN (30 µL) was drawn and frozen for use as the first control (to assess reduction) (Figure 4.14, lane 3) and (2) another sample of reduced IFN (30 µL) was incubated for 16 h without the GSH 26: GSSG 27 mixture at ambient temperature without agitation. This solution was used as the second control (Figure 4.14, lane 4). The reduced IFN was found to re-oxidise in the presence of 0.5 mM GSH 26: 0.5 mM GSSG 27 whereas the second control solution of reduced IFN (without the GSH 26: GSSG 27) remained in the open form even after incubation for 16 h in 50 mM sodium phosphate buffer, pH 7.8 at ambient temperature. The analysis also confirmed that shaking or the different temperature incubations had no effect on the re-oxidation of the reduced IFN as there was no visible difference in the IFN bands. The re-oxidation study on reduced IFN suggested that the 0.5 mM GSH 26: 0.5 mM GSSG 27 oxidation process could be used for the DB-PEGylation process.

This study showed that the 1:1 ratio of GSH 26: GSSG 27 was efficient enough for refolding the reduced IFN. Therefore, a solution (1 mL) of 50 mM GSH 26: 50 mM GSSG 27 was prepared in 20 mM sodium acetate buffer, pH 4.0 (Glutathione refolding solution – GRS). This stock solution was frozen and stored at -20 °C. After thawing of the stock solution, the required volume (typically 25 – 50 µL) was drawn each time and the solution frozen back for storage.

![Figure 4.14](image-url). SDS-PAGE analysis showing the effect of 0.5 mM GSH 26: 0.5 mM GSSG 27 on the re-oxidation of reduced IFN; Novex 12% bis-tris gel of the reactions stained with colloidal blue, lane 1 – protein molecular weight standards, lane 2 – unreduced IFN α-2b, lane 3 – reduced IFN α-2b, lane 4 – reduced IFN α-2b incubated for 16 h at ambient temperature, lane 5 – reduced IFN α-2b incubated still with 0.5 mM: 0.5 mM GSH 26: GSSG 27 for 16 h at ambient temperature, lane 6 – reduced IFN α-2b incubated shaken with 0.5 mM: 0.5 mM GSH 26: GSSG 27 for 16 h at ambient temperature, lane 7 – reduced IFN α-2b incubated still with 0.5 mM: 0.5 mM GSH 26: GSSG 27 for 16 h at 4 °C, lane 8 – reduced IFN α-2b incubated shaken with 0.5 mM: 0.5 mM GSH 26: GSSG 27 for 16 h at 4 °C.
4.4.7 DB-PEGylation with PEG mono-sulfone \(15a\) and with GRS

DB-PEGylation process was then conducted with the addition of the glutathione re-oxidation step. After 2 h of the reaction period between the reduced IFN and the 10 kDa PEG mono-sulfone \(15a\), the mixture was incubated with 0.5 mM GRS for a further 16 h at 4 °C before the cation exchange chromatography and SEC-HPLC isolation steps. This time, the unreacted reduced protein was found to be re-oxidised as observed in the SDS-PAGE analysis (Figure 4.15, lane 4).

For use in the DB-PEGylation process, the volume of GRS stock solution (25 \(\mu\)L) that gave a concentration of 0.5 mM GSH\(^26\), 0.5 mM GSSG\(^27\) in the DB-PEGylation solution (2.5 mL) was taken and added after the reaction period (2 h) between reduced IFN and PEG mono-sulfone \(15a\). The addition of GRS terminates the DB-PEGylation reaction since the remaining PEG mono-sulfone \(15a\) if any, would react with the cysteine of GSH\(^26\). However, PEG mono-sulfone \(15a\) reacts quickly with the reduced protein and the chances of any remaining PEG mono-sulfone \(15a\) are expected to be less (due to 1 eq. stoichiometric reaction). The reaction mixture was then subjected to the ion exchange and SEC-HPLC purification (Figure 4.16) to isolate the monoPEG-IFN conjugate and the protein for biological characterisation. As hypothesised, aggregation significantly reduced to \(\approx 2\%\) (Figure 4.16) from \(\approx 15\%\) (Figure 4.9).
As observed in the SDS-PAGE and antiviral activity studies (Appendix 3), GRS treatment of the mixture caused the re-oxidation of the reduced IFN to its native form. The re-oxidation step can potentially make the overall DB-PEGylation process highly efficient because it becomes possible to recycle unreacted IFN. However, the re-oxidation of the reduced disulfide present in monoPEG-IFN could not be confirmed by SDS-PAGE. Re-oxidation was a likely occurrence since the conjugate showed better antiviral activity (Section 4.4.20). Also, aggregation significantly decreased as observed in the SEC-HPLC (Figure 4.16), which again suggested re-oxidation of the reduced disulfide. The re-oxidation was later confirmed during the peptide mapping of tryptic digests of IFN-PEG conjugate (Section 4.4.16).

4.4.8 DB-PEGylation with 1.5 eq. of PEG mono-sulfone 15a

Typically, the reduction of IFN and reaction with 1 eq. PEG mono-sulfone 15a would give the monoPEG-IFN as the major product (≈ 65 %) and the diPEG-IFN (≈ 18 %) as a by-product. The preparation of monoPEG-IFN was the major objective of the study. However, it was also
interesting to learn if the diPEG-IFN would show antiviral activity. Therefore, it was considered to prepare the diPEG-IFN as the major product in the reaction. A simple solution for this was to increase the stoichiometry of PEG mono-sulfone 15a for reaction with the reduced IFN.

When 1.5 eq. of PEG mono-sulfone 15a were used for the reaction, diPEG-IFN was the major product (61%) as observed both in the SDS-PAGE and SEC-HPLC (Figure 4.17, lane 4 and Figure 4.18 respectively). No unreacted protein was observed in the SDS-PAGE gel or in the chromatogram. Also, there was less soluble aggregates (<1%) found in the chromatogram even with no GRS re-oxidation step. This suggested that the aggregation observed in the earlier 1 eq. reaction could mainly be induced by the unreacted completely reduced IFN rather than the reduced monoPEG-IFN.

When 1.5 eq. PEG mono-sulfone 15a was used for the reaction with reduced IFN, the peak of the diPEG-IFN in SEC-HPLC also showed ‘front tailing’ or ‘shouldering’ (Figure 4.18, peak with its maximum at 60.00 min). This could have been due to the presence of triPEGylated IFN which could also be observed as a faint band in the SDS-PAGE (Figure 4.17, lane 4). This is because both the disulfides of IFN upon reduction expose the thiols widely for attack by the reactive PEG mono-sulfone 15a.

![Figure 4.17. SDS-PAGE analysis of 10 kDa PEG mono-sulfone 15a reaction with IFN; Novex bis-tris 12 % gel stained with colloidal blue; lane 1 – protein molecular weight standards, lane 2 – native IFN, lane 3 – reduced IFN, lane 4 – 1.5 eq. of 10 kDa PEG mono-sulfone 15a reaction.](image-url)
Figure 4.18. SEC-HPLC chromatogram of ion exchange purified reaction mixture of reduced IFN and 1.5 equivalent of 10 kDa PEG mono-sulfone 15a: peak ‘fronting’ or ‘shouldering’ indicated the possibility of triPEGylation due to the exposed thiols of the disulfide bonds.

Since IFN has two accessible disulfides, these studies reveal that the stoichiometry of PEG mono-sulfone 15a employed in the reaction is important to obtain the IFN conjugate of interest. The main product of the DB-PEGylation of reduced IFN with 1 eq. of PEG mono-sulfone 15a was monoPEG-IFN conjugate (~60 % of the purified reaction mixture, SEC-HPLC analysis). When 1.5 eq. of the PEG mono-sulfone 15a was used, the diPEG-IFN was the major product (~61 %).

4.4.9 PEGylation of higher concentration of IFN with PEG mono-sulfone 15a followed by GRS re-oxidation

The experiments described above used IFN at a concentration of 0.2 mg/mL (1 mL) for the reduction and subsequent DB-PEGylation studies. Reactions were also carried out with IFN at a relatively higher concentration of 0.5 mg/mL (1 mL). These were conducted to prepare pure PEG-IFN conjugate for subsequent studies such as circular dichroism (CD). Since a high
concentration of PEG-IFN was required for this analysis, 1 mg of total IFN was required (i.e., 2 × 0.5 mg reactions). This was a considerable amount of IFN for an academic lab. Two reactions were carried out separately employing similar conditions for both (total IFN amount used – 1 mg). DB-PEGylation was carried out for 4 h followed by the re-oxidation step for a further 16 h. Upon completion of the reaction (including the re-oxidation step), the solutions were analysed by SDS-PAGE (Figure 4.19), buffer exchanged separately to 20 mM sodium acetate buffer, pH 4.0 and then pooled (total volume – 5 mL). The reaction mixture was purified by cation exchange and the conjugates isolated by SEC-HPLC (Figure 4.20).

Figure 4.19. SDS-PAGE analysis of higher scale reaction (starting IFN conc - 0.5 mg/mL) of 1 eq. PEG mono-sulfone 15a with reduced IFN; Novex bis-tris 12 % gel stained with colloidal blue; lane 1 – protein molecular weight standards, lane 2 – native IFN, lane 3 – reduced IFN, lane 4 – 1 eq. of 10 kDa PEG mono-sulfone 15a reaction incubated with 0.5 mM. 0.5 mM GSH 26, GSSG 27. Reaction was similar to the 0.2 mg/mL starting IFN conc. scale.
Figure 4.20. SEC-HPLC of ion exchange purified reaction mixture of reduced IFN (0.5 mg/mL reaction scale) and 1 eq. of 10 kDa PEG mono-sulfone 14a.

The solution was collected manually from the SEC-HPLC was concentrated using Vivaspin® 20 mL concentrator to 2 mL volume. This solution was made up to 2.5 mL with deionised water and then buffer exchanged to acidified water, pH 2.85 using a PD-10 desalting column. Buffer exchange to acidified water, pH 2.85 was conducted to avoid spectral interference from buffer salts during CD spectroscopy analyses. The 3.0 mL elution after the load volume (2.5 mL) was collected and concentrated again using a Vivaspin® 6 mL concentrator to 1 mL. The final protein content was assayed by microBCA and was found to be 0.275 mg/mL (27.5 % of the total protein used). The solution obtained was used for the CD spectroscopy studies (Section 4.4.10).
4.4.10 CD spectroscopy studies of DB-PEGylated IFN

CD spectroscopy is a method to analyse the secondary structure of a protein. It gives information regarding the protein's secondary structure such as the α-helices and β-sheets. Therefore, this technique is used mainly to compare the secondary structure of a modified protein to that of the native protein. Morehead et al. have studied the differences in the α-helical conformation of native IFN-α and sulfitolysed IFN-α (oxidative cleavage of the disulfides in IFN-α) by far UV CD spectroscopy (Morehead et al. 1984). In their study, they found that the alpha helices of sulfitolysed IFN-α were similar to the native IFN-α. This meant that the conformation of the disulfide cleaved sulfitolysed IFN-α was dependant mainly on other non-covalent interactions within the protein structure. Based on their studies, it was assumed that the α-helical structure of DB-PEGylated IFN also could be analysed by far UV CD spectroscopy. Initially, the IFN and monoPEG-IFN samples were prepared in acidified water, pH 2.85 with approximate concentrations of 0.175 mg/mL and 0.11 mg/mL respectively. These samples were then analysed by UV spectroscopy at the wavelength of 280 nm with acidified water, pH 2.85 as the control. Differences in the absorbance of samples at 280 nm were noted in the spectra (Figure 4.21) due to concentration difference. Difference was also observed in the CD spectra (Figure 4.22).

![UV spectra in acidified water, pH 2.85](image)

Figure 4.21. UV absorbance spectra of IFN and PEG-IFN acquired in deionised water, pH 2.85.
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Figure 4.22. Far UV CD spectra of IFN and PEG-IFN based on the UV$_{280\text{nm}}$ non-normalised spectra

Figure 4.23. UV$_{280\text{nm}}$ normalised UV spectra of IFN and monoPEG-IFN; an increase in absorbance for the DB-PEG-IFN can be observed which indicates that the aromatic ring in the conjugated PEG contributes to the protein’s absorbance.
The concentration of the solutions were then normalised (or calibrated) to 280 nm using calculations based on the molar extinction coefficients of tyrosine, tryptophan and disulfide bonds in IFN. This is because the $\text{UV}_{280\text{ nm}}$ absorbance of a protein is predominantly due to tyrosine, tryptophan and disulfide bonds (Pace et al. 1995). Some differences in the UV spectra were observed with respect to the degree absorption (Figures 4.23). There was a shifted maximum UV absorbance for monoPEG-IFN to 265 nm. The native protein displayed a maximum at 280 nm. This difference could be due to the presence of the aromatic moiety in the PEG linker.

The samples were then normalised to 210 nm. At this wavelength, all the peptide bonds in the protein would absorb. The absorbance of native and the monoPEG-IFN was found to be similar at this wavelength. Determining the protein concentration and thus the CD spectra was then relatively easier. CD spectral peaks based on this concentration were observed to overlap with each other (Figure 4.24). It could be inferred from these CD studies that the monoPEG-IFN retains the native IFN’s alpha-helical structure. The CD experiment requires a considerable amount of IFN and therefore was conducted only once. It was more important to determine if the monoPEG-IFN was biologically functional. Therefore, the available IFN stock was used mainly to prepare conjugates for biological assays.

![Figure 4.24. Far UV CD spectra of IFN and DB-PEG-IFN based on the normalised UV spectra at 210 nm](image-url)
4.4.11 Molecular modelling studies

Molecular computational modelling of protein structure is useful to gain insight about its tertiary structure with time. Many proteins have been modelled based on experimental structural information gained from NMR spectroscopy of the protein in solution and by X-ray crystallographic studies. Many experimentally derived protein structures are available on the protein data bank (PDB). Each protein is denoted with an alpha-numerical identity tags (PDBid). For example, the NMR derived structure of IFN α-2a is available in PDB and its PDBid is 1itf. Freely available software such as Rasmol can be used to view these structures. However, for the computational experiments that were conducted for IFN, the software that was used was Maestro®. This is available as a part of the Schrödinger® modelling suite that operates on a Linux operating system.

The initial model of IFN α-2b for study of the conformational changes induced by the replacement of a disulfide bond with a 3 carbon-bridge was obtained with homology modelling. Two different structures of IFN α-2b were constructed (Figure 4.25 b & c). The first structure had the three carbon methylene bridge between the cleaved disulfide bond Cys1–Cys98 and the second structure had the three carbon methylene bridge between the cleaved disulfide bond, Cys29–Cys138. Stochastic dynamic simulations were carried out on these structures to determine the structural effects of the 3-carbon methylene bridges in the place of the native disulfides. Stochastic dynamic simulation was used for this study since it implicitly takes into account the effect of solvent (water) on protein structure. The use of implicitly defined water was further validated by comparing the simulation with the simulation of a system with explicitly defined water (Godwin et al, 2006).

The final conformations of the two 3-carbon bridged IFN isomers were compared to the NMR derived structure of IFN α-2a (pdb id=1itf) and X-ray structure of IFN α-2b (pdb id=1rh2). The RMSD values derived for the Cys1-CCC-Cys29 structure and Cys29-CCC-Cys138 structures were 2.49 Å and 2.28 Å respectively. The distance between the cysteine sulfur atoms increased from 2.03 Å to 4.90 Å for the modified structures. The RMSD values for the residues involved in receptor binding (Piehler et al, 2000) were 2.79 Å and 2.74 Å for Cys1-CCC-Cys98 and Cys20-CCC-Cys138 isomers respectively. These values observed were not significantly different from the RMSD values for the native IFN α-2a which were within 1.4 – 2.2 Å. It was inferred from this experiment that the structure around the receptor binding site of IFN did not alter due to the presence of 3-carbon bridge in either of the disulfide cysteine thiols.
Both of the IFN disulfide bonds were then subjected to modifications by 3-carbon bridges with the benzoic acid side chains and the modified structures were used for simulations of 2,000 ps. The conformations were recorded every 20 ps resulting in a trajectory that was analysed by calculating the RMSD values for the backbones of the starting model of IFN and for each conformation of the modified protein at the different time frames. A plot of the calculated RMSD values versus time indicated that the overall structures deviated by < 4 Å from the native conformation (Figure 4.26A). All of the conformations were then superimposed and a ribbon plot created with the positions of the linkers at different time points shown in CPK representation (Figure 4.26B). The main secondary structural features (i.e., helices) were preserved. This was consistent with the observations from the CD experiments.

The 3-carbon bridge between CYS29-CYS138 had a lower conformational flexibility than the bridge between CYS1-CYS98. This may be due to the restricted movements on the inter-helical connecting loops. However, the aromatic moiety of the 3-carbon bridge between CYS1-CYS98 had greater freedom to move because the N-terminal cysteine was not constrained by inter-barrel interactions. This could result in greater conformational space sampling by the benzoic acid residue leading to different interactions with the surface of the protein. These results suggest that the insertion of a 3-carbon bridge that is linked by thio-ether bonds displays greater conformational mobility than the native disulfide bond. This additional mobility may enable the modified protein to maintain its tertiary structure.
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Figure 4.26. Stochastically simulated 3-carbon disulfide double bridged IFN (2,000 ps); A) The RMSD plot of the backbone molecules of the modified IFN was calculated using the native conformation of IFN α-2a. B) Backbone plots of the chemically modified IFN α-2b in CPK representation. The 3-carbon disulfide bridges are shown in blue.

Figure 4.27. Stochastic simulation derived structures of DB-PEGylated IFNs; A) Cys29-CCC-Cys138 monoPEGylated IFN isomer (PEG 10 kDa), B) Cys1-CCC-Cys98 monoPEGylated IFN isomer (PEG 10 kDa), C) DiPEGylated IFN (PEG 10 kDa).
4.4.12 PEGylation of IFN with PEG bis-sulfone 14a

Since the PEG mono-sulfone 15a required an extra synthetic step and was derived directly from the PEG bis-sulfone 14a, conjugation studies were also conducted with the PEG bis-sulfone 14a. Other possible limitations of the PEG mono-sulfone 15a include its inherent reactivity. It was certainly possible for this compound to undergo reaction with solvent under certain conditions (Chapter 2, section 2.4.7). During conjugation, PEG mono-sulfone 15a could be formed in situ from PEG bis-sulfone 14a by elimination of one of the sulfone moieties. The generated PEG mono-sulfone 15a would then undergo reaction with reduced IFN. The IFN concentration of 0.5 mg/mL was used for all the reactions with PEG bis-sulfone 14a. These reactions were conducted with one equivalent of PEG bis-sulfone 14a. The reaction workup was similar as that which was used for PEG mono-sulfone 15a reaction. PEG bis-sulfone 14a reaction was also found to be efficient with the SEC-HPLC yields of PEG-IFN typically ranging between 50 to 60% (Figure 4.28). Similar to PEG mono-sulfone 15a, no conjugation occurred when PEG bis-sulfone 14a was incubated with native IFN (control incubation).

