The Synthesis and Conjugation Chemistry of Polymeric Precursors for Medicine

by

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Abstract

This thesis is concerned with the development of physiologically soluble polymers for drug conjugation. As for any medicine, there is a need for polymer-drug conjugates to be structurally defined especially in terms of molecular weight (MW) and molecular weight distribution (MWD).

To develop polymeric precursors for the synthesis of polymer-drug conjugates possessing better optimised MW profiles and expedient conjugation chemistry, a first strategy involved the preparation of novel $N,O$-acetal protected $N$-(2-hydroxypropyl)methacrylamide (HPMA) monomers for anionic polymerisation. However, these monomers exhibited poor stability and anionic polymerisation was not achieved (other uses for these monomers are described based on free radical polymerisation).

A second more versatile strategy was adopted that utilised Atom Transfer Radical Polymerisation procedures to prepare a narrow MWD active ester homopolymer ($M_w/M_n = 1.1$ to $1.3$, $M_n = 2,000$ to $50,000$ g·mol$^{-1}$), and believed to be the first such example. Using the known active ester monomer, $N$-methacryloxysuccinimide, the reaction conditions had to be carefully controlled to ensure a successful polymerisation.

The conjugation chemistry (aminolysis) of the homopolymer was systematically evaluated and could be monitored using infra-red spectroscopy. The utility of this strategy was exemplified by the preparation of (1) model copolymer-drug conjugates with tailored drug loading and solubilising pendent chains and (2) methacrylic acid (co)polymers with incremental differences in structure for a cytokine and chemokine release study. A propensity for competitive hydrolysis and imide formation during conjugation was identified and experimental conditions were found to minimise/eliminate this problem.

It was concluded that such a narrow MWD active ester homopolymer has improved versatility over current precursors for the preclinical preparation and development of methacrylamide based polymer-drug conjugates and other methacrylamide speciality polymers for medicine.
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Chapter 1

Polymer-Drug Conjugation and the Need for Improved Synthetic Methods

1 Introduction

The covalent conjugation of a drug to a physiologically soluble polymer can improve the drug efficacy by taking advantage of the intrinsic biological properties of large molecules in solution.

The challenge undertaken in this thesis was to develop a more definitive, practical synthetic route to soluble polymer-drug conjugates that addresses a clear need for conjugates with better optimised molecular weight (MW) characteristics and improved structural definition.

2 Macromolecular Conjugation

The idea of conjugating biologically active molecules (drugs) to macromolecules slowly developed over the last century gaining momentum until Ringsdorf (1975) crystallised earlier ideas and proposed the first clear concept for polymer-drug conjugates. The Ringsdorf model (adapted in Figure 1.1) essentially describes a polymeric backbone to which is conjugated (1) pendent drug molecules via a biodegradable linker and (2) pendent chains to solubilise the macromolecule. Practically, the solubilising pendent chains relate to a physiologically soluble polymer or comonomer repeat units. The original model also included the co-conjugation of a
targeting moiety capable of mediating cell-specific targeting and more recent applications of polymer-drug conjugation have not required the linker between the polymer and drug to be biodegradable (Advanced Medicines, 2001; see also multivalency in section 7).

Figure 1.1. Model for polymer-drug conjugates also known as macromolecular prodrugs (adapted from Ringsdorf (1975)).

2.1 Biological rationale of polymer-drug conjugation

Unlike low MW drugs in the bloodstream, which either systemically diffuse into tissue or are readily excreted through the kidneys into the urine (renal ultrafiltration), macromolecules by virtue of their size exhibit prolonged circulation. The rate of renal excretion is dependent on the size of a molecule in solution (section 3) and so macromolecules are not so readily excreted. Macromolecules also cannot easily escape by diffusion from the blood stream into the extracellular fluid of healthy tissue because the endothelium (the cell lining of blood and lymph vessels) is unbroken, and so for these reasons, circulation half-life is increased.
While macromolecules cannot diffuse readily into healthy tissue, inflamed or diseased tissue sites, such as solid tumours, have been shown to be hyperpermeable because of a discontinuous endothelium (Jain, 1989; Maeda, 1992). This enables long circulating large molecules to escape from the blood capillaries and extravasate passively into inflamed or diseased tissue. Furthermore, a feature of solid tumours is a lack of effective lymphatic drainage, which in combination with the hyperpermeability, can lead to the accumulation of large molecules. This phenomenon has been termed the Enhanced Permeability and Retention (EPR) effect (Matsumura & Maeda, 1986). Once at the site of a tumour, the mode of cellular uptake of macromolecules is limited to the process of endocytosis (the cellular mechanism responsible for particulate and macromolecule uptake - see Appendix I). Endocytosis provides the opportunity to release the drug intracellularly in diseased cells (see also Appendix I). For these reasons, polymer anti-cancer drug conjugates have been well researched (Putnam & Kopecek, 1995).

Because of the covalent bonding, polymer-drug conjugates are considered ‘new chemical entities’ by regulatory authorities (Duncan et al., 1996). Covalent bonding can ‘inactivate’ the drug, reducing toxicity until the drug is released by the biodegradable linker at the site of action (e.g., inside the tumour cell). Another reason for the increased efficacy of these constructs derived from conjugation, particularly in the delivery of sparingly soluble drugs, is the more favourable physiochemical properties that exploit the properties of large hydrophilic molecules, i.e., increased drug solubility.

In addition to low MW drugs, higher MW drugs such as peptides and proteins have also been conjugated to polymers including enzymes, antibodies, hormones and cytokines (Nucci et al., 1991; Burnhamn, 1994; Duncan et al., 1996). The conjugation
of proteins to hydrophilic soluble polymers is aimed at improving the protein stability and the modification of the protein pharmacokinetic profile (Veronese & Morpurgo, 1999). Therapeutic proteins can suffer from short body residence times due to renal ultrafiltration and/or degradation by enzymes. In a similar way to the conjugation of low MW drugs, polymer-protein conjugation can prolong blood residence times, protect the protein from enzymatic degradation and prevent the protein from inducing immunological and antigenic reactions. The area of bioactive molecule conjugation to macromolecules has been extensively reviewed by researchers including Kataoka, 1997; Monfardini & Veronese, 1998; Brocchini & Duncan, 1999; and Kopecek et al., 2000. The advantages of drug conjugation to a polymer are summarised in Table 1.1.

---

**Table 1.1. Advantages of conjugation of bioactive compounds to macromolecules**

(adapted from Veronese & Morpurgo, 1999).
3 The Influence of Molecular Weight on the Fate of Soluble Polymers In-Vivo

The process of renal excretion for macromolecules in the bloodstream has a threshold value dependent on the size of the macromolecule in solution. Macromolecules with a hydrodynamic volume above the renal threshold are not excreted and are retained in the body. Yamaoka and co-workers (1994) studied the distribution of labelled poly(ethylene glycol) (PEG) after intravenous injection to mice using samples of MW between 3,000 and 190,000 g·mol⁻¹ and low polydispersities of about 1.2. The polydispersity of a polymer sample can be defined as the ratio of the weight average molecular weight (M_w) to the number average molecular weight (M_n), i.e. \( M_w / M_n \), and is a measure of the molecular weight distribution (MWD) (see Appendix II). PEG is a water-soluble non-ionic polymer and it was reported that high MW PEG was retained in the blood circulation for a longer period than low MW PEG. The half-life in circulation extended from 18 min to 24 h as the MW increased from 6,000 to 190,000 g·mol⁻¹. Renal clearance decreased with increasing PEG MW whereas liver clearance increased with increasing PEG MW after passing a minimum at around 50,000 g·mol⁻¹.

\[
\text{PEG:} \quad \overset{\text{HO\text{-\text{CH\text{-}\text{CH}_2\text{-O\text{-}H}}}}}{n}
\]

\[
\text{PHPMA:} \quad \overset{\text{CH}_2\text{-CO \text{-NH \text{-CH}_2 \text{-CHOH \text{-CH}_3}}}{n}
\]

For poly(N-[2-hydroxypropyl]methacrylamide) (PHPMA), another watersoluble non-ionic polymer, the renal threshold was identified at approximately 45,000 g·mol⁻¹ (Seymour et al., 1987) using fractionated PHPMA containing 1 mol%
methacryloyltyrosinamide (for radio-labelling) of MW 12,000 to 778,000 g·mol⁻¹. Fractions with a higher MW were lost only slowly from the bloodstream by extravasation. When the 778,000 g·mol⁻¹ fraction was given subcutaneously approximately 20% of the dose remained at the site of injection 21 d later. This increased retention was not seen for smaller fractions that moved readily into the bloodstream where they were lost in the urine or gradually penetrated into other tissues and organs.

\[
\text{CH}_2\text{CH}_2\text{OH} \quad \text{PVA}
\]

Yamaoka and co-workers (1995) also studied the effect of MW on body distribution using ¹²⁵I labelled poly(vinyl alcohol) (PVA) of MW ranging from 14,800 to 434,000 g·mol⁻¹ in mice. PVA is a also non-ionic, water-soluble polymer and it was found that the blood plasma half-life period was prolonged from 90 min (MW 14,800 g·mol⁻¹) to 25 h (MW 434,000 g·mol⁻¹). The renal excretion rate of PVA was rapidly reduced at around 30,000 g·mol⁻¹ as the MW increased. PVA was located in most organs but with very low accumulation, indicating insignificant interaction of PVA with cell components such as macrophages.

Yamaoka and co-workers studied the tumour accumulation of PVA when injected intravenously into tumour-bearing mice (Tabata et al., 1998), showing that PVA accumulated in tumour tissue to a significantly greater extent than in normal tissue which was attributed to the MW. PHPMA (and copolymers) has also been shown to accumulate in tumour tissue due to the EPR effect (Duncan et al., 1992; Noguchi et al., 1998). In both PVA and PHPMA studies it has been reported that smaller polymers that are excreted readily accumulate less well in tumour tissue than higher MW polymers. For narrow MWD ¹²⁵I-labelled PHPMA ranging from 4,500 to
800,000 g·mol\(^{-1}\) administered intravenously to tumour bearing mice (Noguchi et al., 1998), higher MW fractions (> 50,000 g·mol\(^{-1}\)) showed significantly increased tumour accumulation after 6 h compared to lower MW fractions (< 40,000 g·mol\(^{-1}\)). While the lower MW fractions accumulated effectively in the tumour tissue quickly after administration (within 10 min), they were rapidly cleared from the tumour due to rapid diffusion back into the bloodstream followed by renal clearance. These studies indicate that, if a polymer-drug conjugate is designed to passively accumulate in tumour or inflamed tissue via the EPR effect, then the MW and MWD has to be optimised to ensure a balance between renal excretion and EPR.

![PVP structure](https://example.com/pvp_structure.png)

The influence of MW on tissue retention was demonstrated by Hespe et al. (1977) using \[^{14}\text{C}\] poly(N-vinylpyrrolidone) (PVP) of similar MW but different MWD. Retention of the sample with a broader MWD was found to be much longer in organs than for the sample with a narrower MWD indicating the effect of large MW macromolecules. HPMA copolymers have also been used to study the effect of size on pinocytosis (Cartlidge, Duncan & Lloyd, 1986; see Appendix I for pinocytosis description). Using cross-linked HPMA copolymers with \(M_w\) ranging from 34,000 g·mol\(^{-1}\) to above 400,000 g·mol\(^{-1}\) it was reported that the rate of pinocytic capture is dependent on the MW. Rat yolk sacs capture lower MW polymers more rapidly whereas, in contrast, rat intestinal cells capture higher MW polymer most efficiently.
For polymers with intrinsic pharmacological activity (see polymeric drugs in section 7 below), the pharmacological activity can also be influenced by MW and MWD. Regelson and co-workers (1975) evaluated anionic polymers for biological activity. Using a hydrolysed pyran copolymer prepared from divinyl ether (DIVE) and maleic anhydride (MA), and often referred to as DIVEMA it was reported that narrow MWD samples possessed lower toxicity than broader MWD samples and retained anti-tumour activity against both Ehrlick adrenocarcinoma and Lewis lung carcinoma in mice.

The above mentioned literature strongly indicates that the polymer solution properties will frequently be the most important factor governing biodistribution, elimination, metabolism and toxicity of a polymer-drug conjugate. The size or hydrodynamic volume of a polymer in solution is related to the MW, chemical functionality and physical properties of the polymer. Consequently parameters such as shape, flexibility and charge influence the pharmacokinetics (Petrak & Goddard, 1989). Therefore, for the biological and toxicological reasons outlined, there is a strong need for polymers suitable for drug conjugation to have optimised MW, narrow MWD and optimised solubilising pendent chains (as depicted in Figure 1.1). This is essential for optimising structure-property relationships in pre-clinical development; ensuring the predictable behaviour required of any medicine and for obtaining stringent regulatory approval.

Ultimately, if the MWD is not controlled then safety is an issue, such as the potential for systemic accumulation leading to storage-disease syndrome toxicity (Seymour et al., 1987). An examination of the polymer conjugation literature reveals that many polymers considered for conjugation have not been optimised with regard to MW and MWD (polymers considered for conjugation are described in section 5 below.
4 Structural Limitations of Polymers for Conjugation

A broad range of polymers have now been investigated for drug conjugation and comprise those that are truly synthetic such as PEG and those that are derived from natural products such as the polysaccharide, dextran. Polymers are further divided into degradable and non-degradable types depending on their stability and fate in the body. Synthetic polymers are attractive for drug conjugation because they can potentially be tailored to the application requirements due to their diversity in chemical functionality, MW and architecture. However, control over these structural parameters and drug conjugation is non-trivial. All polymers for pendent drug conjugation can have structural limitations:

i) Control of the MW characteristics of synthetic polymers can be hard to achieve (e.g., polymers prepared by free radical polymerisation). This is essential to ensure predictable biological behaviour, to avoid undesirable biological responses and to prevent accumulation of high MW conjugates.

ii) The polymer may be limited by a lack of conjugation sites (i.e., functionality). A suitable polymer must provide sufficient carrying capacity with respect to the known potency of the drug it will deliver.

iii) Functional groups along a polymer molecule suitable for conjugation can be random (e.g., statistical copolymers) and therefore drug conjugation along a polymer molecule is unpredictable and therefore non-reproducible.

It is also important that the suitability of the polymer-drug conjugate for large-scale manufacture in terms of reproducibility, characterisation and overall cost is a consideration early in the development process.
Structure and synthetic issues are exemplified by HPMA based polymer-drug conjugates. HPMA copolymer-drug conjugates have been developed systematically over the last two decades and are one of the most studied class of linear, non-degradable synthetic polymer conjugates (Kopecek et al., 2000). However, it is only recently that HPMA copolymer conjugates have reached clinical evaluation as anti-cancer treatments due to the time taken to establish the biological rationale and the arduous process of chemical and biological optimisation (Vasey et al., 1999).

Currently HPMA copolymer-drug conjugates are prepared by a reaction involving a HPMA copolymer precursor with pendent p-nitrophenyl ester functionality (Scheme 1.1) and a low MW drug (e.g., doxorubicin) (Duncan & Kopecek, 1984). This type of reaction is known as a polymer analogous reaction, which is defined as the specific transformation of functional groups in a polymer without a change in the degree of polymerisation (Strohriegl, 1993; this definition is used throughout this thesis).

The use of the copolymeric precursor shown in Scheme 1.1 (and other analogous HPMA copolymers) has been essential to prepare HPMA based polymer-drug conjugates. From this one precursor, many polymer-drug candidates can potentially be prepared by conjugating different drug molecules. This is highlighted by the five HPMA copolymer anti-cancer drug conjugates currently under Phase I/II clinical evaluation in humans: HPMA copolymer-doxorubicin (PK1), HPMA copolymer-paclitaxel, HPMA copolymer-camptothecin, HPMA copolymer-platinate and HPMA copolymer-doxorubicin-galactosamine (PK2) bearing a cell specific targeting residue for hepatocellular uptake. These are the biggest polymer class of polymer anti-cancer drug conjugates in clinical evaluation (Duncan, 2001).
Scheme 1.1. Preparation of a HPMA copolymer-drug conjugate. Known as PK1 this HPMA copolymer conjugate is currently in Phase II clinical evaluation.
However, there are several potential limitations with this copolymer approach. The HPMA copolymer is typically prepared using the free radical initiator 2,2'-azo-bis-isobutyrylnitrile (AIBN) in acetone solution at 50 °C. This is a precipitation polymerisation which aims to limit the MW to below the renal threshold. For free radical prepared polymers the MWD is considered broad (Odian, 1991; Allcock 1990). Subr and co-workers (1992) as part of a drug release study using the anti-cancer drug adriamycin (doxorubicin) prepared a set of HPMA copolymer precursors with different p-nitrophenyl terminated comonomers, similar to the precursor in Scheme 1.1. Values of $M_w$ for these polymers varied from 13,000 to 23,000 g·mol$^{-1}$ (Table 1.2), which is well below the renal threshold for PHPMA ($\sim$ 45,000 g·mol$^{-1}$) and therefore not optimised for tumour accumulation (as described in section 3). The anti-tumour HPMA copolymer platinate in clinical trials has $M_w$ 24,400 g·mol$^{-1}$ (Stewart et al., 2000), therefore this polymer is also not optimised for tumour accumulation. Increasing the p-nitrophenyl content by increasing the comonomer concentration, led to a 6,000 g·mol$^{-1}$ difference in $M_w$ (entries 5 & 6, Table 1.2).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Spacer</th>
<th>mol% ONp</th>
<th>$M_w$</th>
<th>$M_w/M_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GlyLeuGly</td>
<td>5.4</td>
<td>23,000</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>GlyPheGly</td>
<td>5.8</td>
<td>19,500</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>GlyPheLeuGly</td>
<td>6.0</td>
<td>23,000</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>GlyLeuPheGly</td>
<td>4.6</td>
<td>18,500</td>
<td>1.4</td>
</tr>
<tr>
<td>5</td>
<td>GlyPheLeuGly</td>
<td>8.8</td>
<td>13,000</td>
<td>1.3</td>
</tr>
<tr>
<td>6</td>
<td>GlyPheLeuGly</td>
<td>4.6</td>
<td>19,000</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Table 1.2. Example characterisation data for HPMA polymeric precursors (data taken from Subr et al., 1992).
Although it can be argued that values for $M_w/M_n$ in Table 1.2 are reasonable for free radical prepared polymers, it is medically imperative that the value of $M_w/M_n$ is as close to unity as possible to ensure reproducible behaviour. Changing the comonomer or comonomer concentration in many cases (where Gly, Phe, Leu, and Gly are not the peptides) can have a dramatic effect on the MWD with values of $M_w/M_n$ over 2.5 (Duncan, 1998). These differences in MW characteristics of the copolymers make it extremely difficult to assess structure activity relationships and draw meaningful comparisons between different conjugates during preclinical evaluation.

Additionally, while $p$-nitrophenyl ester groups of the precursor react readily with compounds containing aliphatic primary amine groups in organic and buffered aqueous solutions (Rejmanová et al., 1977; Rihová and Kopecek, 1985) competitive hydrolysis due to the short half-life of this active ester (Hermanson, 1996) can be a problem. Many low MW anti-cancer drugs, for example, are hydrophobic. Hence, to ensure a homogenous reaction mixture with the hydrophilic polymer, DMSO and DMF are typically used as conjugation solvents. Since these solvents are very hygroscopic it can be difficult to exclude water from the reaction and prevent competitive hydrolysis of the $p$-nitrophenyl ester occurring. If not all the active ester groups of the copolymer precursor are required for conjugation to a bioactive molecule, then the remaining ester groups have to be quenched with a simple amine (e.g., $1$-amino-$2$-propanol), therefore adding another layer of structural heterogeneity to the molecule in terms of redundant pendent chains. Quenching of the remaining reactive groups is preferred to the preparation of another precursor with fewer reactive pendent chains because of the time needed to optimise a new polymerisation and the unavoidable differences in MW and MWD. Overall, the polymer analogous reaction depicted in Scheme 1.1 actually produces conjugates that have pendent chains terminated with either drug, carboxylate, or quenching amine (Figure 1.2).
Figure 1.2. Pendent chain heterogeneity associated with HPMA copolymer conjugates.

For the five HPMA copolymer conjugates in clinical evaluation, a precursor containing a Gly-Phe-Leu-Gly linker is incorporated (shown previously in Scheme 1.1) which is designed to undergo degradation by lysosomal enzymes (Duncan et al., 1984; Subr et al., 1986). The rate of degradation of a linker has been shown to be dependent on the polymer and drug combination (Soyez et al., 1996). Hence, the optimal combination of linker, drug and polymer may not be the same for every drug chosen. In principle, the HPMA copolymer methodology is limited by the need to make a new copolymer precursor for each new drug to be conjugated since the only parameter that can be readily changed in the final conjugate is the mol% of the drug.
5 Polymers for Conjugation

This section is devoted to the most common polymers investigated for drug conjugation, ending with a more complete summary with chemical structures in Table 1.3.