![Figure 4.28. SEC-HPLC of ion exchange purified reaction mixture of reduced IFN (0.5 mg/mL reaction scale) and 1 eq. of 10 kDa PEG bis-sulfone 14a.](image-url)
In the chromatogram of the PEG bis-sulfone 14a reaction with reduced IFN (Figure 4.28), there was reduced ‘shouldering’ of the diPEG-IFN peak as observed with the PEG mono-sulfone 15a reactions with reduced IFN (Figures 4.18 and 4.20). In the case of PEG mono-sulfone 15a, it undergoes rapid reaction with thiol upon addition to the solution containing the reduced protein. When PEG mono-sulfone 15a is added to the solution containing reduced IFN, a higher concentration of a reactive Michael acceptor is available for reaction. This can cause a greater chance of two molecules of PEG mono-sulfone 15a to react separately with both thiols derived from a single reduced disulfide. Separate PEGylation of both the exposed thiols of the reduced disulfide can occur and this side reaction can compete with the desired mono-PEGylation. PEG bis-sulfone 14a must first undergo elimination before thiol addition can occur. By controlling this first elimination it is possible to tailor the concentration of the PEG mono-sulfone 15a in the reaction mixture. This can prevent separate mono-PEGylation of the two thiols of a reduced disulfide.

4.4.13 Recovery studies of IFN conjugates

There are several steps involved in the DB-PEGylation process which could each lead to the loss of valuable protein (Scheme 4.1). In non-denaturing conditions, the typical protein recovery using PD-10 column was as high as 96 % as shown in the experiment with the model protein, RNase. This was also confirmed during DB-PEGylation of L-asparaginase. Each PD-10 desalting/ buffer exchange step led to a loss of \( \approx 5\% \) of the initial protein content. Typically, \( \approx 90\% \) of the initial protein content was available for the required ion exchange chromatography after the buffer exchange step. The recovery of IFN and conjugates using PD-10 was also found to be consistent (as assayed by UV\( _{280\text{ nm}} \) and microBCA assays).

Although, most reactions were conducted with 1 eq. of PEG mono-sulfone 15a or PEG bis-sulfone 14a, there is always a chance of presence of traces of unreacted compounds in the reaction mixture. Therefore, prior ion exchange purification is necessary to isolate the pure conjugate/s in the subsequent SEC-HPLC step. The ion exchange purification step has to be carefully conducted. This is because the wash volume (fresh buffer used to remove trace unreacted PEG from the reaction mixture) had a significant effect on the recovery of the pure mixture for conjugate isolation by SEC-HPLC (Table 4.2). The typical protein yield after 2 mL of wash (including the 1 mL eluent volume that is discarded) was found to be 74.40 %. This volume (2 mL) of wash was sufficient to remove any PEG species (as determined by SEC-HPLC analysis).
Size exclusion chromatography was a useful technique in the separation of the conjugates from the unreacted IFN as well as from the soluble aggregates. The volume of each conjugate (except aggregates) collected manually from the SEC-HPLC ranged 5 - 8 mL. This volume was independent of the amount of protein or conjugate present in the reaction mixture. Typically, the fractions had to be concentrated using spin concentrators for SDS-PAGE or MALDI-TOF analyses.

Scheme 4.1. Purification steps involved in the DB-PEGylation process with their typical protein recovery yields; Filled arrow – major protein recovery, Non-filled arrow – minor protein loss.
Chapter 4. DB-PEGylated interferon α-2b

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<th>Wash volume including 1 mL elution volume</th>
<th>Recovery (microBCA assay)</th>
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<td>59.92%</td>
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<tr>
<td>2 mL</td>
<td>74.40%</td>
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</table>

Table 4.2. The effect of wash volume on the recovery of IFN during cation exchange chromatography.

4.4.14 10 kDa PEG bis-sulfone 14a stoichiometry studies

When a higher stoichiometry of 10 kDa PEG bis-sulfone 14a was used for the PEGylation (2 eq.), diPEG-IFN was the major product (Figure 4.29) with no PEG-IFN observed in the SDS-PAGE gel. When more equivalents of 10 kDa PEG bis-sulfone 14a was used for the reaction, a band identified as triPEGylated IFN was observed along with diPEGylated IFN. The triPEGylation observed could be due to the formation of PEG mono-sulfone 15a at concentrations high enough to compete with the second elimination reaction. This led to two PEGs attaching to the thiols of the same reduced disulfide. This reaction was not observed with L-asparaginase even with addition of 50 equivalents of PEG mono-sulfone 15a to a reduced disulfide (Chapter 3). This could be because, unlike IFN α-2b, the disulfide bond in L-asparaginase is not terminally located for easy access to the PEG mono-sulfone 15a.

Figure 4.29. SDS-PAGE analysis of the DB-PEGylation of IFN using higher stoichiometry of PEG bis-sulfone 14a; Novex Bis-Tris 12% gel stained with colloidal blue (left) and barium iodide (right); lane 1 - protein molecular weight standards, lane 2 - native IFN, lane 3 - reduced IFN, lane 4 - IFN incubated with 2 eq. of 10 kDa PEG mono-sulfone 15a for 16 h at ambient temperature, lane 5 - reduced IFN incubated with 2 eq. of PEG bis-sulfone 14a, lane 6 - reduced IFN incubated with 3 eq. of PEG 10 kDa bis-sulfone 14a, lane 7 - IFN incubated with 4 eq. of 10 kDa PEG bis-sulfone 14a, lane 8 - IFN incubated with 5 eq. of 10kDa PEG bis-sulfone 14a, lane 9 - 10 kDa PEG bis-sulfone 14a.
These higher stoichiometry studies with fully reduced IFN showed that diPEG-IFN was the main product even with 5 eq. of 10 kDa PEG bis-sulfone 14a. This observation suggested that the formation of the 3-carbon bridge between the thiols to 'rebridge' the cysteines of the reduced disulfide. Theoretically, if the 3-carbon bridging had not occurred, all the four thiols (from two disulfides) would have undergone PEGylation separately to yield a quadPEGylated IFN as the major product.

4.4.15 3-carbon disulfide bridging studies

PEG bis-sulfone 14a and PEG mono-sulfone 15a are designed to undergo sequential thiol addition reactions to form a 3-carbon bridge between the cysteines of a reduced disulfide bond. This reaction was studied with GSH 26 (Chapter 2). Also, the higher PEG mono-sulfone 15a stoichiometry reactions with L-asparaginase (Chapter 4) suggested that the 3-carbon disulfide bridging reaction did occur upon DB-PEGylation. Although, all these observations indicated that the bis-alkylation occurred, it was necessary to further confirm this reaction on IFN and to ascertain, if possible, what effect disulfide rebridging might have on the biological function of IFN.

The carboxylic acid bis-sulfone 13a was used for these experiments. For this, the reduced IFN in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 was reacted with 10 eq. of carboxylic acid bis-sulfone 13a (5 eq. to one reduced disulfide bond). Since the carboxylic acid bis-sulfone 13a was not soluble in aqueous buffers, a solution of the carboxylic acid bis-sulfone 13a in acetonitrile was prepared for this reaction. The reaction was conducted in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8. The 13a acetonitrile solution was added to the reduced IFN and the reaction mixture incubated without agitation for 2 h at ambient temperature. The percentage of acetonitrile in the reaction mixture typically did not exceed 1%.

SDS-PAGE analysis of the reaction at the 5 min and 2 h time points were carried out to determine the extent of the 3-carbon bridging reaction. The reformation of the IFN native disulfides (in controls) or the disulfide thiols with 3-carbon bridge (in reactions) could be monitored by the protein’s migration in the SDS-PAGE. Since IFN showed distinct native or reduced bands in the electrophoretic analysis, this study was ideal to confirm the 3-carbon bridging reaction. Ideally, the reformed native disulfides or the disulfide thiols with 3-carbon bridges would attain a closed structure similar to the native protein. SDS-PAGE analysis of the reactions suggested the possible formation of the 3-carbon bridge between the thiols (Figure 4.30, lanes 5 and 7). The controls showed no reformation of the disulfide bonds throughout the
reaction period (Figures 4.30, lanes 3 and 4). In contrast, the reaction mixture at the 5 minute time point indicated significant ‘rebridging’ of the disulfide thiols with the 3-carbon bridges (Figure 4.30, lane 5). The 2 h time point showed complete ‘rebridging’ of the disulfide thiols with the 3-carbon bridges (Figure 4.30, lane 7).

Figure 4.30. SDS-PAGE analysis of the 3-carbon bridging reaction of carboxylic acid bis-sulfone 13a with reduced IFN; Novex bis-tris 12% gel stained with colloidal blue; lane 1 – protein molecular weight standards, lane 2 – native IFN, lane 3 – reduced IFN, lane 4 – reduced IFN incubated for 5 min at ambient temperature, lane 5 – reduced IFN incubated with 10 eq. of carboxylic acid bis-sulfone 13a for 5 min at ambient temperature, lane 6 – IFN incubated for 2 h at ambient temperature, lane 7 – reduced IFN incubated with 10 eq. of carboxylic acid bis-sulfone 13a for 2 h at ambient temperature.

These experiments indicated that the carboxylic acid bis-sulfone 13a at very low concentrations is soluble enough in aqueous buffers and could be applied for further bridging and characterisation studies. Since multiple PEGylation of IFN disulfide bond was observed with PEG bis-sulfone 14a (Section 4.4.14), the possibility of contamination with multiple mono-alkylated IFN was also expected in the reaction product. The carboxylic acid bis-sulfone 13a is smaller in size compared to the PEG bis-sulfone 14a. Therefore, the smaller size would structurally allow easy access for two carboxylic acid bis-sulfones 13a to undergo separate mono-alkylation reactions to the both the thiols of a reduced disulfide. In contrast, the mono-alkylated IFN product which should be visually similar in the gel to partially or completely reduced IFN was not observed in the colloidal blue stained SDS-PAGE gel (Figure 4.30, lane 7). Since the second elimination/ addition reaction of PEG bis-sulfone 14a was observed to be thermodynamically driven (Chapter 2), the reaction of carboxylic acid bis-sulfone 13a with the two thiols derived from a reduced disulfide could be fast enough to avoid the mono-alkylation reactions. However, a more sensitive staining technique such as silver staining was required to confirm this observation.
The 3-carbon bridging reaction was conducted with increasing carboxylic acid bis-sulfone 13a stoichiometry (1, 2, 4, 6, 8 and 10 eq.) to the reduced protein. The reactions after 2 h of incubation at ambient temperature were analysed by SDS-PAGE with silver staining of the gel. As observed before, the reactions showed re-formation of the disulfide thiols with the 3-carbon bridge whereas the control protein solution remained reduced after 2 h (Figure 4.31A). Complete reaction (dibridging) was observed only with 6 eq. of carboxylic acid bis-sulfone 13a in 2 h. The reaction with 1 eq. had a mixture of completely reduced IFN, single bridge IFN and dibridged IFN. The reaction with 2 eq. had a mixture of single bridged IFN and dibridged IFN with no reduced IFN. The reaction with 4 eq. of carboxylic acid bis-sulfone 13a showed predominantly dibridged IFN with some amount of single bridged IFN. Treatment of all the reactions (especially the 1, 2 and 4 eq. reactions) with the GSH 256 yielded completely reformed IFN (Figure 4.31B). All these reactions indicate the absence of a mono-alkylated IFN product.

The glutathione reformed reaction mixtures were purified by simple desalting to deionised water, concentrated using Vivaspin® 6 mL concentrators and analysed by MALDI-TOF MS.
Reaction with 1 equivalent of the carboxylic acid bis-sulfone 13a showed a mass increase of 190 Da (19,499 Da) (Figure 4.32B) to the native IFN’s mass (19,304) (Figure 4.32A). This suggested the presence of a single bis-alkylated product. Such a product was derived from one molecule of IFN which suggests that a single 3-carbon bridge had formed on one of the reduced disulfides in the protein. The reaction with 8 eq. showed a mass increase of 380 Da (19,686 Da) (Figure 4.32C) which suggested two molecules of the bis-sulfone having undergone bis-alkylation with the same IFN molecule. Therefore this suggested that two 3-carbon bridges had formed. However, since MALDI-TOF MS is not a quantitative analytical method, these results do not imply the presence of pure single or double 3-carbon bridged IFNs in the reaction mixtures.
Figure 4.32. MALDI-TOF spectrum of carboxylic acid bis-sulfone 13a reaction with IFN; A) native IFN, B) reaction with 1 eq. showing predominantly single bridged IFN and C) reaction with 8 eq. showing the double bridged IFN; a mass increase of 190 Da to the native IFN’s mass (19,304 Da) indicates the presence of a single 3-carbon bridge (19,499 Da) and a mass increase of 380 Da indicates the presence of double 3-carbon bridge (19,686 Da).
A method to determine the site of PEGylation is tryptic digestion followed by peptide mass mapping with high resolution MALDI-TOF (Courchesne and Patterson, 2002). Trypsin is commonly used as the proteolytic enzyme for protein digestion. The enzyme cleaves the arginine (R) and lysine (K) amide bonds in proteins (Figure 4.33). Sequencing grade trypsin is available commercially. This enzyme is methylated to prevent autolytic digestion (Promega). The optimum pH for trypsin activity is pH 8.0 and the enzyme is inactive in acidic pH (Stone and Williams, 2002).

![Figure 4.33](image.png)

**Figure 4.33.** Amino acid sequence of IFN including the N-terminal methionine with a total of 166 amino acids (* = site of tryptic cleavage).

The aim of the tryptic digestion studies was to identify the disulfide containing peptides by accurate MALDI-TOF analysis. This was achieved by the tryptic digestion of native IFN followed by MALDI-TOF analysis. To confirm if the identified peptide was the disulfide containing fragment, tryptic digestion and MALDI-TOF analysis was also conducted on the reduced IFN. 3-carbon bridging reaction (bridging of both disulfides) with carboxylic acid bis-sulfone $\text{13a}$ was then conducted on reduced IFN. After isolation of the reaction product from the excess carboxylic acid bis-sulfone $\text{13a}$, it was subjected to tryptic digestion and MALDI-TOF analysis. Due to the formation of 3-carbon bridge between the cysteine containing peptides, the mass of the disulfide fragment (observed with tryptic digestion of the native IFN and MALDI-TOF analysis) increased by 190 Da (the mass of the bridge with the benzoic acid moiety).
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Table 4.3. Theoretically calculated peptide segments of IFN tryptic digestion with their molecular mass; the N-terminal methionine is also included (literature provided by Shantha Biotechnics, India).

Initially, the native IFN was subjected to tryptic digestion followed by high resolution MALDI-TOF MS. The obtained mass spectral peaks (Figure 4.34) were then matched with the theoretically calculated tryptic digest peptide masses of IFN (Table 4.3). The peak masses of interest were 2,120 Da composed of peptides # 7 & # 9 and approximately 4,600 Da composed of peptides # 1 & # 6 (minus the methionine residue not present in IFN α-2b).

![Figure 4.34. High resolution (reflectron mode) MALDI-TOF spectrum of tryptic digested native IFN in 10 mM sodium phosphate buffer, pH 8.0; the expected peptide fragment # 2 was not observed.](image-url)
Figure 4.35. MALDI-TOF spectra of tryptic digests showing the Cys29 – Cys138 containing peptide; A) native IFN containing the Cys29 – Cys138 peptide, B) reduced IFN with absence of Cys1 – Cys98 peptide; the peptide containing disulfide Cys29 – Cys138 is the peak with mass 2,118 Da (spectrum A) which is not observed with the reduced IFN (spectrum B). This peak could be useful for determining the 3-carbon bridge reaction on IFN Cys29 – Cys138. Ideally, the Cys29 – CCC – Cys138 should have a molecular weight of 2,308 Da.

The peptides containing the disulfides were the peptides # 1 & # 6 (Cys1 – Cys98) and # 7 & # 9 (Cys29 – Cys138) which should show up ideally in the MALDI-TOF spectrum at 2,120 Da and 4,598 Da respectively. The disulfide peak disappeared if the IFN was completely reduced before tryptic digestion and MALDI-TOF analysis (Figure 4.35B). The high resolution reflectron mode MALDI-TOF MS analysis of the tryptic digests did not show the peaks above the mass of 2,500 Da (Figure 4.35). The glutathione reformed DB-PEGylated IFN upon tryptic digestion and analysis by low resolution MALDI-TOF MS showed both the disulfide containing peptides (Figure 4.36). This supported the hypothesis of reformation of the unreacted disulfide in PEG-IFN. The spectrum achieved with the low resolution linear mode showed peaks with higher molecular masses (Figure 4.36). The linear mode MALDI-TOF MS was observed to be less accurate in comparison with the reflectron mode MALDI-TOF MS.
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Figure 4.36. MALDI-TOF spectra of tryptic digested single 3-carbon bridged 10 kDa PEG-IFN showing the Cys29 – Cys138 (2,114.59 Da) and Cys1 – Cys98 (4,651 Da) containing peptides. The Cys1 – Cys98 peak mass was not exact due to possible non-specific site of tryptic cleavage.

Since both the disulfides were observed in the mass spectrum of the tryptic digest of DB-PEG-IFN, this experiment did not confirm the 3-carbon disulfide bridging reaction. Tryptic digestion of diPEG-IFN for this experiment would also not yield fruitful results since both the disulfides would disappear in the MALDI-TOF spectrum. Furthermore, since the PEG usually displays a broad peak in the MALDI-TOF spectrum, analysis of the PEGylated peptide fragments would not be conclusive enough to confirm the 3-carbon bridging reaction. The 3-carbon bridging reaction had to be therefore confirmed with the help of tryptic digested dibridged IFN (without PEG). Therefore, the reduced IFN was reacted with 8 eq. of carboxylic acid bis-sulfone 13a for 2 h at ambient temperature to yield the dibridged IFN product (Figure 4.38A). The modified IFN was then purified by buffer exchanging with a PD-10 Desalting column to 10 mM sodium phosphate buffer, pH 8.0. The solution obtained was concentrated with a Vivaspin® column and subjected to trypsin digestion as explained earlier. Acetonitrile was added after 14 h to aid the solubility of the any hydrophobic peptides formed during the tryptic digestion. The solutions obtained were subjected to MALDI-TOF analysis (Figure 4.37B & C and Figure 4.38A & B).
Figure 4.37. 3-carbon bridging and tryptic peptide sequencing studies A) SDS-PAGE gel (Novex Bis-Tris 12%) 3-carbon bridging reaction of IFN α-2b; lane 1 – protein molecular weight standards, Lane 2 – native IFN, lane 3 – Completely reduced IFN, lane 4 – IFN incubated with 8 eq. of carboxylic acid bis-sulfone 13a for 2 h at ambient temperature. B) Reflectron mode high resolution MALDI-TOF spectrum of tryptic digests of native IFN showing the Cys29 – Cys138 containing peptide (2,119 Da); C) Reflectron mode high resolution MALDI-TOF spectrum of tryptic digests of 3-carbon bridged IFN showing the Cys29 – CCC – Cys138 containing peptide (2,309 Da).
No reaction to amine groups on the protein/peptide was observed with both PEG mono-sulfone 15\text{a} and the carboxylic acid bis-sulfone 13\text{a} at near neutral pH conditions (pH 7 – 8.6). This observation is in contrast to what was observed by Liberatore et al with carboxylic acid bis-sulfone 13\text{a} reaction to disulfide reduced antibody (Liberatore et al, 1990; del rosario et al, 1990). They conducted all the reactions with 10 molar excess of carboxylic acid bis-sulfone 13\text{a} in a mixture of 5 % v/v DMSO in phosphate buffer containing 2 mM EDTA, pH 7 which might
possibly have had an influence in the different reaction behaviour of the carboxylic acid \textit{bis}-sulfone 13a with their protein.

4.4.17 PEGylation of IFN using ester PEG \textit{bis}-sulfone 14b

DB-PEGylation was also attempted using the ester PEG \textit{bis}-sulfone 14b to study the effect of PEG and carboxylic acid \textit{bis}-sulfone 13a linkage on the reactivity and conjugate stability. Recall that the linkage was found to have an influence on the urethane PEG \textit{bis}-sulfone 14b reactivity (Chapter 2). Reduced reactivity was again observed with the ester PEG \textit{bis}-sulfone 14b. Satisfactory reaction with reduced IFN was observed only with 25-fold molar excess of the compound (Figure 4.39). This could be due to the electron interference or the conformation adopted by the compound in the aqueous buffer. Immediately after dissolution, a few molecules undergo elimination reaction which could be the reason for the observed reactivity with IFN. However, with time the PEG might be adopting a conformation which is unfavourable for the elimination reaction to occur.

![Figure 4.39](image.png)

The conjugates were purified from the excess PEG \textit{bis}-sulfone 14b by ion exchange chromatography and the PEG-IFN was isolated by SEC-HPLC. The manually collected eluate was then concentrated using Vivaspin concentrator to 1 mL, and subjected to PD-10 buffer exchange to 50 mM sodium phosphate buffer, pH 8.0. The solution obtained was again concentrated using Vivaspin concentrator to 250 µL and subjected to incubation at 37 °C at pH 7.8 for 10 days. The linkage was found to be quite stable with little IFN being released (Figure 4.40, lane 5). However, the IFN released was observed to be similar to the native IFN in the SDS-PAGE analysis which suggests that there is formation of the 3-carbon bridge between the thiols of the PEGylated disulfide and also the re-oxidation of the non-PEGylated disulfide.
4.4.18 Reactions with amide 14a, urethane 14c, carbonate 14d PEG bis-sulfones.

A simple comparison study of the DB-PEGylation reaction with amide 14a, urethane 14c and carbonate 14d PEG bis-sulfone was conducted to determine the effect on PEGylation reactivity of the PEG linking functionality. PEG bis-sulfones (2 eq. of each) were allowed to react separately to completely reduced IFN. As expected, the amide PEG bis-sulfone 14a and the carbonate PEG bis-sulfone 14d displayed diPEGylation whereas the urethane PEG bis-sulfone 14c displayed low reactivity as observed in the SDS-PAGE (Figure 4.42). Low reactivity was also observed with the ester PEG bis-sulfone 14b as described (Section 4.4.17).

These differences in reactivity could be a consequence of the masking of the bis-sulfone site by the PEG molecule. Since the carbonate PEG bis-sulfone 14d showed good reactivity with reduced IFN and also was found to undergo detachment from PEG (Chapter 2), the compound could be useful to study the biological effects of single or double 3-carbon bridges in IFN. This could be conducted by the DB-PEGylation, purification and isolation of either monoPEG-IFN or diPEG-IFN; the conjugate obtained could be subjected to PEG detachment in neutral to basic buffers followed by SEC-HPLC to collect the pure single or dibridged IFNs.
4.4.19  DB-PEGylation using 20 kDa PEG bis-sulfone 14a

DB-PEGylation with amide 20 kDa PEG bis-sulfone 14a was carried out to prepare a conjugate for the animal in vivo half-life studies. The reaction conditions employed for PEG 20 kDa bis-sulfone were similar to the PEG 10 kDa bis-sulfone reactions. However, with the help of preliminary experiments, it was found that 2 eq. of 20 kDa PEG bis-sulfone 14a was required to maximise the yield of monoPEGylated IFN (Figure 4.42). A total of 2 mg IFN (0.5 mg/mL reactions x 4) was used for the reaction. The reactions were pooled after exchanging them individually to 20 mM sodium acetate buffer, pH 4.0 and ion exchange carried out using SP-FF column to purify the conjugate from any excess PEG compound. High yields of monoPEG-IFN could be isolated from the SEC-HPLC (Figure 4.44). The amounts of aggregated IFN, diPEG-IFN and unreacted IFN were considerably low.

Unlike the reactions with 10 kDa PEG bis-sulfone 14a, 2 equivalents of the 20 kDa PEG bis-sulfone 14a was required to obtain a yield of 70% monoPEG-IFN (Figure 4.43 and 4.44). This is because the initial elimination reaction of higher molecular weight PEG bis-sulfones 14a (20 kDa or more) does not occur efficiently as compared to lower molecular weight PEG bis-sulfones 14a (10 kDa or less). This could be due to the lower solvent accessibility to the bis-sulfone moiety in the higher molecular weight PEG bis-sulfones 14a. Immediately after dissolution of PEG bis-sulfone 14a in the buffer containing reduced IFN, elimination occurs with half the amount of the added PEG bis-sulfones 14a. These molecules undergo reaction with reduced IFN. The remaining molecules might adopt a conformation unfavourable for the initial elimination and therefore do not undergo reaction with reduced IFN. This might be the reason why 2 equivalents of PEG bis-sulfone 14a are required for efficient yields of 20 kDa.
Also observed is that the amount of diPEG-IFN is lesser with 20 kDa PEG bis-sulfone 14a reaction. This could also be attributed to lower accessibility of the PEG bis-sulfone 14a to reduced IFN.

Figure 4.42. SDS-PAGE analysis of reduced IFN reaction with 2 eq. of 20 kDa PEG bis-sulfone 14a: Novex bis-tris 12% gel stained with colloidal blue; lane 1 – protein molecular weight standards, lane 2 – native IFN, lane 3 – reduced IFN, lane 4 – reduced IFN incubated with 1 eq. of PEG bis-sulfone 14a for 8 h at 4 °C and refolded with GRS, lanes 5, 7 and 9 – reduced IFN, lanes 6, 8 and 10 – reduced IFN incubated with 2 eq. PEG bis-sulfone 14a at 4 °C.

Figure 4.43. SDS-PAGE analysis of SEC-HPLC (Superose 12) isolated IFN 20 kDa DB-PEG conjugates; A) Novex bis-tris 12% gel (Courtesy: Dr. Antony Godwin and Dr. Ji-won Choi) stained using silver stain; lane 1 – Molecular weight standards, lane – native IFN, lanes 3 & 4 – diPEG-IFN, lanes 6 & 7 – PEG-IFN, and B) western blot analysis of the conjugates to confirm purity; lane 1 – IFN, lanes 3 & 4 – diPEG-IFN, lanes 6 & 7 – PEG-IFN.
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Figure 4.44. SEC-HPLC of ion exchange purified reaction mixture of reduced IFN and 2 eq. of 20 kDa PEG bis-sulfone 14a: Peaks were collected and identified by SDS-PAGE and MALDI-TOF analyses.

As with the elution of diPEG10 kDa IFN in the DB-PEG 10 kDa reactions, the monoPEG 20 kDa IFN had its peak maxima at approximately 60 min in the Superdex 200 prep grade column at an eluent flow rate of 1 mL/min. The diPEG20 kDa was observed to have its peak maxima at 52.67 min. The aggregated protein mixture eluted at the same time as observed with the DB-PEG 10 kDa reactions.

The separated compounds SEC-HPLC were collected and analysed by SDS-PAGE to confirm their identity (Figure 4.45). The 20 kDa monoPEG-IFN solution isolated from SEC-HPLC (Figure 4.44) had a protein concentration of ≈ 40 µg/mL. This solution was used without further concentration processes for the animal *in vivo* half-life studies.
4.4.20 IFN antiviral activity studies

IFN α-2b has several distinct biological functions that can be measured in vitro; they include prevention of infection of the human lung carcinoma cells A549 by encephalomyocarditis (EMC) virus (Grace et al. 2005b). Initially, the relative activity of the native IFN α-2b was determined by comparing the dose (concentration) of the sample which displays 50% prevention of infection in cells or (50% effective dose – ED$_{50}$) in vitro to the dose of the NIBSC reference standard IFN α-2b. In this antiviral assay, the ED$_{50}$ for the NIBSC standard IFN α-2b was 4.8 ± 1.8 pg/ml and for the native IFN α-2b obtained from Shantha Biotechnics, India was 8.1 ± 3.0 pg/ml (Figure 4.47).
Figure 4.46. Microscopy pictures of A549 cells incubated with decreasing concentrations of IFN α-2a (NIBSC) and challenged with EMCV A) 1,000 ppm IFN, B) 500 ppm IFN, C) 250 ppm IFN, D) 125 ppm IFN, E) 62.5 ppm IFN, F) 31.75 ppm IFN, G) 16.32 ppm IFN and H) 8.15 ppm IFN.
Figure 4.47. Representative graph showing antiviral activity of the IFN α-2b standard from NIBSC (UK) (Red line, ) and the IFN α-2b from Shantha Biotechnics (Blue line, ) as observed in an A549 - EMCV antiviral assay; IFN α-2b (NIBSC): \( ED_{50} = 4.8 \pm 1.8 \) pg/mL and IFN α-2b (Shantha): \( ED_{50} = 8.1 \pm 3.0 \) pg/ml.

The monoDB-PEGylated IFN conjugates (10 kDa and 20 kDa) that had been prepared were initially characterised for purity by SDS-PAGE with colloidal blue staining followed by barium iodide staining and silver staining (Figure 4.48). The samples were then subjected to antiviral assays. In the assay, the monoPEG 10 kDa IFN showed an \( ED_{50} \) of 265 ± 75 pg/mL (≈ 5.8 %) and the monoPEG 20 kDa showed an \( ED_{50} \) of 175 ± 35 pg/mL (≈ 8 %) (Figure 4.49).

Figure 4.48. SDS-PAGE analysis of 10 kDa and 20 kDa monoPEG-IFN F3a : Novex bis-tris 12% gel stained with colloidal blue (left), barium iodide (middle) and silver stain (right); lane 1 - protein molecular weight standards, lane 2 – native IFN, lane 3 – 10 kDa PEG-IFN, lane 4 – 20 kDa PEG-IFN.
Figure 4.49. Representative graph showing antiviral activity of IFN (black line, ▲), 10 kDa monoPEG-IFN (Blue line, ▼) and 20 kDa monoPEG-IFN (red line, ○) as observed in an A549 - EMCV antiviral assay; IFN: ED$_{50}$ = 8.1 ± 3.0 pg/mL, 10 kDa monoPEG-IFN: ED$_{50}$ = 273 ± 67 pg/mL and 20 kDa monoPEG-IFN: ED$_{50}$ = 175 ± 35 pg/mL.

Figure 4.50. Representative graph showing antiviral activity of IFN (black line, ▲) and process unreacted (re-oxidised) IFN (blue line, ■) as observed in an A549 - EMCV antiviral assay; IFN: ED$_{50}$ = 8.1 ± 3.0 pg/mL and process unreacted (re-oxidised) IFN: ED$_{50}$ = 14.1 ± 3.0 pg/mL.
Figure 4.51. Representative graph showing antiviral activity of IFN (black line, ▲), 10 kDa PEG-IFN (red line, ◆) and 20 kDa diPEG-IFN (blue line, ■) as observed in an A549 - EMCV antiviral assay; IFN: $ED_{50} = 8.1 \pm 3.0$ pg/mL, 10 kDa diPEG-IFN: $ED_{50} = 550 \pm 95$ pg/mL and 20 kDa diPEG-IFN: $ED_{50} = 678 \pm 93$ pg/mL.

In the case of the two diPEGylated IFN species, the 10 kDa and 20 kDa PEGylated IFN displayed an $ED_{50}$ of $550 \pm 95$ pg/mL and $678 \pm 93$ pg/mL respectively (Figure 4.51). Despite having markedly lowered antiviral activity, both dibridged and diPEG IFN analogues achieved complete inhibition of cell death from EMCV infection at saturating doses. These results indicate that the monoPEGylated IFN and diPEGylated IFN maintained an activity range of 5-8 % and 1-2 % respectively in this assay. It was learned from colleagues at Shantha Biotechnics that a similar antiviral assay displayed results of about 30% activity for the mono PEGylated IFN analogues.

The reduction in biological activity of PEGylated IFNs was mostly due steric shielding of PEG, which is thought to interfere with receptor binding; this decrease in the in vitro biological effects of IFN has also been observed with other PEG-IFNs that have been described (Monkarsh et al. 1997; Bailon et al. 2001; Grace et al. 2001; Youngster et al. 2002; Grace and Cutler, 2004; Dhalluin et al. 2005; Grace et al. 2005b). IFN is known to bind to its receptor (IFNAR1 and IFNAR2) in a complex manner with two ligand-receptor interactions required (Piehler and Schreiber, 1999; Piehler et al. 2000). The PEG present on the IFN surface has a relatively large hydrodynamic volume in solution (SEC-HPLC of PEG bis-sulfone 14a, chapter 3). Reduction in a protein’s receptor-ligand association has been also observed with PEG-antibody fragments.
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(Kubetzko et al. 2005). The observed decrease for the PEG-antibody fragment is thought due to decreased on rates for binding. This seems to be true even though the PEG is covalently bound to the antibody fragment at a site that is far from the CDR binding region. In contrast off rates do not seem to be as adversely affected by the presence of PEG. One interpretation of these results is that the presence of PEG would be expected to generally reduce in vitro biological responses.

In the case of DB-PEGylated IFNs, the presence of two molecules of PEG (10 kDa or 20 kDa) reduced the activity of IFN. The presence of three-carbon bridges in place of the native disulfides in IFN could be another possible reason for reduced biological activity. It was therefore necessary to study the effect of the three carbon bridges without PEG on the IFN’s biological activity. Dibridged IFN (without PEG attached) was prepared for this purpose and analysed for biological activity using antiviral assays. The presence of two three-carbon bridges in IFN was observed to reduce the biological activity of IFN; an ED_{50} value of 104 pg/ml was observed with antiviral assay (Figure 4.52). Assays conducted on predominantly single bridged IFN (prepared by 2 equivalents of carboxylic acid bis-sulfone 13a reaction and re-oxidation with glutathione) showed an ED_{50} of 41 pg/mL in the antiviral assay. For reference the ED_{50} of native IFN was 8 pg/mL. In contrast to the experiments with all the PEG-IFN conjugates that were prepared, it was not possible to confirm that there was no native IFN present with the mono-bridged IFN. In the case of dibridged IFN, the chances of contamination with unreacted IFN were low due to the higher amounts (8 eq.) of carboxylic acid bis-sulfone 13a used in the reaction process. It was clear that the reduced IFN reannealled in the presence of the bis-sulfone 13a, while the control reduced IFN remained open throughout the reaction period.
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Figure 4.52. Representative graph showing antiviral activity of IFN (black line, ▲), mono-3-carbon bridged IFN (red line, ○) and di-3-carbon bridged IFN (blue line, ■) as observed in an A549 - EMCV antiviral assay; IFN: ED$_{50}$ = 8.1 ± 3.0 pg/mL, monobridged IFN: ED$_{50}$ = 46.8 ± 4.7 pg/mL and dibridged IFN: ED$_{50}$ = 104.5 ± 9.6 pg/mL.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>ED$_{50}$ (pg/ml)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN</td>
<td>8.1 ± 3</td>
<td>5</td>
</tr>
<tr>
<td>SEC-HPLC recovered IFN</td>
<td>14.1 ± 3</td>
<td>6</td>
</tr>
<tr>
<td>Monobridged IFN</td>
<td>46.8 ± 4.7</td>
<td>5</td>
</tr>
<tr>
<td>Dibridged IFN</td>
<td>104 ± 9.6</td>
<td>4</td>
</tr>
<tr>
<td>10 kDa monoPEGylated IFN</td>
<td>273 ± 67</td>
<td>6</td>
</tr>
<tr>
<td>20 kDa monoPEGylated IFN</td>
<td>175 ± 35</td>
<td>7</td>
</tr>
<tr>
<td>10 kDa diPEGylated IFN</td>
<td>550 ± 95</td>
<td>4</td>
</tr>
<tr>
<td>20 kDa diPEGylated IFN</td>
<td>678 ± 93</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 4.4 ED50 values observed in the antiviral assays of IFNs shown as mean ± S.E.M.
The reduction in bioactivity of IFN due to the 3-carbon bridges present was not entirely unexpected. Although molecular modeling studies indicated only very minor change in the protein’s secondary structure it was clear from Liberatore et al and del Rosario et al that reaction of a protein with a non-PEG bis-sulfone analogue resulted in considerable intermolecular and non thiol specific reaction. This was presumably due to the presence of DMSO in the solution which may have destabilised the reduced protein. As these experiments with IFN were conducted in the presence of 1% acetonitrile (for solubilising the carboxylic acid bis-sulfone 13a), there is a slight possibility of structural disruption due to the organic solvent. Another possibility could be that the hydrophobic 3-carbon bridge (without PEG) that was present between the thiols might render a different geometrical constraint to the helical chains. This minor conformational change might be sufficient enough to cause some loss of in vitro activity of the protein.

It may also be that once the 3-carbon bridge forms between the cysteines, the protein would have more mobility to adopt the conformation required for activity. Increased mobility was observed while conducting the molecular modeling experiments. Therefore, a possible reason for decreased activity could be the presence of the protruding benzoic acid structure present in the linker (as shown in Figure 4.26). The steric bulk of this moiety could possibly interfere with the binding activity of the protein. Since proteins are known to display significant differences in activity with replacement of even one amino acid (Kerry et al. 1988), the presence of a possibly hydrophobic structure especially between the Cys29 and Cys138 (one of the receptor binding domains) sulfurs might interfere with the receptor binding of IFN. One possibility for future studies would be to utilise compounds that have minimal substituents located off the bridge. It may also be appropriate to form the single and double 3-carbon bridges with PEGylation compounds that can undergo mild decoupling of the PEG to isolate the pure single or double bridged IFNs (e.g. carbonate DB-PEG 14d).

The DB-PEGylated IFN did not appear to reduce the in vitro activity to any greater extent than was observed for other PEGylated IFNs that have been described in the literature. Therefore, the main reduction in DB-PEGylated IFN’s biological activity was mainly due to the steric shielding from the conjugated PEG molecule to IFN.