5.1 Polysaccharides

Potentially biodegradable polymer-drug conjugates such as polysaccharides are used in the hope that the polymer will be broken down into smaller MW units that can be readily eliminated. Partially hydrolysed and fractionated dextran is used as a blood plasma expander and was proposed as a drug carrier by Schacht (1987). This compares to native starch, which is degraded by amylases in the blood too rapidly to be used as a blood plasma expander. Dextrans are polysaccharides consisting of α-D-glucose units joined predominately by 1-6 linkages and are cleaved by enzymes (dextranases) which are not found in the blood.

Work by Vercauteren and co-workers (1990) has shown that the rate of degradation of dextran decreases with increasing degrees of modification. In the study dextran was modified by three methods: partial periodate oxidation and subsequent reduction of aldehyde groups; succinylation; and chloroformate activation with subsequent reaction with 2-hydroxypropylamine, ethylenediamine, and tris(2-aminoethyl)amine. It was found that the type of modification had no significant effect on the rate of degradation. It has also been found that lysosomal enzymes degraded dextran and its derivatives slower than was achieved by dextranases and that only minor quantities of glucose was liberated (Schacht et al., 1998). The results from the Vercauteren and Schacht studies suggest that the modification of polysaccharides may have serious consequences if they are to be used as biodegradable polymers for conjugation.
5.2 Polyaminoacids and proteins

Polyaminoacids have also been investigated for drug conjugation. With regard to proteins, albumin has been popular and continues to be investigated (Ohkwada et al., 1993; Yasuzawa & Tomer, 1997). The very narrow MWD of proteins is very appealing but proteins and polyaminoacids have similar drawbacks to polysaccharides. The degradation of proteins depends strongly on their structure, and like polysaccharides their degradation can lead to many different oligopeptide fragments being released (oligosaccharide fragments for polysaccharides). The biological activity of these fragments, resulting from nonspecific degradation, is hard to assess but may possess unwanted side effects such as immunogenicity. Hence, from a biocompatibility viewpoint, synthetic non-degradable polymers having a suitable MWD (i.e., narrow) may be preferable to degradable polymers because the products released into the bloodstream can be better defined as to their chemical structure and biological properties, resulting in more predictable behaviour and limited side effects.

5.3 PEG

PEG is a commercially available polymer that is generally recognised as safe and is frequently used for protein modification (Nucci et al., 1991). So called 'pegylated proteins' (e.g., enzymes, antibodies) exhibit prolonged blood circulation, reduced immunogenicity and a greater resistance to proteolytic enzymes compared to the unconjugated protein. A potential limitation of PEG for drug conjugation is an insufficient drug-carrying capacity due to only two potential conjugation sites at the terminal ends of the polymer (see PEG, Table 1.3 for structure). If a targeting moiety is also required, this leaves just one possible binding site for the drug. To overcome this, researchers have prepared PEG block copolymers to increase the number of possible conjugation sites. Zalipsky and co-workers (1992) synthesised a PEG-lysine copolymer that they used as a carrier for doxorubicin with MW up to 100,000 but the
urethane bonds between the lysine and PEG were not degraded by proteolytic enzymes. Pechar and co-workers (2000), investigated a multi-block PEG based drug carrier. In their system, PEG blocks were connected by diamine oligo peptide linkages enabling the attachment of two or three drug molecules to each repeating block. Using doxorubicin as the drug they demonstrated in-vitro release of doxorubicin and the degradation of the polymer chain by cathepsin B. In other systems, alternating PEG copolymers and block PEG copolymers that form micelles have been investigated (see also section 7).

5.4 Styrene-maleic acid/anhydride (SMA) copolymer

SMA conjugated with the potent anti-tumour protein neocarzinostatin (NCS) has been approved for clinical use as an anti-cancer treatment for patients with liver cancer in Japan. SMANCS was originally developed to increase the plasma half-life of NCS and to improve tumour and lymph node localisation.

The conjugate, known as SMANCS (reviewed in Maeda, 1991), is prepared using a proportion of the maleic acid units (30-50%) in reactive anhydride form. Two SMA molecules of about 1,500 g mol\(^{-1}\) are allowed to react with the free amino groups of NCS (Ala-1 and Lys-20) to give a conjugate of about 16,000 g mol\(^{-1}\). Some of the free carboxyl groups are butylated to increase the hydrophobicity to aid targeting to the lymphatics. Administered as an oily formulation using biocompatible lipids, SMANCS showed increased stability in circulation and high tumour accumulation with low systemic side effects.

5.5 PVP

PVP is a hydrophilic polymer that has been used to conjugate peptide and proteins through functionalised end groups. Semi-telechelic PVP containing −OH and −COOH end groups can be prepared by the use of chain transfer agents in the
polymerisation of N-vinylpyrrolidone (e.g., isopropoxyethanol; Sartore et al., 1994). Due to the broad MWD’s of the resulting polymers a fractionation step is required to prepare polymer of a convenient MW and MWD. Fractionation has been achieved using gel filtration and by solvent precipitation (Veronese et al., 1990). The activation of the functional end groups of PVP, e.g., by conversion to 4-nitrophenyl carbonate derivatives (Scheme 1.2), for protein conjugation has resulted in low yields of polymer conjugates (Sartore et al., 1994).

![Scheme 1.2. Activation of PVP using 4-nitrophenyl chloroformate (Sartore, 1994).](image)

**Scheme 1.2.** Activation of PVP using 4-nitrophenyl chloroformate (Sartore, 1994).

5.6 PHPMA

PHPMA is a hydrophilic, biocompatible polymer (Kopecek & Sprincl, 1974) that was originally developed for medical use in Czechoslovakia in the context of cross-linked polyacrylamides for biomedical hydrogels (Kopecek et al., 1973). The good biocompatibility suggested PHPMA would be an ideal candidate for drug conjugation (Kopecek et al., 2000). HPMA copolymers have been instrumental in the elucidation of chemical and biological properties of polymer-drug conjugates including: biocompatibility (e.g., Rihova et al., 1983); the development of lysosomally degradable oligopeptide polymer-drug linkers (e.g., Rejmanová et al., 1985); factors governing body distribution and EPR (e.g., Seymour et al., 1987); cell uptake/subcellular distribution (e.g., Flanagan et al., 1989); conjugates containing anti-tumour agents (e.g., Duncan et al, 1992); and the clinical development of polymer-drug conjugates (Vasey et al., 1999).
**Table 1.3.** Common examples of polymers used for drug conjugation ($R = \text{pendent chain}$).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Example Polymer Structures</th>
<th>Typical characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Nondegradable Synthetic polymers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHPMA</td>
<td><img src="image" alt="PHPMA structure" /></td>
<td>Pendent chain; peptidic or acid-labile for lysosomal release of covalently conjugated drugs. Example $M_n$ is around 23 kg mol$^{-1}$, $M_w/M_n \geq 1.4$ (Subr et al., 1992)</td>
</tr>
<tr>
<td>PEG</td>
<td><img src="image" alt="PEG structure" /></td>
<td>Both peptidic and acid-labile linkers have been used, but PEG also directly conjugated to drug (i.e. via an ester bond). Conjugation via end groups. Example MW's are 2 to 40 kg mol$^{-1}$ (Greenwald et al., 1996. No MWD data given)</td>
</tr>
<tr>
<td>DIVEMA</td>
<td><img src="image" alt="DIVEMA structure" /></td>
<td>Proteins can be conjugated through acid anhydride groups. Metal chelates (e.g. platinum) can be derived from carboxylic acid form of anhydride groups. Example MW is 30 kg mol$^{-1}$ and $M_w/M_n$ is 1.4 (values from fractionated samples, Hirano et al., 1994).</td>
</tr>
</tbody>
</table>
PVP

\[
\begin{array}{c}
\text{Fractionation normally required to obtain narrow MWD (Satore \textit{et al.}, 1994).}
\end{array}
\]

SMA

Proteins with free amine groups can be conjugated through reaction with anhydride groups, e.g., neocarzinostatin. Typical \( M_w \) is 1.5 to 6 kg mol\(^{-1}\), more than one molecule may be conjugated to the protein. No MWD data given (Maeda, 1991).

II. Potentially Degradable Polymers

Most of these polymers are naturally occurring or can be synthesised from natural metabolites.

Polysaccharides

Dextran
Chitosan
Carboxymethyl chitin
Carboxymethyl pullulan
Alginate

Polymer side chain usually derivatised to carboxylate or amine then conjugated with the drug. Can be conjugated with an appropriately derivatised drug to give either peptidic or acid-labile pendent chain for lysosomal drug release.
Poly(α,β-aspartic acid) block

Formation of polymeric micelles capable of both drug conjugation and entrapment. Linker potential similar to poly(α-ω)amino acids.

PEG-Lysine alternating polymer

Provides functionality to allow conjugation to predominantly PEG polymer. $M_w$ is typically 50 to 100 kg mol$^{-1}$ (e.g., Pechar et al., 2000; No MWD value given but GPC chromatogram is extremely broad)

PEG-Lysine

Pendent chain options are similar to poly(α-ω)amino acids.

Polymer side chains tend to be derivatised analogous to polysaccharides to give pendent chain conjugation, which can be either peptidic or acid-labile.
Chapter 1: General Introduction

6 Preparation of Polymer-Drug Conjugates

The conjugation of bioactive molecules to a polymer is typically achieved using two distinct strategies, which can be classified as follows (Putnam & Kopecek, 1995):

1. Copolymerisation of a polymerisable drug derivative with suitable monomer(s) to confer physiological solubility (Option 1, Scheme 1.3). This is a two-step method because the polymerisable drug derivative must first be synthesised.

2. Attachment of the drug to a pre-formed polymeric precursor via a polymer analogous reaction (Option 2, Scheme 1.3).

Scheme 1.3. General methods for the preparation of polymer-drug conjugates (adapted from Putnam & Kopecek, 1995).

Polymerisable drug derivatives (Option 1)

The potential advantage of this method compared to the polymer analogous strategy is that low MW monomers can be prepared first, which can be easier to characterise compared to the characterisation of the polymer-drug conjugate. Polymerisable drug derivatives can also limit problems associated with reactions on a
polymer, such as uniform pendent chain structure (see Figure 1.2). However, it is imperative that the drug is stable during polymerisation.

Ulbrich and co-workers (Rihová et al., 1990) used this strategy to prepare a HPMA copolymer using a polymerisable derivative of daunomycin, N-methacryloylglycylphenylalanyl-leucylglycyl-daunomycin (MA-Gly-Phe-Leu-Gly-DNM). The synthesis of this methacryloyl-oligopeptide comonomer and other similar comonomers terminated with a p-nitrophenyl ester (-ONp) used for the preparation of HPMA copolymers, such as MA-Gly-Phe-Leu-Gly-ONp, is a multi-step procedure involving the conjugation of dipeptides (Scheme 1.4). The p-nitrophenyl ester was originally chosen because of the successful use of such esters in peptide chemistry and also for reactive polymers (Ferruti et al., 1972).

**Scheme 1.4.** Preparation of comonomer for preparing HPMA copolymer precursors containing a Gly-Phe-Leu-Gly polymer to drug linker (adapted from Putnam & Kopecek, 1995).
Ulbrich and co-workers copolymerised the daunomycin derivative monomer with HPMA and MA-Gly-Gly-ONp in an analogous way to the preparation of PK-1 shown in Scheme 1.1, resulting in a HPMA copolymer possessing bound drug (via a lysosomally degradable tetrapeptide linker) and an p-nitrophenyl ester reactivity for the binding of targeting moieties. Interestingly, this HPMA copolymer requires a polymer analogous reaction for the preparation of the final conjugate. The final conjugate contains two bioactive agents (daunomycin drug and the targeting moiety). Another example is the copolymerisation l-(metha)acryloyloxymethyl-5-fluorouracils with monomers such as acrylic and methacrylic acid, methyl acrylate and methyl methacrylate. The conjugates prepared all possessed biological activity (Ozaki et al., 1989) indicating the drug molecules were stable during the polymerisation. Further examples are can be found in the review by Duncan & Kopecek (1984).

Use of polymer analogous reaction(s) (Option 2)

For this strategy, a preformed polymer (polymeric precursor) that then undergoes reaction/conjugation with the desired bioactive molecule is used, e.g., HPMA copolymers as shown in Scheme 1.1. In this manner, many different polymer-conjugates can be prepared from the one polymeric precursor by conjugating different bioactive molecules. This potentially eliminates the need for optimising a new polymerisation. A suitable polymeric precursor could be designed therefore to prepare families of polymer-drug conjugates that share the same MW characteristics derived from the backbone of the precursor. This is a significant advantage when investigating the relationship between structure and biological activity.

In all cases for Options 1 & 2, a prerequisite for conjugation is suitable functionality on both the polymer (or monomer for Option 1) and the conjugating molecule. If this functionality is not present then one or both of the reactants have to be modified to allow conjugation. Many possible drug candidates may therefore be
unsuitable for conjugation. For example, the choice of drugs conjugated to HPMA copolymer precursors such as in Scheme 1.1 has been influenced by the convenience of those drugs containing a single primary amine.

Polymers can be activated by coupling reagents or by chemical modification. Activation of carboxylic acid groups on pendant chains for example, has been achieved using carbodiimides with drugs that also possess a suitable amine group, e.g., \( N \)-ethyl-\( N' \)-(3-dimethylaminopropyl)carbodiimide was used to couple to poly-\( \alpha,\beta \)- (DL-aspartic acid) (Giammona et al., 1989).

In addition to HPMA copolymer precursors, the activation of HPMA homopolymer has also been investigated using \( p \)-nitrophenylchloroformate (Scheme 1.5; Lääne et al., 1983) and cyanogen bromide (Scheme 1.6; Chytry and Kopecek, 1983). Both methods can be used for activation of any hydroxyl-containing polymer. Another example in the literature for introducing functionality for conjugation, is the use of chain-transfer agents during free radical polymerisation to produce semi-telechelic polymers (Lu et al., 1998).

\[
\begin{align*}
\text{Scheme 1.5. Activation of HPMA homopolymer for drug conjugation using } p- \\
\text{nitrophenylchloroformate.}
\end{align*}
\]
Scheme 1.6. Activation of hydroxyl containing polymer using cyanogen bromide.

Although many polymer conjugates have been synthesised and studied to date, clinical progress has been slow. The biological and chemical rationale has however, now been more or less established and so with the next generation of polymer conjugates the process of optimisation has to begin. An examination of more efficient preparation methods for the next generation of macromolecular therapeutics is a necessary next step.

7 Complementary Uses of Polymers Suitable for Drug Conjugation

Soluble polymers developed for bioactive molecule conjugation may also be separately developed for other drug delivery and drug therapy applications, and in similar ways to polymer-drug conjugates, control over MW and chemical functionality is important for optimal performance. In addition to the polymer-drug and polymer-protein conjugates mentioned so far in this introduction, other examples of soluble therapeutic polymeric systems include polymeric drugs possessing intrinsic biological activity and polymeric micelles.

Polymeric drugs

While some synthetic polymers are biocompatible and make ideal candidates for drug conjugation, many polymers show intrinsic biological activity and are known as polymeric drugs. The majority of these polymers are polyelectrolytes. Polycations
have electropositive groups attached to the polymer chain or pendent groups and have been shown to be antibacterial agents. Poly-L-lysine, for example, is active against E. coli (Ottenbrite, 1980). A problem with polycations is that they also tend to show inherent toxicity, limiting their medical use. As an aside to polymeric drugs, polycations are becoming increasingly investigated as non-viral vectors to condense anionic plasmid DNA for gene therapy, e.g., poly-L-lysine, polyethyleneimine and the polysaccharide, chitosan (Garnett 1999). Polyanions on the other hand are generally much less toxic and have been shown to exhibit a broad range of biological activity including anti-viral, anti-tumour, anti-inflammatory and immunological effects (reviewed by Baird et al., 1980 and Seymour, 1991). Examples of polyanions showing biological effects include poly(meth)acrylic acid and copolymers, the hydrolysed form of DIVEMA and maleic anhydride copolymers (Ottenbrite & Kaplan, 1985). The sulphated polysaccharide, dextrin-2-sulphate has shown potential in early clinical trials as an anti-AIDS treatment (Shaunak et al., 1998). It is increasingly being demonstrated that the molecular structure and stereochemistry of polymeric drugs is critical to the biological activity and toxicity observed. Consequently, there is a need for structurally defined polymers with respect to MW, copolymer properties, chain coiling, cross-linking, tacticity and electrolytic character to establish structure-activity relationships for polymeric drugs.

Polymeric micelles

Polymeric micelles have been proposed as drug delivery systems through both active and passive targeting (Bader et al., 1984; reviewed by Jones & Leroux, 1999). A polymeric micelle is a macromolecular assembly forming from block or graft copolymers with a spherical inner core and an outer shell (Figure 1.3). Drugs can be incorporated into the micelles using chemical conjugation or/and physical entrapment.
The main advantage of polymeric micelles compared with other soluble drug delivery systems is their large (10-80 nm) yet relatively uniform size, which can be used to prolong blood circulation and to passively accumulate in tumour tissue due to the EPR effect (Yokoyama et al., 1991) and their large drug-carrying capacity (Kataoka et al., 1993).

![Diagram of drug loaded polymeric micelle](image)

**Figure 1.3.** Drug loaded polymeric micelle formed from hydrophilic-hydrophobic block copolymer (The drug shown (red circles) is conjugated to the block copolymer in this case but physical entrapment can also be used).

Since micelles are supra-molecular assemblies of non-covalent interactions the polymer chains can theoretically dissociate into single polymer chains that are small enough to undergo renal excretion. Poly(ethylene glycol-aspartate) block copolymer containing the anti-cancer drug adriamycin by conjugation and physical entrapment, is an example of a micelle system designed for passive tumour targeting (Yokoyama et al., 1999).

**Other drug delivery applications**

There are several methods of drug delivery that involve the use of polymers (Table 1.4), although generally as insoluble materials rather than as soluble molecules.
Polymer application | Example(s)
--- | ---
Soluble systems | Self assemblies e.g., micelles, aggregates, polyelectrolyte complexes,
Macromolecular prodrugs e.g., polymer-drug conjugates
Particulate systems | Hydrogels
Microparticles > 1 μm
Nanoparticles < 1 μm
Hybrid systems | e.g., nanoparticles surface modified with PHPMA (Kamei et al, 1995)
Implants
Excipients

Table 1.4. Example applications of functional polymers in drug delivery.

These include drug release matrices such as micro- and nanoparticles which are often made from degradable polylactide or poly(lactide-co-glycolide) (co)polymers via precipitation or phase separation emulsion techniques. Good control over MW and chemical functionality is essential for the performance of the particle.

\[
\begin{align*}
\underset{\text{CH}_3}{\text{O}} & \underset{\text{H}}{\text{C}} \underset{\text{O}}{\text{C}} \underset{\text{H}}{\text{C}} \underset{\text{O}}{\text{C}} \underset{\text{H}}{\text{C}} \underset{\text{O}}{\text{C}} \underset{\text{H}}{\text{C}} \underset{\text{O}}{\text{C}} \underset{\text{H}}{\text{C}} \underset{\text{O}}{\text{C}} \underset{\text{H}}{\text{C}} \underset{\text{O}}{\text{C}} \\
\text{poly(lactide-co-glycolide)}
\end{align*}
\]

Poly(lactide-co-glycolide) is also used as a subcutaneous polymer implant under the name Zoladex® for the delivery of a luteinizing hormone analogue over 28 days as a treatment for prostate cancer. Hydrogels, water-swollen cross-linked
polymer networks, can be used for a wide range of applications including contact lenses, wound dressings and drug delivering implants. The neutral ester 2-hydroxyethyl methacrylate is the most widely used monomer for hydrogel preparation but other monomers include N-vinylpyrrolidone, acrylamide derivatives and acrylics (Peppas et al, 2000).