Grace et al have shown that the His34 PEG positional isomer in PEGINtron® is the most favourable site of PEG conjugation for the IFN to retain its antiviral and antiproliferative activities through the JAK/STAT signaling pathway (Wang et al. 2002; Grace et al. 2005b). However, this is contradicting to other reports which suggest that Leu30, Arg33 and His34 are important residues involved in receptor interactions (Camble et al. 1986; Uze et al. 1994; Uze
et al. 1995; Piehler and Schreiber, 1999; Piehler et al. 2000; Chill et al. 2004; Quad-t-Akabayov et al. 2006). Also, IFN α-2a PEGylated using a 40 kDa branched PEG (PEGASYS®) on Lys31 showed significantly lower antiviral activity (Foser et al. 2003; Dhalluin et al. 2005). Other studies on IFN-IFNAR interactions have shown that the residues involved in receptor binding are residues Met16 to Ser28 (known as domain A), Cys29 to Phe36 (known as domain AB), Glu78 to Asp95 (known as domain C) and Tyr122 to Ala139 (known as the domain D) (Figure 4.53) (Fish, 1992; Waine et al. 1992; Mitsui et al. 1993; Uze et al. 1994; Klaus et al. 1997). Taken together all these results, the high activity observed with His34 isomer of PEGIntron® is not well understood. In the case of DB-PEGylated IFN, there is a high possibility that the Cys29-CCPEGC-Cys138 isomer has markedly lower activity compared to the Cys1-CCPEGC-Cys98 isomer. However, our diPEGylated species did display activity suggesting that both mono-PEGylated IFNs each had activity. It has been shown that the N-terminally PEGylated Cys1 isomer of PEGIntron® showed 11% biological activity in the antiviral assay involving A549 cells and EMCV (Wang et al. 2002). Therefore, it is highly likely that Cys1-CCPEGC-Cys98 isomer of DB-PEGylated IFN α-2b is structurally more favourable for the biological activity.

Figure 4.53. Structure of IFN α-2a showing the receptor binding domains (coloured red) modeled using Maestro® (Figure adapted and modified from Klaus et al. 1997); the structure suggests that DB-PEGylation on Cys29 and Cys138 could adversely affect the biological activity of IFN.
Unlike other studies which report that the activity of PEG-IFN predominantly depends on the size of the PEG (Wang et al. 2002; Caliceti and Veronese, 2003; Grace et al. 2005b), the biological activities of 10 kDa and 20 kDa monoDB-PEGylated IFNs were similar. The reason for this observation might be that due to the site-specific nature of the DB-PEGylated IFNs which are more homogenous than the amine PEGylated IFNs. The claimed activities displayed by PEGINtron® (27 %) and PEGASYS® (7 %) are basically an average of the activities of many positional isomers (Wang et al. 2002; Foser et al. 2003; Grace and Cutler, 2004).

It is also interesting to note that monoDB-PEG20 kDa-IFN has slightly better activity to the monoDB-PEG 10 kDa-IFN. The possible explanation could be that DB-PEG 10 kDa has more access to both the reduced disulfides and therefore could form more of the Cys29-CCPEGC-Cys138 isomer which leads to reduction in activity of the mixture. Since the Cys29 and Cys138 thiols structurally has lower accessibility relative to the N-terminal Cys1 and Cys98 thiols, PEG20 kDa due to its bulkiness and steric hindrance would tend to form more of the Cys1-CCPEGC-Cys98 isomer; the mixture therefore displays better biological activity.

The biological activities shown by the IFN might be different in assays using different cell lines (Hu et al. 1993). Hu et al have shown that the Daudi cell lines have higher sensitivity to IFNs than AU937 cells due to more number of binding sites on the Daudi cell. Therefore it is likely that the biological activity results observed with A549 cells might be different with other cell lines.

Pharmacologically, the decrease in biological activity in vitro is compensated by the increase in the in vivo half-life of the PEGylated protein (Rajender et al. 2002). The in vivo half-life studies on the 20 kDa PEGylated IFN were therefore conducted to study its pharmacokinetic parameters. Subcutaneously injected DB-PEG 20 kDa-IFN showed an elimination half-life (t1/2) of 12.3 h (Figure 4.54) while the native IFN showed an elimination t1/2 of only 0.7 h (Figure 4.54). DB-PEGylation using 20 kDa DB-PEG led to an increase of approximately 18-fold in the half-life of IFN. The DB-PEGylated IFNs, especially the monoPEGylated species, could be used for further clinical studies and development.


<table>
<thead>
<tr>
<th>Dose</th>
<th>20 kDa DB-PEG-IFN</th>
<th>IFN</th>
</tr>
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<tbody>
<tr>
<td>Amount injected</td>
<td>1,280,000 pg (320,000 IU)</td>
<td>1,280,000 pg (320,000 IU)</td>
</tr>
<tr>
<td>$t_{max}$</td>
<td>3.1 h</td>
<td>1 h</td>
</tr>
<tr>
<td>$t_{1/2}$ (abs)</td>
<td>0.9 h</td>
<td>Not determined</td>
</tr>
<tr>
<td>$C_{max}$</td>
<td>2,726 pg/mL (682 IU/mL)</td>
<td>1,688 pg/mL (423 IU/mL)</td>
</tr>
<tr>
<td>$t_{1/2}$ (elim)</td>
<td>12.3 h</td>
<td>0.9 h</td>
</tr>
<tr>
<td>AUC</td>
<td>43,381 pg/mL (10,970 IU h/mL)</td>
<td>2,022 pg h/mL (506 IU h/mL)</td>
</tr>
</tbody>
</table>

Table 4.5 Details of the half-life studies on IFN and 20 kDa monoPEG-IFN

Figure 4.54. Representative graph showing half-life of IFN (red line, •), and 20 kDa PEG-IFN (blue line, ■) as observed in an A549 - EMCV antiviral assay; IFN half-life - < 1 h and 20 kDa PEG-IFN: 12.3 h.

4.5 CONCLUSIONS

The work described in this chapter was the DB-PEGylation of IFN α-2b. The main emphasis was on the preparation and characterisation of disulfide bridged mono-PEGylated IFN. IFN like most other cytokines has two disulfides which are important for its structural stability. The disulfides in IFN were found to undergo reduction with 100 mM DTT in non-denaturing conditions while the protein retained its tertiary structure in solution. DB-PEGylation of the reduced IFN was found to be efficacious with respect to reactant stoichiometry and reactivity. The characteristic disulfide bridging reaction was confirmed with the help of tryptic digestion and MALDI-TOF studies. Aggregation was observed with the reduced IFN during the purification process which was found to decrease upon re-oxidation. The effect of attachment of
a PEG with a three carbon bridge across one of the native disulfide bonds of IFN a-2b was also studied. Molecular modelling suggested that the tertiary structure of IFN is preserved after the insertion of a single 3-carbon methylene bridge with or without the PEG. Circular Dichroism data further supported the theoretical modelling observations and showed that the IFN retained its alpha-helical structure after DB-PEGylation process. The reduced \textit{in vitro} biological activities (~ 8 \% relative to the native IFN) observed with the PEG-IFNs were mainly due to the steric shielding of PEG. DB-PEG 20 kDa attached to IFN had an \textit{in vivo} half-life of 12.3 h which suggested possible development of this molecule for therapeutic use.
Chapter 5

Tailoring the DB-PEGylation process –

DB-PEGylation of reduction unstable leptin
5.1 INTRODUCTION

Leptin or 'OB protein' is mainly produced by the fat cells and secreted into the blood stream (Zhang et al. 1997; Fruhbeck et al. 1998). It is a "pluripotent" molecule with "many lives" but mainly functioning as a mediator regulating the adiposity in the body (Banks, 2004). A high concentration of leptin in the blood indicates the brain regarding high body fat content. This results in a decrease in appetite and increase in energy expenditure. In contrast, low concentrations of leptin in the blood cause an increase in appetite and a decrease in energy expenditure. Therefore, low leptin concentrations mainly initiate conservation of energy reserves for extreme conditions (Flier, 1998). Like other cytokines, leptin is reported to be 'pleiotropic' and have several physiological functions such as reproduction, bone formation, blood pressure control, immune responsiveness, haematopoiesis and angiogenesis (Fruhbeck, 2001).

5.1.1 Structural features of Leptin

Leptin (ob protein) is produced in the body as a 167 amino acid polypeptide encoded by the obesity (ob) gene; this polypeptide is further cleaved to an active 146 amino acid cytokine protein (Madej et al. 1995). The X-ray crystallography determined structure of leptin variant, E100 (single amino acid substitution at the 100th position - glutamic acid for tryptophan) showed that the protein is a four helix bundle with a single disulfide bond between the residues Cys96 in the beginning of the CD loop and the C-terminal Cys146 (Figure 5.1) (Kline et al. 1997; Zhang et al. 1997). The lone disulfide bond in leptin is reported to be important for the structural stability and biological function (Rock et al. 1996; Zhang et al. 1997; Boute et al. 2004). In contrast, the studies reported by Imagawa et al show that the C-terminal deletion or mutation of the cysteines involved in the disulfide formation does not affect the activity of leptin (Imagawa et al. 1998). Hence one reason to do these experiments is to understand if the disulfide bond could be reduced and PEGylated while the protein maintains its tertiary structure and biological function.
The native form of leptin was observed to have a high propensity to aggregate whereas the E100 mutant was stable and readily crystallisable (Zhang et al. 1997). Despite this advantage, the structure published by Zhang et al for E100 is not completely resolved with the structural data of the sequences 1-2 and 25-39 still unknown (Figure 5.2). This could have been due to flexible nature of these peptide segments.

Figure 5.1. Structure of leptin variant, E100 (pdb id 1AX8) showing the disulfide bond between Cys96 and Cys146 (Drawn using Maestro® Schrodinger suite).

Figure 5.2. Sequence details of leptin variant, E100 (pdb id 1AX8, Zhang et al, 1997); missing sequences shown in faded black (Figure adapted from protein data bank).
5.1.2 Mechanism of action of leptin

Leptin acts via leptin receptor (OB-R) which is classified under the class I cytokine receptor family that comprises receptors for G-CSF, IL-6 (Tartaglia, 1997). Several isoforms of OB-Rs (OB-Ra to OB-Rf) have been identified which are similar in their extracellular and transmembrane domain structures (Sweeney, 2002; Tartaglia et al. 1995). These receptors are present mainly in the hypothalamus (Tartaglia, 1997). Leptin acts via the Janus Kinases (JAK) and transcription activators (STAT) pathway (Sweeney, 2002) similar to the other class I receptor activators like interferons and interleukins. This in turn induces the gene expression in the nucleus which is followed by the lipostatic effects.

5.1.3 Rationale for DB-PEGylation of leptin

The rationales for selecting leptin as model protein to further examine the DB-PEGylation are:

1. Leptin has a single disulfide bond between the residues Cys96 and the C-terminal Cys146. The presence of potentially solvent accessible single disulfide bond may be ideal for DB-PEG conjugation studies.

2. The disulfide bond is reported to be important for the protein to maintain its biological activity (Zhang et al. 1997) since protein structure is easily lost on disulfide reduction or modification. Unlike IFN, the disulfide bond in leptin is present between the flexible CD loop and the loop of D helix (Figure 5.1). Disulfide reduction is an important step in the DB-PEGylation process. Therefore, this study would help in understanding the biological effects of disulfide reduction followed by DB-PEGylation of this structurally important disulfide bond.

3. Leptin has a short in vivo circulation half-life (Zeng et al. 1997). PEGylation of leptin has been reported to extend the protein’s half-life and improve the clinical efficacy in humans (Hukshorn et al. 2000). Since leptin has only one disulfide bond, reduction and DB-PEGylation of leptin would give a homogenous product of defined activity.

4. This study would further help in the evaluation of DB-PEGylation approach in preparing clinically relevant PEGylated proteins.

5.2 MATERIALS

1. Recombinant leptin (Sigma-Aldrich, cat. no. L4146)
2. Dithiothreitol (Sigma-Aldrich, cat. no. 43819, m.w – 154)
3. Sodium dihydrogen orthophosphate dihydrate (Na$_2$H$_2$PO$_4$·2H$_2$O, Fisher Scientific UK cat. no. S/3760/53)
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4. L-arginine (Sigma-Aldrich, cat. no. W381918)
5. Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich-Fluka, cat. no. 03682)
6. Reduced L-glutathione (Sigma-Aldrich, cat. no. G4251)
7. Oxidised L-glutathione (Sigma-Aldrich, cat. no. G4376)
8. PD-10® column (GE Healthcare, cat. no. 17-0851-01)
9. 1 mL HiTrap SP FF (GE Healthcare, cat no. 17-5054-01)
10. HiLoad 16/60 Superdex 200 pg column (GE Healthcare, cat. no. 17-1069-01)
11. Novex Bis-tris 12 % gels (Invitrogen, Cat. no. NP001 BOX)
12. Colloidal blue staining kit (Invitrogen, Cat. no. LC6025)
13. 0.1 M Perchloric acid
14. 5 % Barium chloride solution
15. MicroBCA assay kit (Pierce, Cat. no. 23235)
16. Glacial acetic acid (BDH)
17. Sulfuric acid, concentrated
18. Eppendorf tubes, 1.5 mL and 2 mL
19. UV spectrophotometer
20. Peristaltic pump
21. HPLC system with UV detector
22. SDS-PAGE system (BDH)

5.3 METHODS

DB-PEGylation process involves the three main steps which are 1) disulfide reduction, 2) DB-PEGylation and 3) purification of the reaction mixture. The lone disulfide bond in leptin was reduced with DTT 16. However, reduction of this disulfide bond was found to result in the precipitation of the protein out of the solution. This was mainly attributed to the increase in intermolecular interactions upon reduction. L-arginine at a concentration of 2 M in the buffer was therefore used to prevent aggregation after reduction. This way, reduction without precipitation was achieved. To prevent aggregation, the modified buffer (with 2 M L-arginine) was used until after the DB-PEGylation step. Reduction was ascertained by SDS-PAGE analysis. Reduced leptin was then allowed to react with 1 eq. of PEG bis-sulfone 14a. The reaction mixture was analysed initially by SDS-PAGE, then purified by ion exchange chromatography and characterised by SEC-HPLC and MALDI-TOF MS. Due to lack of time in the project, biological characterisation of the prepared conjugate could not be conducted. However, chemically the DB-PEGylation process could be further optimised for a protein which was unstable upon disulfide reduction.
Human recombinant leptin expressed in *E.coli* was procured from Sigma-Aldrich as a lyophilised product with phosphate buffer salts. When 20 mM sodium acetate buffer, pH 4.0 was added directly to the lyophilised product, the solids were found to be insoluble. This insolubility could have been due to ionic interactions between protein molecules. Therefore, the protein was dissolved initially in acidified water followed by adjusting the pH to 5.2 for storage purposes (Sigma-Aldrich product literature).

5.3.1 Modified reduction and DB-PEGylation of leptin with PEG bis-sulfone 14a

To a solution of leptin (0.5 mg/mL, 1 mL) in 50 mM sodium phosphate buffer containing 2 M arginine and 10 mM EDTA, pH 7.8 was added DTT 16 (100 mM, 15.4 mg). The solution was incubated for 30 min at ambient temperature and the DTT 16 was then removed with a PD-10 Desalting column. The solution formed was incubated at ambient temperature (at still and dark conditions) for 30 min and the DTT 16 then removed with a PD-10 Desalting column. For this, the leptin-DTT 16 solution (1 mL) was loaded onto the PD-10 Desalting column (pre-equilibrated with 25 mL of 50 mM sodium phosphate buffer containing 2 M arginine and 10 mM EDTA, pH 7.8) while discarding the eluent (1 mL) from the bottom. After this, fresh buffer (2 mL of 25 mL of 50 mM sodium phosphate buffer containing 2 M arginine and 10 mM EDTA, pH 7.8 – wash volume) was added and the eluent (2 mL) discarded again. Fresh buffer (2.5 mL of 25 mL of 50 mM sodium phosphate buffer containing 2 M arginine and 10 mM EDTA, pH 7.8) was added to the column and the eluent (2.5 mL) collected in a 15 mL Corning® centrifuge tube carefully letting the droplets trickle through sides of the tube. The collected solution was analysed for its UV absorbance at 280 nm (relative to the buffer containing arginine). Reduction was ascertained by SDS-PAGE analysis.

To the reduced protein solution (~ 0.2 mg/mL as measured by UV \textsubscript{280 nm} 2.5 mL), was added PEG bis-sulfone 14a (1 equivalent, ~ 20 µL from a 13.4 mg/mL solution of 10 kDa PEG bis-sulfone 14a in sodium acetate buffer) and gently swirled for 1 min. The reaction was kept still at 4 °C for a time period of 8 h and then GRS (50 µL of 50 mM GSH 26; 50 mM GSSG 27 in 20 mM sodium acetate buffer) was added to the reaction mixture and allowed to react for a further 16 h.

5.3.2 Ion exchange purification of leptin conjugates

The reaction solution (2.5 mL) was loaded onto a PD-10 Desalting column that was pre-equilibrated with 25 mL of 20 mM sodium acetate buffer, pH 4.0. The PD-10 Desalting column was then eluted with fresh 20 mM sodium acetate buffer, pH 4.0 (3.5 mL) while collecting the
eluent in the same Corning® tube used for the conjugation reaction. This solution was then drawn into a 5 mL syringe with the help of a needle. The tube was washed again with fresh 20 mM sodium acetate buffer, pH 4.0 (1.5 mL) and pooled to the 3.5 mL reaction solution previously drawn. The total solution (5 mL) was then loaded manually into the ion exchange column with the help of a syringe at a flow rate of 1 mL/min. The column was washed with 20 mM sodium acetate buffer, pH 4.0 (2 mL) at a flow rate of 1 mL/min with the help of a peristaltic pump. A fresh syringe was loaded with 20 mM sodium acetate buffer, pH 4.0 containing 1 M sodium chloride (5 mL) and attached to the ion exchange column. The buffer in the syringe was then injected manually at a flow rate of 1 mL/min. The initial 1 mL eluent was discarded. The next 2 mL eluent (which contains the protein and the conjugation mixture) was then collected in a 2 mL eppendorf tube. The ion exchange purified solution (1.9 mL) was then injected into SEC-HPLC for isolation of the conjugates and the protein.

5.3.3 Stability of DB-PEG attached to leptin

MonoPEGylated leptin (10 kDa PEG) was collected (~ 8 mL) from the SEC-HPLC and concentrated to 1 mL with a Vivaspin® concentrator. The resulting solution was buffer exchanged to 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 using a PD-10 Desalting column. This gave 2.5 mL which was further concentrated to 250 µL using Vivaspin® concentrator. From the resulting solution, 30 µL was frozen and stored for reference and the remaining 220 µL was incubated at 37 °C for 7 days (168 h) with constant shaking. Both solutions were further analysed by SDS-PAGE for stability of the PEG linker attached to the protein.

5.3.4 Characterisation of leptin conjugates

All the techniques used for the characterisation of leptin conjugates such as SDS-PAGE, SEC-HPLC, Sample concentration using Vivaspin® 6 mL, MALDI-TOF-MS and microBCA were exactly as described for IFN conjugates (Chapter 4).