Polymers are also widely used as excipients (pharmaceutically inactive matrix material used to guarantee good tablet formation, integrity, and strength) in drug formulation, e.g., cross-linked poly(acrylic acid) (carbomer) is used as a release-modifying agent, tablet binder, viscosity-increasing agent, emulsifying agent and bioadhesive (Kibbe, 2000). Other polymer excipients include PVP, PEG (including derivatives) and various cationic and anionic polymethacrylates, such as the product of methyl methacrylate (MMA) copolymerised with methacrylic acid (MAA) (Kibbe, 2000).

\[
\text{\text{poly(MMA-co-MAA)}}
\]

In addition to drug therapy systems, other uses of bioactive molecules conjugated to suitable polymers and stimuli-response polymers have been investigated. These include applications in diagnostics, biosensors, affinity separations, enzyme bioprocesses and cell culture processes (Hoffman 1998). Other uses include the reduction of protein and cell adherence to biomaterials, and to provide a biologically compatible interface with the surrounding tissue leading to a reduction in foreign body response, e.g., by conjugation of PEG to the biomaterial surface.
Functional polymers can also be used as sequestering agents to selectively bind and eliminate target substances from the intestinal tract. For example, Renagel® containing cross-linked poly(allylamine hydrochloride) is used for serum phosphorus removal for patients with end-stage renal disease (Geltex Pharmaceuticals, 2001).

Another emerging area for functional polymers are polyvalent therapeutic agents that aim to take advantage of the synergistic effect of multiple binding in biological processes such as antibody-antigen interactions. Whitesides and co-workers (Sigal et al., 1996) studied the effect of multivalent polyacrylamides on the agglutination of erythrocytes by Influenza virus, concluding there was a strong inhibition reflecting the enhanced binding that occurs through co-operative polyvalent interactions. In the study polyacrylamides bearing pendent sialoside groups were prepared by free radical polymerisation. Fractionation by Gel Permeation Chromatography (GPC) was necessary to test the MW dependence of the polymeric inhibition. Different MW fractions were laboriously isolated on significantly less than 1 mg scale from 20 GPC runs using an analytical column. This is a good example of the need for more expedient methods for preparing functionalised polymers that possess narrow MWD’s. Whitesides recently argued that the polydisperse nature of polymers and the difficulty in characterising polymers possessing multiple functionality is likely to slow their progress through clinical practice (Borman, 2000).
8 General aim of this study

From the studies described and the considerations outlined in this introduction, it becomes clear that the increased and more widespread adoption of polymer conjugation requires new polymers that must be able to:

- conjugate efficiently to the bioactive molecule(s)
- have better uniformity in structure
- have optimised MW and narrow MWD
- be amenable to a conventional pre-clinical development programme, e.g., enable fast and efficient preparation of polymer-drug conjugates.

In addition biocompatibility should be retained. The aim of this thesis was to prepare narrow MWD synthetic polymers that possess suitable functionality to be exploited as improved, versatile and expedient precursors for the preclinical preparation and development of biologically relevant polymer-drug conjugates.

In Chapters 2 and 3, experiments were made to find an appropriate strategy, i.e., monomer and polymerisation conditions, in order to prepare suitable narrow MWD polymers. Chapter 4 describes a systematic study into the conjugation chemistry of the polymers that were prepared in the preceding chapters for their applicability as precursors to (multi)-functional medicinal polymers, including methods of characterisation and limitations. Precursors for conjugate preparation may also have utility for the preparation of many other medicinal speciality polymers (e.g., polymeric drugs). Hence, in the final experimental chapter, Chapter 5, carboxylate polymeric drugs with incremental changes in structure were prepared and assessed for their effect on chemokine and cytokine release from peritoneal macrophages in order to further investigate the precursor strategy chosen.
Chapter 2

Polymeric Precursors Derived from N,O–Acetal Protected (Meth)Acrylamide Monomers

1 Introduction: Choice of Polymer and Polymerisation Conditions

When considering the preparation of an appropriate polymer for use as a narrow MWD polymeric precursor, biological considerations are paramount. Perhaps the most thoroughly studied synthetic polymers for low MW drug conjugation are PHPMA 1 and related copolymers (refer also to Scheme 1.1, Chapter 1), which have been shown to be excellent for drug conjugation in terms of their solution properties and biocompatibility (see also section 5, Chapter 1). These factors are exemplified by the five HPMA copolymer conjugates that form the biggest class of polymer-drug conjugate anti-cancer treatments in clinical evaluation (Duncan, 2001).

A limitation however of PHPMA-based conjugates has been their non-optimised MW characteristics. It therefore seemed appropriate to develop narrow MWD precursors based on a PHPMA 1 model. In this study, anionic polymerisation was explored as a route to narrow MWD PHPMA homopolymer through the preparation of novel (meth)acylamide monomers suitable for the anionic polymerisation process.
1.1 Anionic polymerisation

An anionic polymerisation is an addition polymerisation in which the propagating chain bears a negative (anionic) charge (Scheme 2.1). Anionic polymerisation is suitable for monomers possessing electron-withdrawing groups, and although significant success has been achieved with styrene and styrene derivatives (Hseih & Quirk, 1996), acrylamide monomers have also been polymerised by this method (e.g., Shields & Coover, 1959; Butler et al., 1960; Xie & Hogen-Esch, 1996; Shiohara et al., 1998).

\[ \text{H}_2\text{C}==\text{CH}_2 + \text{Nu}^- \rightarrow \text{Nu} \text{CH}_2\text{CH}_2\text{OH}^- + \text{monomer} \rightarrow \text{Nu} \left[ \text{CH}_2\text{CH}_2\text{OH}^- \right]_n \text{CH}_2\text{CH}_2\text{OH}^- \]

\[ R = \text{electron withdrawing substituent} \]
\[ \text{Nu}^- = \text{anionic (nucleophilic) initiator} \]

Scheme 2.1. Anionic polymerisation – suitable for monomers with electron withdrawing substituent(s).

Anionic polymerisation can proceed with an absence of chain transfer and termination reactions (see Appendix III) on the polymerisation time-scale and this has been termed ‘living’ (Szwarc, 1956; 1983). Providing that there is fast initiation, narrow MWD polymers can be produced \((M_w/M_n < 1.25)\). Another important aspect of anionic polymerisation is that providing there is quantitative initiation, the monomer to initiator molar ratio defines the molecular weight of the polymer (Hseih & Quirk, 1996). This is important because it would ensure the reproducible synthesis of any polymer intended for drug conjugation. The addition of further monomer in a living anionic polymerisation results in continued propagation, allowing the preparation of
Chapter 2: Protected (meth)acrylamide monomers

block copolymers. Block copolymers would be useful for defining the location of drug in a polymer-drug conjugate and also for the formation of drug-carrying micelles since drug molecules are often hydrophobic in nature (see also section 7, Chapter 1). For appropriate monomers, anionic polymerisation has been shown to be a very reliable and versatile method for the synthesis of well-defined polymers and copolymers (Hong et al., 1999).

An experimental limitation of living anionic polymerisation is an intolerance to acidic protons that can react with the initiating or propagating anion leading to chain transfer and termination reactions. Acidic protons are found in monomers possessing functionality such as hydroxyl groups and in impurities such as water. For example, residual moisture terminates propagating carbanions by proton transfer. The resulting hydroxide ion is usually not sufficiently nucleophilic to reinitiate polymerisation (Odian, 1991). To enable anionic polymerisation of monomers containing acidic protons, it is necessary for those protons to be protected during the polymerisation process. For example, hydroxyl groups have been protected using dimethyl-tert-butylsilyl groups and monomers with primary and secondary amines functionality with trimethylsilyl groups to enable the anionic polymerisation of functional styrene derivatives (Hirao & Nakahama, 1998).

Therefore, to prepare a narrow MWD PHPMA precursor by anionic polymerisation requires a strategy that involves the protection of the hydroxyl and amide protons present in the HPMA 2 monomer (Scheme 2.2). The choice of protecting group(s) is dictated by the need for the facile, selective reaction with only the required protic functional groups. Furthermore, the protecting group(s) must be stable and unreactive during polymerisation but be amenable to complete deprotection without side reaction(s) occurring on the polymer, e.g., cleavage of other functional groups. The two acidic protons of HPMA 2 can be protected either simultaneously with a single protecting group, or individually with the same or different protecting
groups (represented by monomers 3 & 4, Scheme 2.2). A simultaneous protecting group (as for monomer 3) was elected for study in the experiments described below, in order to simplify the protection/deprotection process.

Scheme 2.2. Protection options for the acidic protons of HPMA 2.

It is crucial to consider the conjugation method of any polymer prepared for use as a precursor. For PHPMA prepared by anionic polymerisation, two methods for enabling conjugation can be envisaged. The first method is to use a comonomer during the anionic polymerisation with suitable reactivity for drug conjugation. Practically, this may also involve the use of another protected monomer, e.g., 5, where the (co)polymer, e.g. 6, derived from this monomer and 3 can be deprotected after polymerisation to reveal the suitable functionality (Scheme 2.3).

Scheme 2.3. Copolymerisation method for introducing conjugation functionality for PHPMA prepared by anionic polymerisation.
An alternative method is to prepare a narrow MWD homopolymer 6 using a protected HPMA monomer, then completely deprotect the polymer to produce PHPMA 1, followed by the activation of a proportion of the hydroxyl groups to product an activated copolymer 8 to enable drug conjugation (Scheme 2.4). This conjugation method has already been utilised for PHPMA 1 prepared by free-radical polymerisation using p-nitrophenylchloroformate or cyanogen bromide (see Schemes 5 & 6 section 6, Chapter 1).

\[
\begin{align*}
6 & \quad \text{deprotection} \quad \rightarrow \quad 1 \\
8 & \quad \text{activation} \quad \rightarrow \quad R_2
\end{align*}
\]

\[R = \text{protecting group} \quad \quad \quad R_2 = \text{activating group}\]

**Scheme 2.4.** Alternative strategy for utilising PHPMA 1 homopolymer (where \(R_2\) is an activation group) prepared by anionic polymerisation.

A homopolymer strategy (Scheme 2.4) was selected over a copolymer strategy (Scheme 2.3) in this study, since only the polymerisation of one monomer has to be optimised and the level of drug conjugation is not dictated by the polymerisation chemistry. This potentially allows the mol\% of drug to be more easily varied over a wide range without the need for further polymerisations, depending on the suitability of the homopolymer 1 activation chemistry. Since the linker and drug molecule are generally of low MW, these molecules can be more easily characterised before
conjugation, and providing the conjugation chemistry is well-defined, uniform pendent chain structure can be achieved. Additionally, using only a homopolymer precursor as a common intermediate, different drug and conjugating pendent chains (linkers) at varying stoichiometries can be utilised to prepare families of conjugates. These families will retain the same well-defined MW characteristics of the precursor, which is essential for the systematic study of polymer-drug conjugates.
2 Results and Discussion.

2.1 Synthesis of HPMA 2

Monomer 2 can be synthesised by the reaction of methacryloyl chloride 9 with 1-amino-2-propanol 10 in dichloromethane (DCM) solution (Kopecek & Bazilova, 1973; Scheme 2.5). Triethylamine 11 is added to the reaction to quench the HCl that is generated and prevent protonation and/or reaction with the vinyl group of the amine 2. The procedure has been used successfully to prepare the kg scale quantities of 2 required for the preparation of the HPMA copolymer drug-conjugates in clinical evaluation.

\[
\begin{align*}
&\text{CH}_2=\text{CHCONH}_2 + \text{H}_2\text{NCH}_2\text{OH} \rightarrow \\
&\text{CH}_2=\text{CHCONHCH}_2\text{OH} + (\text{CH}_3\text{CH}_2)_3\text{N}+\text{HCl}
\end{align*}
\]

Scheme 2.5. Literature procedure for the synthesis of HPMA 2.

A problem with this procedure is the complete removal of triethylamine hydrochloride side product 12, which can lead to the requirement for multiple purification steps, e.g., by recrystallisation. As an alternative to this procedure it was found that 2 can be synthesised by allowing methacryloyl chloride 9 to react with 1-amino-2-propanol 10 in aqueous NaOH (Schotten-Baumann conditions; Scheme 2.6 and experimental section 2.1, Chapter 7).

\[
\begin{align*}
&\text{CH}_2=\text{CHCONH}_2 + \text{H}_2\text{NCH}_2\text{OH} \rightarrow \\
&\text{CH}_2=\text{CHCONHCH}_2\text{OH} + \text{NaCl} + \text{H}_2\text{O}
\end{align*}
\]

Scheme 2.6. Schotten-Baumann conditions: an alternative method found for the synthesis of HPMA 2.
Chapter 2: Protected (meth)acrylamide monomers

The product, HPMA 2, was extracted into ethyl acetate (EtOAc) leaving the NaCl side product in the aqueous phase. Recrystallisation from acetone afforded analytically pure 2 (68% yield).

2.2 Synthesis of protected HPMA monomer 2

2.2.1 Di-tert-butylsilylene protection group

While there are many established procedures for the protection of isolated alcohols, 1,2-diols (e.g., substituted methyl and ethyl ethers, acetals, ester and silyl esters) and isolated amides (e.g., N-acyl derivatives), literature describing the simultaneous protection of an amide and an alcohol using just one protection group is less abundant (Greene, 1981). A first attempt at protection was made using di-tert-butyl dichlorosilane 13 (Scheme 2.7). This protection group reacts well with diols and can be removed by treatment with pyridinium hydrofluoride (Trost & Caldwell, 1981). The reaction of 2 with 13 in acetonitrile solution in the presence of triethylamine and 1-hydroxybenzotriazole (HOBT) as a catalyst for silyl transfer did not yield any of the desired product 14 after several separate attempts.

![Scheme 2.7. Attempted di-tert-butyl silylene protection of HPMA 2.](image-url)
Both NMR and TLC analysis of the product was indistinguishable from the starting compound, 2 (see section 2, Chapter 7) indicating that no reaction had occurred. It is possible that the desired product was not formed in the reaction due to the bulky nature of the protection group and rigid structure of the intended cyclic amide structure. In later experiments described below, steric hindrance becomes an important consideration during the synthesis and polymerisation chemistry of other protected monomers being developed. This reaction was not continued and attention was focused on N,O-acetal protection since this type of protection has lower steric demands and is easier to remove than a di-tert-butyl silyene protection group.

2.2.2 Acetal protection and the formation of oxazolidines

Oxazolidines are cyclic N,O-acetals and are the condensation products of β-amino-alcohols and aldehydes or ketones. Similar to cyclic O,O-acetals prepared from 1,2 and 1,3 diols, oxazolidines can be cleaved under mild acidic conditions (Buur & Bundgaard, 1984). The formation of N,O-acetals are therefore an excellent protection method, and moreover, acetal bonds have been found to be stable under anionic polymerisation conditions (Hirao & Nakahama, 1998).

Saavedra (1985) condensed a primary alkanolamine with a range of ketones to prepare oxazolidines by stirring in the presence of anhydrous K₂CO₃ to aid water removal. In this study, similar conditions were examined for the reaction of 1-amino-2-propanol 10 with acetone 15 in DCM solution in the presence of anhydrous K₂CO₃ to synthesise 2,2,5-trimethyl-oxazoline 16 (Scheme 2.8).

The formation of 2,2,5-trimethyl-oxazoline 16 in this reaction is an equilibrium process with the Schiff base 17. The oxazolidine is generally always the predominant form in the equilibrium in this type of reaction (Bergamann, 1953) and this was confirmed for 16 by $^{1}$H NMR where the integral of one proton of 16 was equal to twice the integral of one proton of 17 (integral values for CH-CH$_3$ proton 15.0 ($\delta$ 3.98) & 7.5 ($\delta$ 3.64) respectively). To prepare the N,O-acetal protected HPMA monomer, N,O-isopropylidene-N-(2-hydroxypropyl)methacrylamide 18 (Scheme 2.8), the solvent was removed from the oxazolidine reaction mixture and the crude product was then allowed to react with methacryloyl chloride 9 under aqueous Schotten-Baumann conditions, in a repeat of the conditions used to prepare HPMA 2 from 1-amino-2-propanol 10. By performing the reaction in aqueous media the hydrophilic side products (methacrylic acid sodium salt) and unreacted amine could easily be separated from the more hydrophobic product by extraction of the protected monomer into an organic phase. A liquid product that solidified on standing was isolated (61% yield). The product was distilled under vacuum to give a crystalline solid 18 on standing that melted with very gentle heating (see experimental section 2.1.2, Chapter 7). As expected for the pure monomer 18, analysis by $^{1}$H NMR (CDCl$_3$) showed the
absence of any signals due to the amide and hydroxyl protons, while two singlets relating to the two isopropylidene methyl protons were present (for NMR signals see section 2.1.2 Chapter 7). After allowing the hydrophobic 18 to react with HCl in aqueous THF, the compound reverted to a solid product on isolation whose NMR spectrum was consistent with that of hydrophilic HPMA 2, indicating the suitability of this protection group. This was an important first indication that polymers derived from this monomer might be deprotected to give PHPMA 1.

To increase the number of monomers available for anionic polymerisation, which may have been important for deprotection and solubility profiles for example, an attempt was made to synthesise 5-methyl-2-phenyl-oxazolidine 20, as a first step in the synthesis of monomer 22 (Figure 2.9). When benzaldehyde 19 was allowed to react with 1-amino-2-propanol 10 the result was the formation of a white solid in high yield (86%) that was purified by recrystallisation.

**Scheme 2.9.** Condensation of 1-amino-2-propanol 10 and benzaldehyde 19, followed by reaction with methacryloyl chloride 9.
While $^1$H NMR confirmed a pure single product, the signals were actually consistent with the Schiff base, $N$-(2-hydroxypropyl)benzylideneamine 21, and not the oxazolidine 20 since a signal was observed at $\delta$ 8.32 with an integral for one proton, which is sufficiently down-field to be indicative of an imine proton, i.e., $N=CH$ (Silverstein et al., 1991; for NMR signals see experimental section 2.1.1, Chapter 7). Furthermore, a Fourier Transform infra-red (FT-IR) spectroscopy spectrum of the solid product exhibited a band at 1646 cm$^{-1}$ of strong intensity, also suggesting the presence of an imine group (Silverstein et al., 1991) The carbonyl band of the starting product benzaldehyde at 1699 cm$^{-1}$ was not present. Also in the IR was a broad band at 3215 cm$^{-1}$ that can be attributed to OH stretching, further indicating that the product was in fact 21. This is plausible since imines with aromatic substituents are known to be quite stable (Furniss et al., 1989). The solid product was allowed to react with methacryloyl chloride 9 under Schotten-Baumann conditions. It was hoped that a reaction with 9 would still occur with the imine 21, resulting in the closure of the ring to form the oxazolidine containing monomer 22, or that a reaction would occur with 20 if present due to the equilibrium with 21. The product 22, was however, not formed. This was also the outcome when the reaction was conducted in DCM in the presence of triethylamine. Further attempts were not made to prepare 22 since the protected monomer 18 had already been prepared and attention was focused on the polymerisation chemistry.
2.3 Anionic polymerisation experiments using \(N,O\)-isopropylidene-\(N\)-(2-hydroxypropyl)methacrylamide 18

This anionic polymerisation study was performed during a two-month period spent at the polymer laboratories of Professor Axel Müller, then of Mainz University in Germany. Using rigorously cleaned reagents and glassware, the anionic polymerisation of monomer 18 was attempted in tetrahydrofuran (THF) solution at \(-78^\circ\text{C}\) using 1,1-diphenylhexyllithium 22.5 as the initiator (Scheme 2.10 and experimental section 2.2, Chapter 7). Additionally, \(n\)-octane was added to the reaction solution as an unreactive standard to evaluate monomer consumption by gas chromatography (GC).

![Scheme 2.10. Conditions for the attempted anionic polymerisation of monomer 18.](image.png)

The characteristic red colour of the initiator solution immediately changed to colourless when the initiator and monomer solutions were mixed. After 30 min, any reaction was stopped by quenching with methanol. GC of the reaction solution quenched with methanol showed a constant ratio of \(n\)-octane to monomer indicating no polymerisation had occurred. This reaction was repeated twice with freshly-distilled monomer with the same outcome. In order to be sure that degradation of
monomer 18 had not occurred during these polymerisation attempts, a \(^1\)H NMR spectrum of the monomer was obtained just after final purification by distillation, immediately prior to the reaction (Figure 2.1). Signals at δ 5.2 and 5.6 were observed in the spectrum (see inset Figure 2.1) providing evidence of vinyl protons relating to the deprotected form (i.e., monomer 2). This level of impurity would be sufficient to inhibit the required polymerisation, although it is possible that degradation occurred in the deuterated chloroform of the NMR analysis since a signal at δ 2.05 is also observed, which is consistent with acetone, a degradation product.

![Evidence of vinyl protons from de-protection](image)

**Figure 2.1.** \(^1\)H NMR spectrum of monomer 18 immediately prior to anionic polymerisation (CDCl\(_3\)).

To prevent any protic impurities being present, the reaction was repeated several times with samples of the monomer that had been vacuum distilled over CaH\(_2\).
twice and then either distilled a further time over calcium hydride, or passed as a THF solution through an aluminium oxide column (neutral, dried in vacuum oven). Again, no evidence of polymerisation was obtained.