5.4 RESULTS AND DISCUSSIONS

5.4.1 Preparation of leptin solution and disulfide reduction

Leptin has a disulfide bond between the residues Cys96 in the CD loop and the C-terminal Cys146. The location of this disulfide bond involving the terminal cysteine suggests that the solvent accessibility is high for which facile reduction could occur. When leptin was subjected
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to disulfide reduction using 100 mM DTT 16, the solution was observed to turn cloudy due to protein precipitation. Reducing the disulfide on the flexible loop may expose the buried hydrophobic residues of the polypeptide chain. This would lead to an increase in the intermolecular hydrogen bonding interactions which along with leptin's innate hydrophobic nature (Zhang et al. 1997) causes precipitation. However, no precipitation was reported with the mutated leptin in which the disulfide cysteines were replaced with serine (Imagawa et al. 1998). It is well known that lyophilised (dehydrated) proteins have altered structural conformation (Prestrelski et al. 1993). Water molecules within the structure are important for the protein to maintain its conformation (Takano et al. 2003). Therefore, lyophilisation could be one factor influencing the precipitation during the reduction of leptin's structurally important disulfide bond.

Due to this instability, significant protein loss would occur during the PD-10 desalting step to remove DTT 16. This is not appropriate for the DB-PEGylation process. An aggregation retardant might prevent such intermolecular interactions. Several amino acids have been observed to impart stability to proteins in solutions (Taneja and Ahmad, 1994). Among them, L-arginine at a non-denaturing concentration of 0.5 – 4 M is reported to be effective in reducing protein aggregation (Rudolph and Lilie, 1996; Shiraki et al. 2002; Arakawa et al. 2003; Arakawa and Tsumoto, 2003; Bondos and Bicknell, 2003; Shiraki et al. 2004). L-arginine has a guanidino side group similar to guanidine HCl which prevents protein intermolecular interactions and allows the protein to remain disaggregated and in solution (Arakawa and Tsumoto, 2003; Shiraki et al. 2002). The lyophilised leptin formulation was found to be completely soluble in 50 mM sodium phosphate buffer containing 2 M L-arginine and 10 mM EDTA, pH 7.8. Furthermore, no instability was observed upon treatment of the protein solution with 100 mM DTT 16 in this modified buffer. Up to 93 % of leptin was recovered after the PD-10 desalting of the DTT 16-leptin solution (as assayed by UV280 nm). MicroBCA was not used for the quantitation in this case since arginine present in the buffer showed interference during the assay.

5.4.2 DB-PEGylation of leptin in the presence of L-arginine

Since no precipitation was observed with reduced leptin in 50 mM sodium phosphate buffer containing 2 M L-arginine and 10 mM EDTA, pH 7.8, this buffer was used throughout the reaction process including the reduction and DB-PEGylation steps. Since PEG bis-sulfone 14a specifically reacts to thiols to form a stable thioether bond, the L-arginine present in the buffer was not expected to compete with thiol addition to 14a. Once the reduced protein was separated from the DTT 16, DB-PEGylation was conducted with 1 eq. of the PEG bis-sulfone 14a for 8 h.
Chapter 5. DB-PEGylation of Leptin

at 4 °C. The disulfide reduction and conjugation efficiency were then analysed by SDS-PAGE using a Novex bis-tris 12% PAGE gel. The reduced protein had a slightly larger apparent size relative to the native leptin in the gel. However, the difference in protein migration in the gel was not very distinct (Figure 5.3, lanes 2 & 3) as observed with the reduced IFN. The control reaction of native leptin and 10 kDa PEG bis-sulfone 14a showed no protein bands other than the native protein (Figure 5.3, lane 4). This suggested no conjugation of PEG bis-sulfone 14a to amine nucleophiles in the protein. The reaction mixture of reduced leptin with 10 kDa PEG bis-sulfone 14a showed the presence of possible monoPEGylated leptin, diPEG leptin and cross-linked PEG-leptin (Figure 5.3, lane 5). The reaction mixture after 8 h of incubation was then exposed to re-oxidising condition using 1 mM GSH 26: 1mM GSSG 27 for further 16 h. Re-oxidation step was mainly conducted in this case to re-oxidise any unreacted leptin in the mixture for SEC-HPLC isolation. Reduced leptin if present in the mixture would induce aggregation during the purification step. If the reduced leptin is completely consumed while PEGylation, the re-oxidation step could be avoided.

Figure 5.3. SDS-PAGE analysis of leptin and 10 kDa PEG bis-sulfone 14a reaction; Novex bis-tris 12% gel stained with colloidal blue (left) and barium iodide (right); lane 1 – protein molecular weight standards, lane 2 – native leptin, lane 3 – reduced leptin, lane 4 – reduced leptin incubated with 1 eq. 10 kDa PEG bis-sulfone 14a for 24 h at ambient temperature, lane 5 – reduced leptin reaction with 1 eq. 10 kDa PEG bis-sulfone 14a, lane 6 – solution with 1 eq. 10 kDa PEG bis-sulfone 14a.

The isoelectric point of leptin is approximately 6.0 (similar to IFN α-2b) (Lo et al. 2005). Cation exchange chromatography was chosen as the method of purification to remove any unreacted PEG from the mixture. The ion exchange purified solution was then subjected to SEC-HPLC for conjugate isolation. The peaks of interest were the ones with the peak maxima at 47.90 min indicating soluble aggregation, 61.05 min indicating diPEG-leptin, 71.28 min...
indicating monoPEG-leptin and 101.98 min indicating the native protein (Figure 5.4). These manually collected peaks (~8 mL) were then desalted and concentrated for SDS-PAGE and MALDI-TOF analyses.

![Figure 5.4. SEC-HPLC chromatogram of ion exchange purified reaction mixture of reduced leptin and 1 eq. 10 kDa PEG bis-sulfone 14a. Peaks observed were collected manually and identified by SDS-PAGE and MALDI-TOF analyses.](image)

The eluent with its peak maximum at 71.28 min appeared near the marker of 39 kDa molecular weight in the PAGE (Figure 5.5, lane 5). This compound was further confirmed as monoPEG-leptin by MALDI-TOF MS analysis. The average molecular mass of this PEGylated protein was found to be 27,010 Da (Figure 5.7), an increase of 10,860 Da to the mass of native leptin, 16.150 Da (Figure 5.6). Interestingly, the eluent in the SEC-HPLC with its peak maximum at 61.05 min was observed to be mainly cross linked leptins with two PEG molecules attached. The calculation is based on the mass observed (53,903 Da) in MALDI-TOF spectrum (Figure 5.8) and appearance in the SDS-PAGE gel (Figure 5.5, lane 4). Also observed in the MALDI-TOF spectrum is the presence of diPEGylated leptin with molecular mass of 38,006 Da (Figure 5.8). The elution with its peak maximum at 47.90 min was found to be protein aggregation (Figure 5.5, lane 3). Another interesting fact is that leptin and its 10 kDa DB-PEG conjugates were found to elute in the SEC-HPLC column at the same time as observed with the 10 kDa DB-PEG conjugates of IFN. Also, the conjugates of IFN and leptin appeared at the same
positions (± 1 min) in the SDS-PAGE gel (Figure 5.5, lane 5). This suggested that once a protein of molecular mass range of ~15 – 20 kDa is PEGylated, elution of the conjugate in SEC-HPLC and migration in SDS-PAGE gel are largely defined by the PEG attached to it.

**Figure 5.5.** Identification of the SEC-HPLC conjugate fractions; A) SEC-HPLC chromatogram showing the conjugate fractions; B) SDS-PAGE gel (Novex Bis-Tris 12%) of leptin and DB-PEG 10 kDa reaction stained with colloidal blue (left) and silver staining (right); Lane 1 – Protein molecular weight standards, Lane 2 – Ion exchange purified mixture, Lane 3 – SEC fraction 47.32 min, Lane 4 – SEC fraction 60 min, Lane 5 – SEC fraction 70 min, Lane 6 – SEC fraction 100 min.

DB-PEG cross-linked leptin molecules were found to be a by-product of 1 equivalent DB-PEGylation reaction. Although this could be considered as further evidence for the bridging reaction, the intermolecular reaction is not usually desired in the DB-PEGylation process. Since the thiols of the reduced disulfide in leptin are completely exposed, two molecules of PEG bis-sulfone 14a could react to each of the thiols. This is evident from the presence of diPEGylated species in the MALDI-TOF spectrum (Figure 5.8). The diPEGylated species which is unable to complete the bridging reaction could react to one more of the reduced leptin molecule making it 2:2 PEG: leptin complex with a mass of 53,903 Da (Figure 5.8). Due to the presence of two PEG molecules present, the complex elutes in the SEC-HPLC at the same time as that of the diPEGylated leptin species. However, in the SDS-PAGE the complex shows up just above the 64 kDa molecular weight marker (Figure 5.5, lane 4). The intermolecular cross-linking reaction is a limitation in the DB-PEGylation of leptin. This could be mainly due to the structural features of the disulfide reduced leptin.
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Figure 5.6. MALDI-TOF spectrum of leptin (16,150 Da)

Figure 5.7. MALDI-TOF spectrum of 10 kDa DB-PEGylated leptin (27,010 Da)
As observed in the SDS-PAGE, the 1 equivalent of 10 kDa PEG bis-sulfone 14a with reduced leptin reaction mainly had monoPEGylated leptin product along with some amount of cross-linked leptin complex. Reduced leptin when treated with 2 equivalents or more of 10 kDa PEG bis-sulfone 14a, showed the diPEG-leptin to be the major product of the reaction (Figure 5.9).

As observed with the IFN reactions, glutathione re-oxidation is a vital step in the DB-PEGylation of leptin as well. Reaction of reduced leptin with 1 equivalent of the PEG reagent when not treated with glutathione was observed to have high amounts of aggregates during
SEC-HPLC analysis (*Appendix 3*). The aggregation is mainly due to the presence of unreacted reduced protein in the solution. Reaction with 1 eq. of PEG bis-sulfone 14a typically leaves some amount of unreduced protein (~ 25%). This reduced protein might also form aggregates with the PEGylated protein during the purification step. Therefore the glutathione re-oxidation is necessary to re-oxidise the unreacted leptin thus maximising the product yield during the SEC-HPLC isolation step (*Figure 5.10*).

![SEC-HPLC of ion exchange purified reaction mixture of reduced leptin and 1.3 equivalent of 10 kDa PEG bis-sulfone 14a.](image)

The amount of unreacted disulfide reduced protein can be reduced by carefully optimising the amount of PEG bis-sulfone required for the reaction. Reaction of the reduced protein with 1.3 eq. of 10 kDa PEG bis-sulfone 14a was observed to show reduction in the amount of the unreacted reduced protein. Due to the less amount of reduced protein in the mixture, the amount of aggregates was also found to be less. However, the amount of diPEGylated species increased in the mixture. An alternative to maximise the amount of monoPEGylated leptin in the mixture would be the addition of 1 eq. PEG bis-sulfone 14a as small equal amount (for example, 0.25 eq.) to the reaction solution at a time interval of 15 min each between additions. However, this hypothesis was not verified during this study.
5.4.3 Stability of DB-PEG attached to leptin

The monoPEGylated leptin (10 kDa PEG) incubated for 7 days at 37 °C was found to be stable to PEG detachment at near physiological pH (pH 7.8). Only very small amount of PEG was found to be cleaved from the leptin as determined by SDS-PAGE analysis and silver stained gel (Figure 5.11). This showed that the PEG attached to the leptin was stable to hydrolytic cleavage at pH 7.8.

![Figure 5.11 SDS-PAGE analysis of the stability of PEGylated leptin incubated for 7 days in 50 mM sodium phosphate buffer, pH 7.8; Novex bis-tris 12 % gel stained with colloidal blue; lane 1 - 10 kDa DB-PEG-leptin, lane 2 - 10 kDa DB-PEG-leptin after 7 days at pH 7.8 and 37 °C, lane 3 - Protein standards.]

5.5 CONCLUSIONS

Leptin has a lone disulfide bond which is required for the maintenance of its tertiary structure. Precipitation was observed when the disulfide bond in leptin was reduced in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8. The precipitation was mainly due to the increased intermolecular interactions upon disulfide reduction. Reduction of the disulfide bond in leptin without precipitation was possible only in a buffer supplemented with 2 M L-arginine. Reduced leptin was then DB-PEGylated with PEG bis-sulfone 14a in the presence of 2 M L-arginine. L-arginine did not decrease or interfere with the conjugation process. PEG bis-sulfone 14a did not undergo conjugation when incubated with the native leptin. DB-PEGylation resulted in the formation of small amounts of cross-linked leptin. However, a high yield of site-specifically monoPEGylated leptin (67 %) was prepared by this method. The prepared DB-PEG-leptin was observed to be stable at pH 7.8 and 37 °C for 7 days. Further in vitro biological characterisation of the disulfide bridged PEG-leptin is required to evaluate the potential clinical utility of the prepared conjugate.
Chapter 6

Tailoring the DB-PEGylation process –

DB-PEGylation of somatostatin at acidic pH
6.1 INTRODUCTION

Somatostatin 96 or ‘somatropin release inhibiting factor’ (SRIF) is a naturally occurring peptide (Brazeau et al. 1973) which acts on the anterior pituitary lobe and inhibits the release of growth hormone (GH), glucagon and insulin (Reichlin, 1983). The peptide is secreted by cells in the GI tract and neurons in the nervous system. Somatostatin 96 is used mainly in the treatment of neoplasms, gigantism and acromegaly (Reichlin, 1983).

Somatostatin 96 is a cyclic peptide comprising of 14 amino acids (Figure 6.1). The residues 7–10 of somatostatin 96 have been identified as its receptor binding site (Veber et al. 1979). The disulfide bond between the residues Cys3 and Cys14 provides a conformational constraint for the peptide to be biologically active (Veber et al. 1981).

Figure 6.1. Structure of somatostatin 96 and reduced somatostatin 97; the residues essential for biological activity are shown in circles.

The main limitation observed with the systemic administration of somatostatin 96 is its short in vivo half-life due to enzymatic degradation (Peters, 1982). Octreotide is an 8-amino acid analogue of somatostatin 96 which has better systemic half-life (Harris, 1994). PEGylation is another method to improve the pharmacokinetics of a peptide by prolonging its systemic circulation time (Harris et al. 2001). Several peptides like insulin (Hinds and Kim, 2002), calcitonin (Lee et al. 1999a; Lee et al. 1999b), growth hormone-releasing hormone (GHRH) (Esposito et al. 2003; Munafo et al. 2005), octreotide (Na and DeLuca, 2005; Na et al. 2005) glucagon (Stigsnaes et al. 2006) have been PEGylated with low molecular weight mPEGs (2–5 kDa) and characterised for their biological activity, physical stability and pharmacokinetics. Somatostatin 96 ‘dextranylated’ using a single chain of 70 kDa dextran molecule was observed to retain its biological activity and have an in vivo half-life of ~ 27 h (Wulbrand et al. 2002). These studies suggest that polymer attachment to smaller peptides could be useful in preventing enzymatic degradation and thus prolonging its systemic circulation. DB-PEGylation technique could therefore be applicable to therapeutically relevant cyclic peptides with disulfide bonds like somatostatin 96 and octreotide.
The rationales for choosing somatostatin 96 as a model for DB-PEGylation process could be listed as:

1. Somatostatin 96 has a single accessible disulfide bond between the residues Cys3 and the C-terminal Cys14. This disulfide bond could be reduced using stoichiometric equivalents of TCEP HCl 90 and then used for DB-PEGylation.

2. It might be possible to analyse the 3-carbon disulfide bridging reaction more accurately by reflectron mode MALDI-TOF-MS studies. Therefore, this study will be mainly a chemical exercise and no biological characterisation of the prepared conjugate will be conducted.

3. Somatostatin 96 is affordable and easily available (1 mg and 5 mg lyophilised powder in vials, Sigma-Aldrich).

6.2 MATERIALS

1. Somatostatin (Sigma-Aldrich, cat. no. S9129, m.w - 1637.88 Da)
2. TCEP, HCl (Sigma-Aldrich, cat. no. 93,284, m.w. 286.65)
3. Trifluoroacetic acid (Sigma-Aldrich, cat. no. 299,537)
4. C18 Phenomenex column (5 μm, 250 x 4.6 mm)
5. Sephadex G-25 (PD-10) desalting column (Amersham-Biosciences)
6. Acetonitrile, HPLC grade (Fisher Scientific)
7. Deionised water
8. HPLC

6.3 METHODS

Somatostatin 96 is a peptide with a molecular mass of 1,637.9 Da. Efficient separation of the disulfide reductant from the peptide by PD-10 Desalting column requires the peptide molecular mass to be higher than 5,000 Da. Therefore, the usual desalting procedure using a PD-10 Desalting column to separate the disulfide reductant from the native polypeptide was not possible. A non-interfering disulfide reductant which does not need to be removed from the reaction solution prior to the DB-PEGylation process would be a good alternative strategy. TCEP HCl 90, a phosphine reductant is reported to reduce protein disulfides at stoichiometric equivalent concentrations. Removal of the reductant from the reaction buffer is usually not necessary since the reductant is irreversibly oxidised and therefore non-nucleophilic and unreactive (Getz et al. 1999). Somatostatin 96 was initially reduced with 2 eq. of TCEP HCl 90 in 50 mM sodium phosphate buffer containing 10 mM EDTA. Since Ellman’s assay for thiols would have interference from the reactive TCEP HCl 90, assessment of the reduction was
possible only by the distinct smell of thiols in the reaction solution. Since separation of the TCEP HCl 90 from the reduced peptide would require tedious purification by RP-HPLC, it was not attempted.

As observed in the case of reduced leptin, upon disulfide reduction, reduced somatostatin 97 precipitated out of the solution. This might be because somatostatin 96 (1 mg) procured from Sigma-Aldrich was also a lyophilised product. But unlike leptin, it was possible to reduce somatostatin 96 in 50 mM sodium phosphate buffer, pH 6.2 using 2 eq. of TCEP HCl 90 without denaturation. However, conjugation with PEG bis-sulfone 14a was not possible at this pH due to its slow elimination (Chapter 2). PEGylation with PEG mono-sulfone 15a is possible at pH 6.0 as observed with the initial interferon conjugation studies (Chapter 4). But PEG mono-sulfone 15a reaction with GSH 26 at pH 6.2 for 2 h (short reaction period) suggested that the second elimination of PEG mono-SG conjugate 78 was inefficient (Chapter 2). Therefore, the experiments conducted with reduced somatostatin 97 were to study if PEGylation with PEG mono-sulfone 15a and the 3-carbon bridging with carboxylic acid bis-sulfone 13a are possible at a slightly acidic pH (≈ pH 6.0) with longer incubation periods.

6.3.1 DB-PEGylation of somatostatin

Preparation of PEG mono-sulfone 15a by RP-HPLC purification: To a 2 mL eppendorf tube containing 50 mM sodium phosphate buffer, pH 7.8 (1 mL), was added PEG bis-sulfone 14a (25 mg, 4.55 μmol; 5,500 g/mol) and mixed to dissolve. The solution was incubated for 20 h and purified by reverse phase HPLC using a C18 Phenomenex column (5 μm, 250 × 4.6 mm) (gradient of 30 - 60% acetonitrile in 30 min at a flow rate of 1 mL/min with UV detection at 215 nm). The collected PEG mono-sulfone 15a fraction (2 mL) was diluted (to 10 mL) with acidified water and subjected to freeze-drying to obtain the solid PEG mono-sulfone 15a (0.7 mg).