Xie and Hogen-Esch (1996) described the anionic polymerisation of the acrylamides, \(N,N\)-dimethylacrylamide (DMA) and \(N\)-acyryloyl-\(N'\)-methylpiperazine in THF at \(-78^\circ C\) in the presence of initiators such as triphenylmethyllithium to give narrow MWD polymers. Interestingly the corresponding methacrylamide analogues of these two monomers did not polymerise under similar conditions (both monomers were readily polymerised by radical initiators at \(50^\circ C\)). To explain this, Xie and Hogen-Esch suggested that insufficient stabilisation of the amide enolate intermediate may be the reason that these monomers do not polymerise. The suitability of a monomer to undergo anionic polymerisation depends on its susceptibility to nucleophilic attack by the initiator and by propagating carbanions. Monomer reactivity increases with increasing ability to stabilise the carbanion charge and so generally the weaker the electron-withdrawing substituents, the stronger the nucleophile needed for polymerisation (Odian, 1991). Noticeable in the \(^1\)H NMR of 18, the vinylic proton signals have shifted up-field in the spectrum compared to unprotected HPMA 2 (5.34 \& 5.73 \(\delta\) for 2 and 5.09 \& 5.19 \(\delta\) for 18) indicating increased electron density because of a weaker electron-withdrawing substituent, and therefore decreasing the susceptibility of the monomer to nucleophilic attack. However, this clearly does not provide sufficient evidence to support an electronic factor being responsible for the fact that 9 did not undergo anionic polymerisation. In addition to the presence of protic impurities through degradation and electronic factors, in the next section (2.3.2), indirect evidence is provided which suggests that the anionic polymerisation of monomer 18 may be sterically inhibited.
2.4 Free radical polymerisation experiments using \( N,O\)-isopropylidene-\( N\)-(2-hydroxypropyl)methacrylamide 18

The conventional free radical polymerisation of the protected monomer 18 was also attempted. It was hoped this would provide information on the polymer solubility and deprotection chemistry. In a first reaction, the monomer was heated at 50 °C with AIBN initiator (5 wt%) in acetone, i.e., similar conditions used in the literature for the precipitation polymerisation of HPMA 2 (Kopecek & Bazilova, 1973). After 24 h it was evident that no polymerisation had occurred from GPC and NMR analysis. Additionally, the radical polymerisation was also attempted using bulk, suspension and emulsion procedures but again no evidence of polymerisation was obtained (experimental section 2.2, Chapter 7). The fact that 18 would not even undergo free-radical polymerisation suggested that there may be a steric hindrance effect preventing attack on the monomer by the propagating chain radical. Kinetically, the propagation step may be extremely slow due to steric interactions between the two \( \alpha \)-substituents of the propagating radical and the two substituents of the incoming monomer, especially perhaps because of the rigid nature of the amide bond and the oxazolidine ring structure. Therefore, to investigate and eliminate this potential problem, attempts were made to synthesise the protected HPMA monomers, \( N,O\)-ethyldene-\( N\)-(2-hydroxypropyl)methacrylamide 23 and \( N,O\)-methylidene-\( N\)-(2-hydroxypropyl)-methacrylamide 24, which would possess slightly less bulky \( N,O\)-acetal protection groups than 18.

![Chemical structures](image-url)
When 23 was prepared from 1-amino-2-propanol in an analogous manner to the preparation of 18, but using acetaldehyde 25 instead of acetone, the yield obtained (19%) was considerably lower than that obtained for the synthesis of 18 (experimental section 2.1, Chapter 7). To increase the yield, the preparation of 23 was also attempted using HPMA 2 and acetaldehyde dimethyl acetal 26 in an acetal exchange reaction in the presence of pPTSA (Scheme 2.11; Garner & Park, 1987; Thaisrivongs et al., 1987; Wohr & Mutter, 1985) and in a second method in the presence of boron trifluoride etherate (Bouffard et al., 1980). A final method involved the direct reaction of acetaldehyde 25 with HPMA 2 in the presence of an acid catalyst, camphorsulphonic acid (Scheme 2.12).

Although the desired product 23 was obtained in all these reactions (experimental section 2.1., Chapter 7), the yields were poor to moderate (15%, 8% and 56% respectively). Moreover, NMR analysis revealed that the liquid products
obtained were never completely pure, even after vacuum distillation. This was attributed to a significant propensity for the monomer to degrade on storage. For example, after storing below 5 °C overnight an orange discolouration was observed. Degradation was confirmed by NMR by the appearance of amide and hydroxyl protons (see NMR signals for 2, section 2.1, Chapter 7).

In addition to the methods used to prepare 23, the synthesis of monomer 24 was also attempted using the condensation of 1-aminopropanol with formaldehyde 27 (37% w/v aqueous solution) shown in Scheme 2.13. The reaction was performed in toluene, under reflux using a Dean and Stark trap for water removal. The yield of 24 (10%) was poor in this reaction and NMR analysis showed that the product was impure. The reaction was repeated using paraformaldehyde, (CH₂O)ₙ, in the presence of para-toluenesulphonic acid (p-TSA) but the yield was only 3% and NMR analysis showed the sample to be a complex mixture of products. Since acetals are degraded in the presence of acid, effort was made to ensure any acid was excluded from these monomers, e.g., any solvents used in the preparation were treated with base (washed with aqueous NaHCO₃ and dried with anhydrous K₂CO₃) but the isolated compounds still showed poor stability.

As an alternative to a less bulky protection group, it was decided to prepare the acrylamide monomer 28 (experimental section 2.1, Chapter 7) that possesses the same protection group as 18, but does not contain the α-methyl group. Monomer 28 was a crystalline solid that, similar to the methacrylamide 18, was easily purified by vacuum distillation, appeared to be pure by NMR analysis, and displayed good stability on storage.

\[
\begin{align*}
\text{\includegraphics[width=1cm]{structure28.png}}
\end{align*}
\]

When an acetone solution of 28 was heated at 50 °C with AIBN (5 wt%) a white polymeric product, poly(\(N,O\)-isopropylidene-\(N\)-[2-hydroxypropyl]acrylamide) 29 (\(M_n = 37,300 \text{ g}\cdot\text{mol}^{-1}\), \(M_w/M_n = 2.24\), PMMA standards, DMF eluent with 0.1% LiCl, 50 °C), precipitated after the addition of the reaction solution to water. The hydrophobic 29 was soluble in acetone, THF, DCM and diethyl ether. The synthesis of polymer 29 supports the hypothesis that a steric effect may be inhibiting the free-radical polymerisation of the monomer 18.

The novel polymer 29 showed good stability with NMR and IR analysis consistent over a one-year period. The addition of a drop of trifluoroacetic acid (TFA) to an aqueous THF solution of the hydrophobic 29 resulted in a product whose NMR, FT-IR and elemental analysis was consistent with the structure of hydrophilic poly(\(N\)-[2-hydroxypropyl]acrylamide) (PHPA 30) (Scheme 2.14, experimental section 2, Chapter 7).
During the course of this study, it became increasingly clear that other strategies were also available to prepare a homopolymer precursor. Therefore, in parallel with the anionic polymerisation experiments, controlled radical polymerisation (CRP) techniques (Chapter 4) were also being examined as a route to narrow MWD homopolymer precursors. Due to the increasing success of a CRP strategy (Chapter 4) the anionic polymerisation of the acrylamide monomer 28 (or the methacrylamide monomers 23 & 24) was not attempted. The N,O-acetal protected monomers were diverted towards the preparation of block copolymers that have the potential for reversible micelle formation, which are being developed in the Biomedical Polymers Group and can be briefly summarised here.

Drug-carrying micelles can take advantage of the large hydrodynamic volume of the micelle to prolong blood residence times and maximise the EPR effect (see also section 7, Chapter 1). It can be envisaged that a micelle entering diseased cells via endocytosis will be subjected to the decrease in intracellular pH observed in the endosomal and lysosomal compartments of the cell (approximately pH 6.5 and 5.5 respectively). This provides the opportunity to design acid-sensitive drug-carrying micelles that dissociate in response to this intracellular change in pH (Figure 2.2).
Figure 2.2. Aim: Micelle-forming hydrophilic-hydrophobic block copolymer that is capable of non-reversible dissociation to hydrophilic polymer chains upon an intracellular decrease in pH. Physically entrapped drug is liberated intracellularly along with hydrophilic chains of sufficiently low MW to be excreted.

The idea is to prepare block copolymers containing a hydrophilic block and a reversibly (through acid sensitive deprotection) hydrophobic block prepared from monomers such as 28. It can be envisaged that a drug-carrying micelle based on this idea would, after entering the endosomal or lysosomal compartments of a cell dissociate to liberate the drug and the resulting wholly hydrophilic polymer chains of sufficiently low MW (controlled by the polymerisation) would be eliminated from the body.

To this end, semi-telechelic polymers 31 derived from monomer 28 have been prepared at different MW’s (e.g., $M_n = 5,300$ g·mol$^{-1}$, $M_w/M_n = 1.5$ and for a second sample $M_n = 2,500$ g·mol$^{-1}$, $M_w/M_n = 1.6$, GPC, PMMA standards) by a supervised undergraduate student using AIBN-initiated polymerisation in the presence of the chain transfer agent, 2-mercaptoethylamine hydrochloride. The formation of a block copolymer 33 prepared by conjugating 31 with the commercially available hydrophilic monomethoxy-PEG $(O-\{(N\text{-succinimidyl}o\text{xycarbonyl)}\text{-methyl}\})-O'$-methyl-polyethylene glycol, MW 5000, 32) (Scheme 2.15) have so far been
unsuccessful. It would be hoped that a copolymer such as 33 would degrade under acidic conditions to the hydrophilic copolymer 34. Of course, issues concerning the rate of degradation, drug-carrying capacity, toxicity and biocompatibility are all key questions to be addressed as this work develops but this preliminary work is aimed at establishing proof of principle.

\[
\begin{align*}
31 & \quad \text{hydrophobic} \\
32 & \\
\text{(CH}_3\text{CH}_2)_3N & \quad \text{Intracellular increase in acidity}
\end{align*}
\]

\[
\begin{align*}
33 & \\
34 & \quad \text{hydrophilic}
\end{align*}
\]

Scheme 2.15. Preparation of hydrophilic-hydrophobic block copolymer 33 and its acidic hydrolysis to give the hydrophilic-hydrophilic block copolymer 34.
3 Conclusions

Novel N,O-acetal protected (meth)acrylamide monomers (18, 23, 24 & 28) based on HPMA were synthesised. The protected HPMA monomer, N,O-isopropylidene-N-(2-hydroxypropyl)methacrylamide 18, did not undergo anionic or free radical polymerisation. The analogous acrylamide monomer 28 did polymerise by free radical initiation, implying steric inhibition may be a factor for 18 not polymerising, although electronic effects and protic impurities were also identified as possible explanations. The hydrophobic poly(N,O-isopropylidene-N-[2-hydroxypropyl]acrylamide) 29 was synthesised by the free radical polymerisation of the acrylamide 28 and was shown to deprotect under acidic conditions to give hydrophilic PHPA 30, providing evidence that the strategy described in Scheme 2.1 may be feasible for monomer 28. However, the aim of the study in this chapter was to prepare a narrow MWD polymethacrylamide PHPMA 1 and not a polyacrylamide (although this is not to imply that monomer 28 and resulting polymers may not be suitable for future research into therapeutic polymers).