DB-PEGylation: To a solution of somatostatin 96 (0.25 mg/mL, 0.15 μmol) prepared in 50 mM sodium phosphate buffer, pH 6.2 was added TCEP HCl 90 (44 μg, 0.15 μmol, 9 μL of a 5 mg/mL solution) for 1 h at ambient temperature. PEG mono-sulfone 15a (0.7 mg, 0.13 μmol, 0.86 eq.) was then added to the reduced peptide. The reaction solution was gently swirled to mix and left overnight at 4 °C. The solution was then subjected to desalting to deionised water using a PD-10 Desalting column. The conjugate fraction (third 1 mL fraction with maximum amount of conjugate) was analysed by MALDI-TOF-MS. A control incubation of PEG bis-sulfone 14a (0.7 mg) with native somatostatin 96 (0.25 mg/mL) in 50 mM sodium acetate buffer, pH 7.8
was also conducted for 16 h at 4 °C. After 16 h, the solution was subjected to PD-10 desalting. The third 1 mL fraction (with the maximum amount of conjugate) was analysed by MALDI-TOF-MS.

6.3.2 Preparation of 3-carbon disulfide bridged somatostatin

To a 5 mL squat vial, was taken carboxylic acid bis-sulfone 13a (5.7 mg) and dissolved in 2:3 (v/v) acetonitrile: 50 mM sodium phosphate buffer (prepared using D$_2$O), pH 8.0 (1 mL). The solution was incubated for 24 h at ambient temperature. The formation of the corresponding mono-sulfone 98 was monitored by $^1$H-NMR (Appendix 2) and the pH then adjusted to 6.2 using 1 N HCl. Somatostatin 96 (0.25 mg/mL, 1 mL) in 2:3 (v/v) acetonitrile:50 mM sodium phosphate buffer, pH 6.2 was disulfide reduced using 2 eq. of TCEP HCl 90 for 1 h at ambient temperature. The solution containing ≈ 1.4 eq. of the mono-sulfone 98 moiety was added to the reduced peptide 97. The reaction solution was gently shaken and left still overnight at 4 °C. The solution was then subjected to freeze-drying to remove solvent. The solids were re-solubilised in water and filtered (0.2 μm). The filtrate was subjected to reversed phase (C$_{18}$) HPLC using acetonitrile/water solvent system. The 3-carbon bridged somatostatin 99 was purified from the reaction solution by reversed-phase HPLC using C$_{18}$ Phenomenex column (5 μm, 250 × 4.6 mm) with HPLC grade water containing 0.05 % TFA as solvent A and HPLC grade acetonitrile containing 0.05 % TFA as solvent B. The gradient used was 20 % to 40 % solvent B in 10 min. The flow rate of 1 mL/min, column temperature of 25 °C and UV detection at 215 nm was used for the analysis. The peptide peak fraction (retention time – 8.56 min) was collected and then characterised by MALDI-TOF MS.

6.4 RESULTS AND DISCUSSION

6.4.1 Disulfide reduction and DB-PEGylation of reduced somatostatin

Somatostatin 96 is a low molecular weight peptide comprising of only 14 amino acids with a disulfide bond between Cys3 and C-terminal Cys14. Disulfide reduction was expected to be relatively easy due to the structurally accessible disulfide bond. However, efficient removal of excess of DTT 16 by PD-10 desalting before the PEGylation step is possible only if the peptide has a molecular weight above 5,000 Da. This is because the exclusion limit for the PD-10 Desalting column is 5,000 Da. TCEP HCl 90, a phosphine compound is known as a stoichiometrically efficient reductant unreactive to thiol-specific reagents (Getz et al. 1999). The use of TCEP HCl 90 was considered therefore ideal for the disulfide reduction of somatostatin 96 and subsequent PEGylation with PEG mono-sulfone 15a 5 kDa. When
somatostatin 96 was reduced with TCEP HCl 90 in 50 mM sodium phosphate buffer containing 10 mM EDTA, pH 7.8 was observed to form cloudy precipitates in solution. This property was not observed when the peptide was reduced in 50 mM sodium phosphate buffer, pH 6.2. This could be because the intramolecular hydrogen bonding interactions stabilises the molecule from denaturation at pH 6.2. The isoelectric point of somatostatin is ~ 9.5 and the peptide is reported to be more stable in acidic pH (Herrmann and Bodmeier, 2003).

The initial elimination reaction observed with PEG bis-sulfone 14a is slow at pH 6.2 (Chapter 2). Therefore, the use of PEG mono-sulfone 15a was considered for reaction with reduced somatostatin 97. The hypothesis is that PEG mono-sulfone 15a could be used for DB-PEGylation at slightly acidic pH depending on the stability of the protein in use. As observed with reduced IFN at pH 6.0, the α, β alkenyl functional group present in PEG mono-sulfone 15a is highly reactive to thiol nucleophiles. Therefore, the initial thiol addition reaction was expected to occur relatively quickly. PEG mono-sulfone 15a was prepared from 5 kDa PEG bis-sulfone 14a in 50 mM sodium phosphate buffer, pH 7.8. A slightly basic buffer was required to initialise the characteristic elimination reaction of PEG bis-sulfone 14a. Pure PEG mono-sulfone 15a was then isolated prior to the PEGylation process by reversed phase HPLC (Figure 6.2) followed by freeze drying of the collected fraction.

![Figure 6.2. RP-HPLC chromatogram of PEG bis-sulfone 14a reaction mixture; eluting peaks of interest are (1) p-tolyl sulfinic acid, (2) product 74, (3) PEG mono-sulfone 15a and (4) PEG bis-sulfone 14a. Fraction containing the PEG mono-sulfone 15a was collected and freeze dried to obtain the pure PEG mono-sulfone 15a (0.7 mg).](image-url)
The thiol addition reaction of PEG mono-sulfone 15a occurs much faster at pH 6.2. This was evident with the PEG mono-sulfone 15a reactions with GSH 26 and IFN in ≈ pH 6.0 buffer (Chapter 2 and Chapter 4 respectively). But the second elimination required to complete the bridging reaction was not an immediate process as observed with the PEG mono-S>G 72 at pH 6.2 (Chapter 2). This is because the elimination reaction is slow at pH 6.0 (Chapter 2). Therefore, reaction of PEG mono-sulfone 15a with reduced somatostatin 96 would require longer incubation periods to undergo completion. The PEG mono-sulfone 15a and the reduced somatostatin reaction mixture at pH 6.2 buffer was therefore incubated overnight at 4 °C. The reaction solution was then buffer exchanged to water by simple desalting by PD-10 Desalting column and analysed by MALDI-TOF MS.

**Figure 6.3.** MALDI-TOF spectrum of monoPEGylated somatostatin; also seen is un-reacted PEG.
Chapter 6. DB-PEGylation of Somatostatin

Figure 6.4. RP-HPLC chromatogram showing the PEG \textit{mono-sulfone} 15a reaction with reduced somatostatin 97; UV detection at 215 nm. Eluting peaks of interest are (1) buffer salts, (2) p-tolyl sulfonic acid 25 and (3) PEGylated somatostatin as determined by MALDI-TOF-MS.

The MALDI spectrum showed the presence of monoPEGylated somatostatin (PEG mass of \(\approx 5,000 \text{ Da} + \text{peptide mass of 1,638 Da} = \approx 6,631 \text{ Da}\)) along with some amount of unreacted PEG (= 5,250 Da) (Figure 6.3). The unreacted somatostatin 97, if any, was removed by the PD-10 desalting procedure. Typically the reaction solution (without PD-10 desalting) when analysed by reversed-phase HPLC showed the presence of both unreacted somatostatin 97 and the PEG-peptide conjugate (Figure 6.4). Interestingly no unreacted PEG \textit{mono-sulfone} 15a was observed in the reaction mixture. This suggested there may have been some discrepancy in the amount of PEG used in the reaction. Therefore, the unreacted PEG observed in the MALDI-TOF spectrum could be a result of incomplete disulfide reduction of the total amount of available peptide and a possible deactivation of PEG \textit{mono-sulfone} 15a by the reactive TCEP HCl 90.

The primary objective of these experiments was to determine if PEGylation of reduced somatostatin with PEG \textit{mono-sulfone} 15a occurred at pH 6.2. It was possible that ‘rebridging’ could have been competitive with bis-PEGylation. However, with this experiment it was not clear whether the 3-carbon bridging reaction between the thiols of reduced somatostatin 97 occurred or not. The presence of no diPEGylated somatostatin in the MALDI-TOF spectrum after the PD-10 desalting process suggests the possibility of 3-carbon bridge formation. This is
Chapter 6. DB-PEGylation of Somatostatin

because a 3-carbon bridged conjugate would have no more thiol available for another PEG mono-sulfone 15\(\text{a}\) to react. However, the presence of only monoPEGylated somatostatin does not necessarily mean completion of the 3-carbon bridging reaction. It is possible that the conjugated PEG sterically prevents the approach of another molecule of PEG mono-sulfone 15\(\text{a}\) to the second thiol. Also, the use of one equivalent of PEG mono-sulfone 15\(\text{a}\) to reduced somatostatin 97 may have favoured the reaction of one peptide with one PEG mono-sulfone 15\(\text{a}\). Therefore, it was necessary to study the 3-carbon bridging reaction at pH 6.2 using carboxylic acid mono-sulfone 98 (Figure 6.5).

![Figure 6.5. Structure of carboxylic acid bis-sulfone 13\(\text{a}\) and carboxylic acid mono-sulfone 98](image)

6.4.2 Preparation of 3-carbon disulfide bridged somatostatin 99

Reaction of a reduced protein with carboxylic acid bis-sulfone 13\(\text{a}\) followed by MALDI-TOF analysis is one method to analyse the 3-carbon bridging reaction. This was shown with the earlier studies with IFN (Chapter 4). Reflectron mode MALDI-TOF MS is an accurate method to analyse a low molecular weight peptide (Courchesne and Patterson, 2002). MALDI-TOF MS analysis on reflectron mode of somatostatin 96 showed an exact mass of 1,638.10 Da (Figure 6.8A). The hypothesis is that it is possible to analyse the 3-carbon bridged somatostatin 99 (Figure 6.6) with carboxylic acid bis-sulfone 13\(\text{a}\) can be accurately analysed by MALDI-TOF-MS.

![Figure 6.6. Schematic representation of the structure of 3-carbon disulfide bridged somatostatin 99](image)

The carboxylic acid bis-sulfone 13\(\text{a}\) is insoluble in aqueous solvents at a high concentration (for example, 5 mg/mL). Reaction of carboxylic acid bis-sulfone 13\(\text{a}\) with reduced somatostatin 97
is not feasible at pH 6.2 due to slow elimination. Therefore, conversion of carboxylic acid bis-sulfone 13a to carboxylic acid mono-sulfone 98 is required for the initiation of the conjugation at pH 6.2. Preparation of pure carboxylic acid mono-sulfone from carboxylic acid bis-sulfone 13a was not known at the time of this experiment. It was later found that the carboxylic acid mono-sulfone 98 could be prepared in methanol with the addition of sodium borohydride – Appendix 2). Elimination of carboxylic acid bis-sulfone 13a does not occur in 100% acetonitrile. The elimination reaction was therefore conducted in 40% acetonitrile in 50 mM sodium phosphate buffer (prepared using D$_2$O), pH 8.0. Monitoring the formation of carboxylic acid mono-sulfone 98 by $^1$H-NMR was necessary to calculate the amount of carboxylic acid mono-sulfone 98 required for the conjugation reaction. For $^1$H-NMR analysis, a concentration of 5 mg/mL would provide spectra with sufficient S/N ratio to observe good peak intensities. The elimination reaction was then monitored using $^1$H-NMR analysis using a carboxylic acid bis-sulfone 13a concentration of 5.7 mg/mL. Elimination was found to be slow in the mixed aqueous-organic solvent. Approximately 30% of elimination was observed with the carboxylic acid bis-sulfone 13a in 48 h (Appendix 2) (as determined by the integrated vinyl peaks in the $^1$H-NMR spectra). Reduction of somatostatin 96 with TCEP HCl 90 followed by bis-alkylation reaction the carboxylic acid mono-sulfone 98 was also conducted in 40% acetonitrile in 50 mM sodium phosphate buffer, pH 6.2. Somatostatin 96 was reduced using 2 eq. of TCEP HCl 90 for 1 h at ambient temperature to ensure complete reduction. Reaction of reduced somatostatin 97 with 2 eq. of carboxylic acid mono-sulfone 98 (the volume of carboxylic acid mono-sulfone 98 solution containing exactly 2 eq. to the reduced peptide 97) was conducted by overnight incubation at 4 °C. The reaction mixture was then diluted with water, freeze dried and redissolved in deionised water to precipitate out the un-reacted carboxylic acid bis-sulfone 13a or carboxylic acid mono-sulfone 98. The filtered solution was then subjected to reversed phase-HPLC (Figure 6.7) to collect the 3-carbon disulfide bridged somatostatin 99 fraction (8.55 min). The collected fraction was then used for reflectron mode MALDI-TOF-MS analysis.
Figure 6.7. RP-HPLC chromatogram of the isolation of the modified somatostatin 99 from the reaction mixture; eluting peaks of interest are (1) buffer salts and p-tolyl sulfinic acid 25 and (3) modified somatostatin 99 as determined by MALDI-TOF-MS.
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Figure 6.8. Reflectron mode MALDI-TOF-MS spectrum of A) native somatostatin 96 (1,638.10 Da) with its isotopes and B) 3-carbon disulfide bridged somatostatin (1,829 Da) 99.

The MALDI-TOF spectrum of the reaction product showed an exact mass increase of 190 Da (1,829.70 Da) (Figure 6.8B) to the molecular mass of the native somatostatin 96 (1,638.10 Da) (Figure 6.8A). The calculated molecular mass of the 3-carbon bridge along with the benzoic acid group is 190 Da. This strongly suggests the formation of the 3-carbon bridge in the peptide. Based on the mass increase (190 Da), only one 3-carbon bridge is possible in the modified somatostatin 99. Therefore, it is highly likely that the 3-carbon bridge is between the thiols. However, this result does not confirm unequivocally whether the 3-carbon bridging is between the thiols or between the amine nucleophiles. Moreover, no control experiment was performed between the native somatostatin 96 and carboxylic acid mono-sulfone 98 to confirm non-reactivity to amines. But, if reaction had occurred to the many amine groups present in somatostatin 96, the molecular mass would have been much higher. Also, intermolecular cross-linking would have been observed in the RP-HPLC and MALDI-TOS-MS analyses. This experiment however indicated that the carboxylic acid mono-sulfone 98 underwent the second
elimination and alkylation after initial addition to one of the cysteine thiols. The result therefore confirmed that the 3-carbon bridging reaction occurred at pH 6.2.

The initial thiol addition of PEG mono-sulfone 15a was fast at pH 6.2 as observed with the PEGylation of reduced somatostatin 97. However, it was not entirely certain if the 3-carbon bridging reaction occurred at pH 6.2. Therefore, a possible plan of work to ensure completion of the 3-carbon bridging reaction would be to increase the pH to 7.8 after 1 h of reaction at pH 6.2. The second elimination and thiol addition occurs efficiently at pH 7.8. An alternative method would be to exchange the reaction buffer (sodium phosphate buffer, pH 6.2) with a PD-10 Desalting column to 50 mM sodium phosphate buffer, pH 7.8. The PEGylated somatostatin can be easily isolated by PD-10 desalting (unlike native 96 or reduced somatostatin 97) due to the attached PEG 5 kDa. The assumption is that the peptide would remain in solution at pH 7.8 (without precipitation) since the PEG is conjugated to the peptide by first thiol addition. Also, the PD-10 desalting will eliminate any nucleophilic TCEP HCl 90 which could interfere with the second thiol addition. This process of shifting the buffer pH to 7.8 after initial conjugation at pH 6.2 would therefore help in the completion of the DB-PEGylation of somatostatin.

6.5 CONCLUSIONS

A PEGylation strategy that exploits the chemical selectivity of the two sulfur atoms derived from native disulfide bonds in proteins was applied to the cyclic peptide, somatostatin 96. Somatostatin 96 was ideal as a reaction model for the 3-carbon bridging and PEGylation studies since it had a single accessible disulfide bond. The reduction of the disulfide bond in somatostatin 96 for the purpose of DB-PEGylation was achieved using 1 – 2 eq. of TCEP HCl 90. PEGylation of reduced somatostatin 97 with PEG mono-sulfone 15a was possible at pH 6.2. The reactions of carboxylic acid mono-sulfone 98 with reduced somatostatin 97 followed by MALDI-TOF analysis suggests the presence of only a single 3-carbon bridge with the benzoic acid moiety in the modified somatostatin 99. This strongly suggests that the 3-carbon bridging is between the sulfurs of the modified somatostatin 99.
Chapter 7

Conclusions and future perspectives
7.1 DB-PEGYLATION APPROACH

This thesis explored the hypothesis that ‘rebridging’ a reduced disulfide bond with a 3-carbon bridge between the cysteine thiols could be achieved as a means for site-specific PEGylation while maintaining protein function. During this study, the bis-thiol specific PEGylation approach was evaluated using 1) tripeptide GSH 26, 2) cyclic peptide somatostatin, 3) tetrameric L-asparaginase, 4) cytokine protein IFN α-2b and 5) leptin as reaction models. DB-PEGylation was found to introduce a 3-carbon bridge with an attached PEG between the cysteine thiols of a reduced disulfide bond. This newly formed 3-carbon bridge (Figure 7.1B) was found to maintain protein tertiary structure (IFN) and the biological activity (IFN and L-asparaginase). Two key advantages of the DB-PEGylation approach are (1) a structurally homogenous PEGylated product is obtained and (2) DB-PEGylation is stoichiometrically efficient.

![Figure 7.1. Partial structures of modelled IFN and DB-PEG IFN; A) native Cys29-Cys138 and B) DB-PEGylated Cys29-CCC-Cys138. The three-carbon disulfide bridge is shown in green.](image)

7.1.1 Limitations of the DB-PEGylation approach

Reduction of the disulfide bond to liberate the cysteine thiols is a pre-requisite for DB-PEGylation. A protein upon reduction can lose its tertiary structure to denature or aggregate. Therefore, the disulfide reduction step is the main limitation of the DB-PEGylation approach. For efficient DB-PEGylation to occur, it is important that the protein maintains its tertiary structure after disulfide reduction. It was observed from some experiments that the reduced protein can precipitate from solution upon agitation or exposure to heat (sunlight). Proteins like L-asparaginase and IFN were observed to remain in solution on disulfide reduction at still and ambient temperature conditions. In contrast, leptin and somatostatin were found initially to readily precipitate once their disulfides were reduced at similar conditions. Hence the reduction
conditions had to be optimised to ensure that protein structure was preserved. Factors such as pH and the presence of an aggregation retardant were found to stabilise reduced proteins so that they could undergo the DB-PEGylation. The reduction step has to be carefully optimised for each protein depending on its solution stability after disulfide reduction.

The DB-PEGylation approach is primarily useful for proteins with solvent accessible disulfide bonds. From the perspective of disulfide reduction, such proteins can in principle be reduced at mild conditions using a suitable reductant in the absence of denaturants. Some proteins which are structurally rigid and compact like bovine pancreatic ribonuclease (RNAse) require the presence of denaturants like 6 M guanidine HCl or 8 M urea for disulfide reduction (unpublished results). In such a case, disulfide reduction often leads to complete disruption of protein tertiary structure (Resnick et al., 1959). For PEG mono-sulfone 15a to form the 3 -carbon bridge between the cysteines of the reduced disulfide, the juxtapositioned thiols (of the reduced disulfide) is a must. Maintaining the protein tertiary structure is therefore critical. The complete unfolding of the protein in the presence of denaturants would lead to intermolecular bis-alkylation reactions, loss of structure and biological function.

Formation of aggregates of reduced proteins was another disadvantage found in the DB-PEGylation process. Aggregation could be significantly reduced by re-oxidation of the reduced disulfides with glutathione refolding solution. However, PEGylation reactions of IFN and leptin typically showed at least 2 % of the aggregates during SEC-HPLC analyses. The glutathione refolding step is important when mono-PEGylation of a protein with multiple reduced disulfide bonds (for example, IFN with two disulfide bonds) is desired.

Location of the disulfide bond is also important for the DB-PEGylation approach. DB-PEGylation of a disulfide bond that is located near a protein’s receptor binding site (as in the case of IFN Cys29–Cys138) might affect the protein’s biological activity. However, there is increasing evidence that suggests (Foser et al. 2003; Grace et al. 2005b; Ramon et al. 2005) that PEGylation far from the binding site still results in diminished in vitro biological activity. Interestingly, the diPEGylated IFN prepared by DB-PEGylation did show some biological activity.

7.1.2 Further work on the DB-PEGylation process

The DB-PEGylation process could be typically completed within 24 h (8 h DB-PEGylation + 16 h re-oxidation when necessary). Further improvement may be required to decrease the time period of reactions. For example, the time period for the re-oxidation could be decreased using
buffers with constituents favouring rapid oxidation. It was observed from some experiments that
the presence of metal contaminants like Cu$^{2+}$ could re-oxidise the reduced disulfide rapidly.
Treatment of a reduced protein solution with 40 μM copper (II) sulfate oxidises the reduced
disulfides rapidly in proteins (Leon et al. 1999; Lu et al. 1992). Therefore, further studies with
a buffer containing copper (II) sulfate, have to be conducted. As an initial study, reduced IFN
solution could be treated with 40 μM copper (II) sulfate. Samples should then be drawn at
equal time intervals (say 15 min) for up to 2 h and analysed by SDS-PAGE. If effective, this
procedure could be adopted for the DB-PEGylation process.

7.2 DB-PEG COMPOUNDS

The main advantage of PEG bis-sulfone 14a and PEG mono-sulfone 15a is that they are specific
to thiols. The interactive mechanism for bis-alkylation makes these compounds ideal for
undergoing reaction with the two thiols derived from a reduced disulfide bond. Therefore, a
more uniform protein-PEG product could be obtained after the DB-PEGylation process. In all
cases examined, PEG bis-sulfone 14a or PEG mono-sulfone 15a did not undergo reaction to a
protein unless the disulfides were reduced. Conjugation to an intact unreduced protein did not
occur at a pH range of 6 – 8.6 even with large excess of the PEG compounds 14a and 15a. This
observation was consistent throughout the studies with many native proteins. Another important
advantage was that the amide PEG bis-sulfone 14a and PEG mono-sulfone 15a were
stoichiometrically efficient. Reaction yields of monoPEGylated proteins were high throughout
the studies with many proteins. Also, PEG mono-sulfone 15a was found to be reactive to thiols
even at pH 6.0. This helped to tailor the PEG bis-sulfone 14a based on the protein’s pH stability
after reduction. For storage purposes, PEG bis-sulfone 14a and PEG mono-sulfone 15a were
chemically stable in pH 4.0 solutions. Solutions of PEG bis-sulfone 14a or PEG mono-sulfone
15a prepared in pH 4.0 buffer could be stored at -20 °C and reused for up to a year.

7.2.1 Disadvantages of DB-PEG compounds

PEG bis-sulfone 14a and PEG mono-sulfone 15a were found to have absorbance at both the UV
wavelengths of 215 nm and 280 nm which are typically used for protein or peptide detection.
Since PEG as such does not show absorbance at UV 215 nm or at 280 nm (PEG can be typically
detected using a refractive index detector), the UV absorbance observed with the DB-PEG
compounds 14a and 15a is advantageous during SEC-HPLC characterisation. However, when
analysing a protein conjugation reaction mixture by SEC-HPLC, the compounds 14a and 15a
can show interference with protein or peptide absorbance.
PEG mono-sulfone 15a undergoes competitive reaction at basic pH values. The Michael acceptor present in the PEG mono-sulfone 15a is susceptible to hydroxyl group addition. This typically would yield either a compound 74 which cannot undergo the 3-carbon bridging reaction (and could result in non-productive mono-thiol addition) or a compound 76 which cannot react to thiols at all. Furthermore, upon addition of either PEG bis-sulfone 14a or PEG mono-sulfone 15a to a solution of reduced protein, a ‘localised’ concentration of these compounds can form initially in the solution before they could uniformly spread to the other parts of the solution. In the case of PEG mono-sulfone 15a, the reaction with thiols is quick. Therefore, a high ‘localised’ concentration of PEG mono-sulfone 15a around a reduced protein could lead to a relatively faster reaction of two PEG mono-sulfones 15a with each of both thiols from a reduced disulfide. The second molecule of PEG mono-sulfone 15a can react with the second thiol even before the first PEG mono-sulfone 15a completes the bridging reaction. The initial thiol addition reaction is slow in the case of PEG bis-sulfone 14a since it has to undergo elimination before it can react with a thiol. To address reactivity, the initial elimination from the PEG bis-sulfone 14a can be matched to a particular protein, in conditions most conducive to its reduction. The use of the PEG bis-sulfone 14a also provides time to ensure complete mixing in the solution before it undergoes elimination and subsequent thiol addition reaction.

Intramolecular thiol bis-alkylation to relink disulfides through a 3-carbon bridge was shown to be possible with the prepared DB-PEG. However, intermolecular reactions (between cysteines of two proteins) could also occur with PEG bis-sulfone 14a or PEG mono-sulfone 15a (as observed with leptin). This intermolecular reaction might be envisaged to prepare bivalent Fabs or di-peptides.

7.2.2 Future work on DB-PEG compounds

Upon protein DB-PEGylation with PEG bis-sulfone 14 or PEG mono-sulfone 15, a new phenyl group is introduced into the protein. The newly attached aromatic ring to the protein could possibly contribute to the protein’s spectral properties as observed during the UV and circular dichroism spectroscopy studies on IFN (Chapter 4). If the PEG detaches from its linkage with the phenyl ring in vivo, the exposed functionality could possibly induce immune responses. Therefore, there is a need to replace the phenyl group with an aliphatic group. An aliphatic based compound (Figure 7.2) would avoid the spectral interference with the protein. However, the aromatic groups as ‘leaving groups’ (SO₂R of 13, 14 and 15) are useful in the compound characterisation by ¹H-NMR and also during elimination studies with ¹H-NMR or RP-HPLC.
Chapter 7. Conclusions and future perspectives

Figure 7.2. Proposed DB-PEG 100 with aliphatic side chain (Y) and aromatic leaving group.

The most important features required for the DB-PEG 100 reactivity are the 1) α, β Michael acceptor formed by the initial elimination reaction, 2) an α, β' leaving group (SO₂R) and the 3) mPEG attached to the side chain (Figure 7.2). These features are required in any further modifications that are done on the compound. As observed with the urethane 14c and ester 14b PEG bis-sulfone, the elimination reaction could be affected by the possible electronic interference or hydrogen bonding interactions after mPEG attachment to the bis-sulfone linker. Successful reaction of the DB-PEG 100 depends on this elimination reaction. Therefore, modifications in DB-PEG 100 have to be carefully designed so as to favour the elimination reaction.

7.3 FURTHER STUDIES ON DB-PEGYLATED PROTEINS

Clinically useful monoPEGylated IFN was prepared successfully with the DB-PEGylation technique. All the structural and biological studies that were conducted indicate the presence of a stable molecule. However, due to the presence of two accessible disulfides in IFN, the formation of two positional isomers is possible. Some reports suggest that the disulfide bond, Cys29 – Cys138 is important for the biological activity of IFN-α (Morehead et al, 1984). But, such disulfide modification reactions are very different to the reactions that result in the incorporation of a three carbon bridge that essentially ‘rebridge’ the cysteines. It is possible that the Cys29-PEG-Cys138 positional isomer may display lower in vitro activity than the Cys1-PEG-Cys98 isomer. Therefore, there may be a future need to separate these two PEG positional isomers to analyse their biological activity profiles. To explore selectivity and reduction parameters, leptin and somatostatin with one disulfide bond each were also PEGylated using the DB-PEGylation approach. Further investigation on the biological activities of these PEGylated peptides has to be conducted.
7.3.1 More proteins

Other proteins with disulfide bonds can be evaluated to study the general applicability of this PEGylation approach. Many therapeutic proteins with accessible disulfides like G-CSF, EPO, IL-6, Fab', TNF-α are potential candidates for DB-PEGylation studies. The high reaction efficiency of the prepared DB-PEG would allow production of cost-effective and more affordable PEGylated proteins. DB-PEGylation could therefore potentially improve the clinical use of life-saving therapeutic proteins.
Appendix I
APPENDIX I

Proteins are primarily made up of L-amino acids linked together via peptide bonds, where the number and sequence of amino acids differ with each protein. The number and sequence of amino acids is specific to a protein and is constructed in a living organism via transcription and translation of the encoded gene. The 20 commonly found amino acids in proteins are shown in the table below.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Abbreviation (3-letter)</th>
<th>Abbreviation (1-letter)</th>
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<tr>
<td>Alanine</td>
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<tr>
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<td>N</td>
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<td>Cys</td>
<td>C</td>
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</table>

List of amino acids and their abbreviations (adapted from Biochemistry, Lehninger)

These amino acids form the building blocks of a protein molecule. Each amino acid has a different side chain with characteristic physicochemical properties. The amino acid side chains do not usually involve in the formation of peptide bonds and are therefore available for interaction with their environment.
Chemistry of amino acids

Amino acids comprise of an amino group and a carboxylic group attached to the same α-carbon atom. The α-carbon atom is also bonded to a side chain group (R), which specifies the amino acid, and a hydrogen atom. Stereochemically, all amino acids found in proteins are L-forms. The 20 common amino acids are classified into four groups based on their polarity (Biochemistry, Lehninger).

1. Non-polar or hydrophobic R groups,
2. Neutral (uncharged) polar R groups,
3. Positively charged R groups and
4. Negatively charged R groups (at pH 6.0 to 7.0).

1. Non-polar or hydrophobic R groups – These amino acids (table 2) have low solubility in water and form the hydrophobic part of any protein. This group comprises of five amino acids with aliphatic hydrocarbon R groups (alanine, leucine, isoleucine, valine and proline), two with aromatic rings (phenyl alanine and tryptophan) and one containing sulfur (methionine). Glycine is also considered as a non-polar amino acid as the functional R group is only a hydrogen atom.

2. Neutral polar R groups – These amino acids are much more soluble in water than the non-polar amino acids. This group consists of amino acids with polar functional groups which can interact with water molecules through hydrogen bonding. These functional groups are hydroxyl groups (serine, threonine and tyrosine), amide groups (asparagine and glutamine) and thiol group (cysteine).

3. Positively charged R groups – These are basic amino acids in which the R groups have a net positive charge at neutral pH. The amino acids classified in this group are lysine which has an amino group at the ε position on its aliphatic side chain, arginine with a guanidinium group and histidine with an imidazolium group.

4. Negatively charged R groups – The amino acids, aspartic acid and glutamic acid contain a second carboxyl group as their R group. These carboxyl groups ionise and impart a negative charge to the amino acid at pH 6 to 7.
Classification of 20 common amino acids based on their polarity and charges

**Nucleophilicity and reactivity of amino acid side chains**

The ionisable amino acids aspartic acid, glutamic acid, lysine, arginine, cysteine, histidine and tyrosine in a peptide or protein can undergo acid-base interactions. Depending on pH and pKa, the ionisable side chains exist as a protonated or an unprotonated form. At pH values below the pKa of a carboxylate group, the acid exists in the protonated and uncharged form. At pH values above the pKa of the carboxylate group, the acid exists in the protonated and negative charged form. The same principle applies to the hydroxyl group on the phenol ring of the amino acid, tyrosine. However, amine groups at pH below their pKa values exist in a protonated and positively charged form. At pH values above their pKa values, they exist in an unprotonated and neutral form.
Appendix II

$^1$H-NMR spectrum of carboxylic acid manich salt 22

IR spectrum of carboxylic acid manich salt 22
$^{1}$H-NMR spectrum of carboxylic acid $\text{bis}$-sulfide

$^{13}$C-NMR spectrum of carboxylic acid $\text{bis}$-sulfide
Appendix H

Current Data Parameters
NAME: bl-02-02-2006-44
RUNNO: 12
PROCNO: 1

E2 - Acquisition Parameters
Date: 20060202
Sample: 0.52
PROBND: 5 mm BR-CR-LR
POLARIS: depth15
SOLVENT: CDCl3
MS: 40
SWM: 20990.81 Hz
FDMD: 0.143414 Hz
AC: 1.364611 sec
SW: 20.850 usec
TE: 6.00 usec
TT: 145.000000
DD: 0.00034128 sec
D1L: 0.00039504 sec
daa: 0.00010000 sec

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M1: 12C
E1: 4.30 usec
E2: 13.00 usec
P1: 0.40 dB
NP1: 100.627629 MHz

--- CHANNEL f2 ===========
CDEPMD: m17316
MEJG: 18
E2: 7.88 usec
E3: 15.76 usec
P1: 100.00 usec
P1L: 22.30 usec
D1L: 22.30 usec
DPOG: 400.1316005 MHz

E2 - Processing parameters
DP: 100.617640 MHz
NOW: 1
TB: 1.00 Hz
TH: 1.40

13C-DEPT NMR spectrum of carboxylic acid bis-sulfide 23

2D-1H-1H COSY spectrum of carboxylic acid bis-sulfide 23
Appendix II

INMSS 48 in 50 50 Acn H2O =0.1%FA
06020703149ES (0.05) F (1.00 1.00) C29:840512

Isotope Model

TOFMSE+ 6.77e12

06020703149ES 3 (0.135) Cn (C16:4, 90.00, Ar), Sm (SG, 2x3.00), Cm (2:12)

TOFMSES+ 4.54e3

10 O i

TOP MS ES+ spectrum of carboxylic acid bis-sulfide

IR spectrum of carboxylic acid bis-sulfide

208
Appendix II

**HOOC-**

**Appendix II**

**A**

**t**

**P**

**s**

**b**

**l**

**-29**

**01**

**-2004**

**27**

10

**PROC**

**NO 1**

**F2 - Acquisition Parameters**

**Data**

**20040129**

**Time**

**13:49**

**Instrument**

**Spect**

**Pulse Program**

**5 mm BBO BR IN**

**T1**

**65.36**

**Solvent**

**CDCl3**

**NS**

**64**

**DF**

**927.15 Hz**

**EVREN**

**0.125614 Hz**

**AQ**

**1.958424 sec**

**R**

**102**

**DM**

**60.400 usec**

**DS**

**0.600 usec**

**E1**

**2.000000000 sec**

**E2**

**0.000000000 sec**

**M1**

**0.030000000 sec**

**CHANNEL 1**

**MOC1**

**1 H**

**F1**

**7.60 usec**

**F11**

**0.000 dB**

**SP01**

**400.132710 MHz**

**F2 - Processing Parameters**

**SB**

**0.30**

**PC**

**1.00**

**Current Data Parameters**

**NAME**

**sbo-02-02-2004-42**

**PROC**

**11**

**FD**

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**DE**

**4.00**

**E1**

**23980.92 Hz**

**AQ**

**1.396479 sec**

**R**

**804**

**JW**

**20.36 usec**

**D1**

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**DJ**

**0.00000000 sec**

**DELTA**

**1.00000000 sec**

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**CD**

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**FL2**

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**FL3**

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**SP02**

**400.131605 MHz**

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**DE**

**4.00**

**D1**

**1.00 Hz**

**DJ**

**1.00**

**PC**

**1.40**

**1H NMR spectrum of carboxylic acid bis-sulfone 13a**

**13C NMR spectrum of carboxylic acid bis-sulfone 13a**
Appendix II

**13C-DEPT NMR spectrum of carboxylic acid bis-sulfone 13a**

**1H-1H COSY-NMR spectrum of carboxylic acid bis-sulfone 13a**
Appendix II

**SBMS49 in 50:50 Acn:H2O + 0.1% FA**

TOF MS ES+ spectrum of carboxylic acid bis-sulfone 13a

IR spectrum of carboxylic acid bis-sulfone 13a
White crispy foam substance formed during rotary evaporation of chloroform solution of carboxylic acid - bis-sulfone 13a

\[
\text{H}_2\text{COOC--}\text{C--S--C--H}
\]

\(^1\)H-NMR spectrum of methyl ester of bis-sulfide 39 by Fischer esterification
Appendix II

\[ \text{\textsuperscript{1}H-NMR spectrum of methyl ester of bis-sulfone 40 prepared by Fischer esterification} \]

\[ \text{TOF MS ES+ spectrum of methyl ester acid bis-sulfone 40} \]
1H-NMR spectrum of methyl ester of bis-sulfone 40 prepared by thionyl chloride method

IR spectrum of methyl ester of bis-sulfone 40
Appendix II

1H-NMR spectrum of amide 5 kDa PEG bis-sulfone 14a

1H-NMR spectrum of 10 kDa PEG bis-sulfone 14a
Appendix II

**Data Parameters**

**PROCNO**: 10

**Acquisition Parameters**

**TIME**: 10.27
**DURATION**: 5 mm SEL 10-11
**PULPROG**: epg0
**TD**: 6536
**SOLVENT**: CDC13
**AQ**: 16.6
**SN**: 2278.146 Hz
**FID**: 0.128514 Hz
**AQ**: 3.943243 sec
**SN**: 68.146 sec
**AQ**: 6.00 usec
**SA**: 0.00 usec
**D1**: 1.0000000 sec

**Processing Parameters**

**F2**: 400.130357 MHz
**SN**: 400.130357 MHz
**SI**: 3.08 Hz
**SF**: 0.5000 db

**NMR spectrum of 20 kDa PEG bis-sulfone 14a**

**C-NMR spectrum of 5 kDa PEG bis-sulfone 14a**
Appendix II

\[ ^{13}\text{C-DEPT-NMR spectrum of 5 kDa PEG bis-sulfone 14a} \]

\[ \text{MALDI-TOF spectrum of 5 kDa PEG bis-sulfone 14a} \]
Appendix H

**Acquisition Parameters**

- **Acquisition Parameters**
  - Name: sh1-10-12-2005-43
  - Data: 20051210
  - Time: 10.10
  - Instrument: spect
  - Frequency: 5 mm BRB 90
  - Pulsed: 190
  - Sample: 50
  - Solvent: CDCl3
  - MAS: 10
  - SW: 278.146 Hz
  - MRES: 0.116114 Hz
  - AQ: 3.9584243 sec
  - DE: 6.50 usec
  - TE: 10.8 sec
  - TD: 1.0000000 sec

**Current Data Parameters**

- **Current Data Parameters**
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  - Data: 20050622
  - Time: 10.06
  - Instrument: spect
  - Frequency: 5 mm BRB 90
  - Pulsed: 190
  - Sample: 50
  - Solvent: CDCl3
  - MAS: 300
  - SW: 278.146 Hz
  - MRES: 0.12534 Hz
  - AQ: 3.9584043 sec
  - DE: 6.00 usec
  - TE: 206.8 sec
  - TD: 0.0000000 sec

**Processing Parameters**

- **Processing Parameters**
  - SI: 32768
  - SF: 400.1500000 MHz
  - WN: KR
  - LA: 0.00 Hz
  - PC: 1.00

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**1H-NMR spectrum of 5 kDa PEG bis-sulfide 42**

**1H-NMR spectrum of 10 kDa PEG mono-sulfone 15a**

218
Appendix II

Sample Ref SB(3127)
PROTON CQ13 (D3) 3127, 14b, 41

Current Data Parameters
NAME: 20015-12-20-20
PROC: 1

F2 - Acquisition Parameters
Time: 10.00
FD (H-H): 5.495 MHz
FIDRES: 0.128 MHz
AG: 3.5596 Hz
DG: 228.1
DE: 6.0 μsec
DS: 0.30 Hz
DD: 1.00 μsec

F2 - Processing Parameters
SF: 400.124710 MHz
SN: 32748
SB: 400.130650 MHz

1H-NMR spectrum of ester 10 kDa PEG bis-sulfone 14b

1H-NMR of 5 kDa urethane PEG bis-sulfide 34
### Appendix II

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**$^1$H-NMR of 5 kDa urethane PEG bis-sulfone 14e**

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**$^1$H-NMR of 5 kDa $p$-nitrobenzyl/chloroformate activated mPEG 38**
Appendix II

\[ ^1H\text{-NMR of 5 kDa carbonate PEG bis-sulfone 14d} \]

\[ \text{HOOS} \]

\[ ^1H\text{-NMR of p-tolysulfinic acid 25 isolated by RP-HPLC} \]
Appendix II

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'\(^1\)H-NMR of PEG mono-hydroxy (mono-sulfone) 74 isolated by RP-HPLC

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**Acqisite zg30**

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'\(^1\)H-NMR of PEG 5 kDa mono-sulfone 15a isolated by RP-HPLC

222
Appendix II

1H-NMR of PEG bis-sulfone 14a and GSSG 27 incubation product with addition of 1 N NaOH

1H-NMR of cys-PEG-cys 82
\(^1\)H-NMR of PEG mono-sulfone 15a and GSH 26 reaction product after 2 h incubation.

\(^1\)H-NMR of 10 kDa urethane PEG bis-sulfone 14e with slight elimination during its preparation.
Appendix II

Current Data Parameters
NAME   cm-02-02-2006-45
EXPNO  10
PROCNO 1

F2 - Acquisition Parameters
Data  7008/02
Time  14.07
INSUM 5 mm BBO-BB
PHTPOT 400/45
T1D  835.36
SOLVENT  D2O
NG  16
DS  5
FDM  827.16 Hz
FIDRES  0.12621 Hz
AQ  2.506/243 sec
R0  454.1
SW  60,000 usec
TE  3.081.0 K
DI  1.00000000 sec
TOO  1

H-NMR of urethane PEG bis-sulfone 14c in D2O.

F2 - Processing parameters
SI  12748
SF  400.1364510 MHz
WDW  10 K
SSB  0
LB  0.30 Hz
GB  0
PC  1.00

H-NMR of urethane PEG bis-sulfone 14c and GSH 26 reaction product.
Appendix II

**F2 - Acquisition Parameters**

- Date: 2021-11-08
- Time: 15:17
- INSTRUM: spect
- SPECTRO: 5 mm MHO 80-16
- POLARIS: ag3
- TE: 60.50
- TR: 1000
- TD: 16
- SD: 2
- D3F: 6275.146 Hz
- FTRES: 1.1261546 Hz
- ACQ: 3.0082543 sec
- BW: 456.1 Hz
- SW: 0.0255181
- DE: 6.00 usec
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**F2 - Processing parameters**

- MHz: 600.00 MHz
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- TM: 0.30 Hz
- TC: 1.00

**1H-NMR of carboxylic acid mono-sulfone and p-tyr sulfinic acid mixture prepared in deuteriated methanol by addition of sodium borohydride.**

Flash reversed-phase chromatographic set-up for the preparation of PEG mono-sulfone 15a.
$^1$H-NMR of the elimination of carboxylic acid bis-sulfone 13a
in 40% acetonitrile in 50 mM sodium phosphate buffer, pH 8.0 – 0 h
Appendix II

Current Data Parameters
NAME: ab1-14-06-2005-13
EXPER. 10
PROCNO 4

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INSTRUM: spect
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DG: 0.000000 second
DG2: 0.30 Hz
DG3: 0.00 Hz
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DG6: 0.00 Hz
DG7: 0.00 Hz
DG8: 0.00 Hz

Data Parameters
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PROCNO 4
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Date: 20050613
Time: 09:39
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DG7: 0.00 Hz
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H-NMR of the elimination of carboxylic acid bis-sulfone 13a
in 40 % acetonitrile in 50 mM sodium phosphate buffer, pH 8.0 – 6 h

H-NMR of the elimination of carboxylic acid bis-sulfone 13a
in 40 % acetonitrile in 50 mM sodium phosphate buffer, pH 8.0 – 24 h
\[ ^1H-NMR \text{ of the elimination of carboxylic acid bis-sulfone 13a} \]

in 40% acetonitrile in 50 mM sodium phosphate buffer, pH 8.0 – 48 h

\[ ^1H-NMR \text{ of the elimination of carboxylic acid bis-sulfone 13a} \]

in 40% acetonitrile in 50 mM sodium phosphate buffer, pH 8.0 – 72 h
Appendix II

RP-HPLC chromatogram of carboxylic acid bis-sulphone 13a (UV 215 nm)

RP-HPLC chromatogram of mPEG amine 24 (UV 215 nm) (Injection: 50 µL, 10 mg/mL)
Appendix III
Ion exchange chromatographic set-up for the purification of the conjugation reaction mixture (left). Ion exchange column with its attachments (right).

SEC-HPLC set up for the characterisation and isolation of protein conjugates.
UV SPECTROSCOPY

UV spectroscopy is widely employed in the quantitation of proteins based on the absorbance value shown at the wavelengths of either 280 nm (Near UV) or 215 nm (Far UV) (Aitken and Learmonth, 2002a). This absorbance is based on Beer-Lambert's law:

\[ A \propto c \]  (Equation 2)

where \( A \) = molar absorption coefficient (M^-1 cm^-1),
\( c \) = concentration in mol/L and
\( l \) = optical path length in cm.

A protein's UV absorbance at 280 nm depends upon the number of Trp, Tyr and disulfide bonds present in the structure (Pace et al, 1995). The molar extinction coefficient, \( \varepsilon \), can be calculated from the UV 280 nm absorbance value of a 1 mg/mL (0.1 % w/v) protein solution. Pace and co-workers have also reported that the molar absorption coefficient, \( \varepsilon \), of a completely folded protein can best be predicted by using the equation 3.

\[
\varepsilon (280) (M^{-1} \text{ cm}^{-1}) = (# \text{ Trp}) (5,500) + (# \text{ Tyr}) (1,490) + (# \text{ disulfide bonds}) (125)
\]  (Equation 3)

The protein's UV absorbance at 280 nm is the simplest and most widely used method. Quartz cuvettes which are transparent at the near UV wavelength are used for the analysis. The UV absorbance measurement at 205 - 215 nm is also employed sometimes especially for detection of peptides during HPLC analysis. At this far UV analysis, the peptide bonds absorb strongly along with other residues like Trp, Phe, Tyr, His, Cys, Met, and Arg.
HPLC pump: Jasco PU-980 Intelligent HPLC pump
Detector: Jasco UV-1575 Intelligent UV/Vis detector
Column used: Superdex 200 prep grade
Mobile phase: 20mM sodium acetate buffer, 150mM NaCl, pH 4.0
Flow rate: 1mL/min
Run time: 120 min
UV detection wavelength: 280 nm
Injection volume: 2000 μL
Concentration: 0.05 mg/mL

SEC-HPLC elution of native IFN

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Recovery of 0.2 mg (1 mL) RNAse recovery in the third and fourth fractions conducted with 3 different PD-10 columns
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<tr>
<td>Run #1</td>
<td>0.136</td>
<td>0.141</td>
<td>0.137</td>
</tr>
<tr>
<td>Run #2</td>
<td>0.134</td>
<td>0.139</td>
<td>0.136</td>
</tr>
<tr>
<td>Run #3</td>
<td>0.135</td>
<td>0.140</td>
<td>0.140</td>
</tr>
</tbody>
</table>

### Protein concentration based on absorbance 280 nm (2.5 mL)

<table>
<thead>
<tr>
<th>#</th>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run #1</td>
<td>0.194 mg/mL</td>
<td>0.201 mg/mL</td>
<td>0.195 mg/mL</td>
</tr>
<tr>
<td>Run #2</td>
<td>0.191 mg/mL</td>
<td>0.198 mg/mL</td>
<td>0.194 mg/mL</td>
</tr>
<tr>
<td>Run #3</td>
<td>0.192 mg/mL</td>
<td>0.200 mg/mL</td>
<td>0.200 mg/mL</td>
</tr>
</tbody>
</table>

### Protein content based on absorbance 280 nm (2.5 mL)

<table>
<thead>
<tr>
<th>#</th>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run #1</td>
<td>0.485 mg</td>
<td>0.503 mg</td>
<td>0.489 mg</td>
</tr>
<tr>
<td>Run #2</td>
<td>0.478 mg</td>
<td>0.496 mg</td>
<td>0.485 mg</td>
</tr>
<tr>
<td>Run #3</td>
<td>0.482 mg</td>
<td>0.5 mg</td>
<td>0.5 mg</td>
</tr>
</tbody>
</table>

### Percentage protein content based on absorbance 280 nm (2.5 mL)

<table>
<thead>
<tr>
<th>#</th>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run #1</td>
<td>95.10 %</td>
<td>98.52 %</td>
<td>95.88 %</td>
</tr>
<tr>
<td>Run #2</td>
<td>93.70 %</td>
<td>97.25 %</td>
<td>95.10 %</td>
</tr>
<tr>
<td>Run #3</td>
<td>94.50 %</td>
<td>97.93 %</td>
<td>98.03 %</td>
</tr>
</tbody>
</table>

Recovery of RNase 0.5 mg (1 mL) in the 2.5 mL fraction after the wash volume (2 mL) conducted with 3 different PD-10 columns
Absorbance 280 nm (3.5 mL)

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.093</td>
<td>0.092</td>
<td>0.094</td>
</tr>
</tbody>
</table>

Protein content based on absorbance 280 nm (3.5 mL)

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.133 mg/mL</td>
<td>0.131 mg/mL</td>
<td>0.134 mg/mL</td>
</tr>
</tbody>
</table>

Total protein content based on absorbance 280 nm (3.5 mL)

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.465 mg</td>
<td>0.458 mg</td>
<td>0.470 mg</td>
</tr>
</tbody>
</table>

Percentage total protein content based on absorbance 280 nm (3.5 mL)

<table>
<thead>
<tr>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>91.17%</td>
<td>89.80%</td>
<td>92.15%</td>
</tr>
</tbody>
</table>

Recovery of RNAse in the subsequent buffer exchange of the 2.5 mL fraction into 3.5 mL conducted with 3 different PD-10 columns.

Reductant and reduced protein separation using a PD-10 column followed by reaction with the DB-PEG.
Calculation of the number of thiols formed using Ellman’s assay

Molar Extinction Coefficient or Molar absorptivity, \( \varepsilon \), of 0.1 mg/ml asparaginase:

According to Beer-Lambert’s law,

\[
A_{280} = \varepsilon \, b \, c
\]

Where, \( A_{280} \) = Absorption at 280 nm,

\( \varepsilon = \) Molar Extinction Coefficient/ Molar absorptivity,

\( b = \) path length in centimeters,

\( c = \) concentration in moles/liter

\( A_{280} \) of 0.1 mg/ml asparaginase was found to be 0.065 (\( A^{0.01%} \))

Molecular weight of asparaginase = 141,000 Da

Molar concentration of asparaginase = \( \frac{0.1 \, \text{g}}{141,000 \, \text{Da}} = 7.092 \times 10^{-7} \, \text{M} \)

Therefore, \( 0.065 = \varepsilon \times 1\,\text{cm} \times 7.092 \times 10^{-7} \, \text{M} \)

\( \varepsilon = \frac{0.065}{7.092 \times 10^{-7} \, \text{M}} = 91,650 \, \text{M}^{-1} \, \text{cm}^{-1} \)

The concentration of asparaginase will be \( A_{280}/91,650 \, \text{M}^{-1} \, \text{cm}^{-1} \)

Calculation of number of thiols formed:

If 200 \( \mu \text{L} \) of the reduced sample and 100 \( \mu \text{L} \) of the Ellman’s solution (4 mg/ml) is used along with 1 ml of buffer and the absorbance of the sample is 0.007, then Ellman’s assay is calculated as:

\[
0.007/14,150 \, \text{M}^{-1} \, \text{cm}^{-1} = 4.946 \times 10^{-7} \, \text{M}
\]

This value represents the concentration in the spectrophotometric cuvette. To calculate the unknown concentration,

Total volume of solution being measured is 1 ml + 200\( \mu \text{L} \) + 100\( \mu \text{L} \) = 1.3 ml.

Therefore, \( 1.3 \times 1 \, \text{L} / 1000 \, \text{ml} \times 4.946 \times 10^{-7} \, \text{M} = 6.43 \times 10^{-7} \, \text{M} \)

This concentration of sulphhydryls was contributed by the original 200 \( \mu \text{L} \) of the sample.

Therefore, the concentration of free sulphhydryl in the original unknown sample is:

\[
6.43 \times 10^{-7} \, \text{M} / 0.2\,\text{ml} \times 1000\,\text{ml/L} = 3.2 \times 10^{-8} \, \text{M}
\]

To calculate the number of thiols formed per protein,

\[
2.2 \times 10^{-8} \, \text{M} / 7.092 \times 10^{-7} \, \text{M} = 4.5 \, \text{thiols/protein}
\]
Interferon preliminary activity studies

Comparison of antiviral activity of native IFN: ED₅₀ = 65 pg/mL, completely reduced IFN: ED₅₀ = N/A and disulfide reduced monoPEG-IFN: ED₅₀ = 3,797 pg/mL (< 1 %)

Comparison of antiviral activity of native IFN: ED₅₀ = 160 pg/mL, GRS treated IFN: ED₅₀ = 110 pg/mL and disulfide reduced monoPEG-IFN: ED₅₀ = 1,841 pg/mL (8.7 %)
MicroBCA using IFN2ab standard curve

Standard curve

<table>
<thead>
<tr>
<th>Conc(µL/mL)</th>
<th>Abs1</th>
<th>Abs2</th>
<th>Abs3</th>
<th>Average</th>
<th>Blank avg</th>
<th>minus blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.122</td>
<td>0.124</td>
<td>0.124</td>
<td>0.123</td>
<td>0.123</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.253</td>
<td>0.254</td>
<td>0.252</td>
<td>0.253</td>
<td>0.123</td>
<td>0.130</td>
</tr>
<tr>
<td>10</td>
<td>0.375</td>
<td>0.382</td>
<td>0.379</td>
<td>0.379</td>
<td>0.123</td>
<td>0.256</td>
</tr>
<tr>
<td>20</td>
<td>0.598</td>
<td>0.609</td>
<td>0.607</td>
<td>0.605</td>
<td>0.123</td>
<td>0.482</td>
</tr>
<tr>
<td>40</td>
<td>1.091</td>
<td>1.098</td>
<td>1.092</td>
<td>1.094</td>
<td>0.123</td>
<td>0.971</td>
</tr>
</tbody>
</table>

Conc  | Abs
---|---
5   | 0.130
10  | 0.256
20  | 0.482
40  | 0.971

Concentration of samples

<table>
<thead>
<tr>
<th></th>
<th>Abs1</th>
<th>Abs2</th>
<th>Abs3</th>
<th>Average</th>
<th>Minus Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBIFN49</td>
<td>0.482</td>
<td>0.488</td>
<td>0.492</td>
<td>0.487</td>
<td>0.364</td>
</tr>
<tr>
<td>SBIFN50(First)</td>
<td>0.596</td>
<td>0.598</td>
<td>0.604</td>
<td>0.599</td>
<td>0.476 0.467</td>
</tr>
<tr>
<td>SBIFN50(PEG)</td>
<td>0.138</td>
<td>0.13</td>
<td>0.129</td>
<td>0.132</td>
<td>0.009</td>
</tr>
<tr>
<td>SBIFN50(2)</td>
<td>0.569</td>
<td>0.574</td>
<td>0.581</td>
<td>0.575</td>
<td>0.452</td>
</tr>
<tr>
<td>SBIFN50-F51</td>
<td>0.428</td>
<td>0.43</td>
<td>0.43</td>
<td>0.429</td>
<td>0.306</td>
</tr>
<tr>
<td>SBIFN50-F52</td>
<td>0.535</td>
<td>0.536</td>
<td>0.541</td>
<td>0.537</td>
<td>0.414</td>
</tr>
<tr>
<td>SBIFN50-F53</td>
<td>0.501</td>
<td>0.505</td>
<td>0.503</td>
<td>0.503</td>
<td>0.380</td>
</tr>
</tbody>
</table>

Conc.(ug/mL) | Total protein content (ug) | Percentage |
---|----------------|-------------|
| SBIFN49   | 14.98   | 299.6      | 59.92     |
| SBIFN50(First) | 19.22   | 480.5      | 96.1       |
| SBIFN50(2) | 18.60   | 372.4      | 74.4       |
| SBIFN50-F51 | 12.59   | 12.59      | 12.59      |
| SBIFN50-F52 | 17.04   | 17.04      | 17.04      |
| SBIFN50-F53 | 15.64   | 15.64      | 15.64      |

An example of microBCA assay result
HPLC pump: Jasco PU-980 Intelligent HPLC pump
Detector: Jasco UV-1575 Intelligent UV/Vis detector
Column used: Superdex 200 prep grade
Mobile phase: 20mM Sodium acetate buffer, 150mM NaCl, pH 4.0
Flow rate: 1mL/min
Run time: 120 min
UV detection wavelength: 280 nm
Injection volume: 1900 uL

Retention time | Retention Area | Percentage Area
---|---|---
47.65 | 6773.02 | 29.95
59.63 | 3869.47 | 17.11
70.68 | 11627.32 | 51.42
100.50 | 342.15 | 1.51
| 22611.96 | 100.00

SEC-HPLC of 1 eq. reaction of PEG bis-sulfone 14a with leptin for 8 h purified by ion exchange without GRS refolding.
Bibliography


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