Another limitation of a HPMA homopolymer 1 precursor strategy is the hydroxyl group activation method for drug conjugation. This may not be as efficient a method for drug conjugation as the direct reaction with an active ester, i.e., as utilised for a typical HPMA copolymer (Scheme 1.1, Chapter 1) where amide bonds are directly formed. Therefore, an improved, more versatile precursor was thought possible if every repeat unit of the polymer was reactive, e.g., a methacryloyl active ester homopolymer. This would allow the preparation of polymethacrylamides by the direct reaction with amines and potentially enables a greater variety of structures to be prepared since even the solubilising pendant chains can be varied. The full advantages and implications of this strategy are described in Chapter 4. Although the use of active ester homopolymers is a well known method for preparing functionalised polymers (Arshady, 1994) the preparation of a narrow MWD active ester homopolymer precursor to give soluble polymer-drug conjugates (copolymers) has not been examined. This is the challenge undertaken in the next chapter.
Chapter 3

Active Ester Homopolymers by Atom Transfer Radical Polymerisation (ATRP) Procedures

1 Introduction

The aim of this study was to prepare a narrow MWD active ester homopolymer 36 using ATRP (see section 1.2 below) for use as a homopolymer precursor. Post polymerisation modification of active ester polymers is a well known strategy for the preparation of functionalised polymers but it can be difficult to control the polymerisation chemistry to ensure narrow MWD (Arshady, 1994).

There are distinct potential advantages of using a narrow MWD active ester homopolymer as a precursor to polymer drug-conjugates (Scheme 3.1) compared with a typical HPMA copolymer (Scheme 1.1, Chapter 1) or the activated PHPMA 1 homopolymer strategy that was attempted in Chapter 2. Firstly, every repeating unit of the active ester homopolymer is a reactive group, negating the need for polymer activation. Activation may not be as efficient a method for drug conjugation as polymeric active ester reactions, since active ester polymers readily under aminolyis with primary amines to produce poly(meth)acrylamides (Arshady, 1994; Batz et al., 1972; Ringsdorf et al., 1972; Strohriegl, 1993). As for the strategy of PHPMA activation in Chapter 2 (Scheme 2.4), the choice and stoichiometry of linker and drug is not defined by the polymerisation chemistry (reaction I, Scheme 3.1).
Scheme 3.1. Strategy for the preparation and use of an ATRP prepared active ester homopolymer precursor 36.
While the reaction (conjugation) of 1-amino-2-propanol with an active ester homopolymer will give HPMA-based polymers, this strategy also allows the solubilising pendant chains to be changed by conjugation of different solubilising amines (i.e., changing 38 in reaction II, Scheme 3.1). It can be envisaged therefore that families of narrow MWD conjugates can be prepared using 36 for assessment of structure-property relationships that possess a far greater diversity in chemical functionality than could have been achieved with the PHPMA activation.

The preparation of polymer-drug conjugates from an active ester homopolymer requires the sequential conjugation of multiple amine reactants (37 and 38 in reactions I and II, Scheme 3.1). If the conjugation of the drug-linker amine 37 is quantitative, or can be made quantitative by the choice of reaction conditions, then this represents a very efficient way of using both the drug and the linker (e.g., an oligopeptide) which are generally the most expensive parts of the conjugate. As with all precursors, the success of this approach relies on the controlled and reproducible chemical modification of the homopolymer to afford the desired drug conjugate and so it is essential that the conjugation chemistry is well understood and suitable for sequential amine conjugation with minimal or no side reactions (Chapter 4).

In this study, the active ester N-methacryloxy succinimide 35 (MOSu) was chosen as a monomer because the resulting polymer, poly(N-methacryloxy succinimide) 36 (PMOSu) would be more hydrolytically stable compared to the p-nitrophenyl ester used typically for conjugation to HPMA copolymer precursors (Hermanson, 1996), and the imide group may give strong carbonyl bands in IR spectroscopy enabling convenient analysis of the polymer and conjugation products/reactions. Since there was the possibility that MOSu 35 may be incompatible with anionic polymerisation due to the presence of acidic methylene protons adjacent to imide carbonyl groups, it appeared more appropriate to attempt to prepare narrow MWD 36 using ATRP.
1.1 ATRP

Over recent years there has been rapid progress in the development of CRP enabling the synthesis of (co)polymers with controlled MW, narrow MWD and well defined architecture. CRP is based on the reversible activation of a dormant species P-X to a polymer radical P* by thermal, photochemical, and/or chemical stimuli (Scheme 4.2; Fukuda and Goto, 1999). The equilibrium is optimised to be strongly in favour of the dormant species so that the concentration of active radicals is very low, thereby limiting radical-radical coupling reactions as the principal mode of termination (Matyjaszewski et al., 1998). CRP requires rapid initiation, fast exchange between dormant and active species and a small proportion of chains involved in termination reactions for control over MW and low MWD’s. The main types of CRP techniques include (1) stable free radical polymerisation (SFRP), which employs stable nitroxyl radicals (Solomon et al., 1985), (2) reversible addition-fragmentation chain transfer polymerisation (RAFT) (Moad et al., 1996), which uses dithioesters and free radical initiators, and (3) atom transfer radical polymerisation (ATRP) which uses transition metal complexes in conjunction with alkyl halides (Kato et al., 1995; Wang & Matyjaszewski, 1995).

\[
P-X \rightleftharpoons \text{P}^* \quad \text{(+ monomer)}
\]

\[
\begin{align*}
\text{P}^* & \quad \text{P-X} \\
\text{Dormant} & \quad \text{Active}
\end{align*}
\]

Scheme 3.2. General scheme for CRP (adapted from Fukuda and Goto (1999)).
Although other CRP processes may also be appropriate, ATRP was chosen for the controlled polymerisation of MOSu 35 since the literature indicates that ATRP is a robust technique because of the large number of monomers polymerisable with excellent MW and MWD control ($1.05 < M_u/M_n < 1.5$). ATRP has been most successfully applied to the preparation of narrow MWD acrylates, methacrylates (Davis et al., 1999; Wang et al., 1997) and styrene and its derivatives (Patten et al., 1996; Percec et al., 1996). Generally, the conditions for ATRP are more tolerant of protic impurities than that required for the anionic polymerisation attempted in Chapter 2 enabling successful polymerisation even in aqueous media (Wang & Armes, 2000).

In ATRP, the dormant chains are capped by halogen atoms which are transferred to metal complexes in the lower oxidation state, generating growing chain radicals where monomer can add (Scheme 3.3; Matyjaszewski, 1997). The halogen atoms can be transferred back to the growing chain radicals from the transition metal complexes (now in a higher oxidation state) regenerating dormant chains, since the redox process of activation is reversible, i.e., ATRP is catalytic. However, the true mechanism is still open to debate and this may well be an over-simplification (Matyjaszewski, 1998, Heuts et al., 1999).

$$P_n^\text{-X} + \text{Cu(I)/2bipy} \underset{k_p}{\overset{\text{monomer}}{\rightleftharpoons}} P_n^\bullet + \text{X-Cu(II)/2bipy}$$

Scheme 3.3. Mechanism of ATRP proposed by Matyjaszewski (1997) for a polymerisation catalysed by a cuprous halide complexed by two 2,2'-bipyridine (bipy) molecules ($X = $ halogen atom).
An ATRP system comprises monomer, an alkyl halide (initiator), a catalysing species (a redox active transition metal in its lower oxidation state and ligand, e.g., copper(I) bromide complexed by two bipy molecules), a deactivator (a transition metal in a higher oxidation state and ligand - either present from the beginning of the reaction or formed spontaneously), solvent (ATRP can be performed in bulk also) and growing polymer chains after commencement of the reaction. The rate of initiation must be faster than, or comparable to, propagation to ensure that the polymers chains start to grow essentially simultaneously, thus producing a narrow MWD and hence controlled MW. The degree of polymerisation (DP) increases linearly with monomer conversion and is predetermined by the molar ratio of monomer to initiator. The fast deactivation of active species to dormant polymer chains is necessary to minimise the contribution of chain termination and to ensure a narrow MWD (Fischer, 1997).

The catalytic species is vital for successful ATRP as the amount and activity of the catalyst controls the stationary concentration of radicals through the dormant-active equilibrium. Many transition metals have been utilised for ATRP, including Cu (Patten et al., 1996), Ni (Granel et al., 1996), Fe (Ando et al., 1997) and Ru (Sawamoto & Kamigaito, 1996). The metal should participate in a one-electron transfer redox cycle and show a high affinity for the halogen atom but a low affinity for hydrogen and alkyl radicals (Matyjaszewki, 1997). The ligand has a three-fold role, helping to solubilise the transition metal, affecting the redox chemistry by their electronic effects and controlling selectivity by steric/electronic effects. Optimisation of the reaction parameters, such as initiating system, monomer concentration, solvent and temperature, is a key factor in ATRP (and all CRP) and must be achieved in order for controlled polymerisation to occur.
1.2 ATRP of (meth)acrylamides

At the beginning of this study, the preparation of poly(meth)acrylamides by ATRP was very much still to be addressed. Since then several reports in the literature have appeared regarding the ATRP of (meth)acrylamide monomers. Haung and Wirth (1999) used benzyl chloride/CuCl/bipy as an initiating system to grow polyacrylamide 41 films from a silica surface, claiming the preparation of 41 with polydispersities as low as 1.15. For solution ATRP, Li and Brittain (1998) did not obtain any polymer from the attempted polymerisation of N,N-dimethylacrylamide 42 (DMA) using 1-bromo-1-phenylethane/CuBr/bipy initiating system at 95 °C in toluene. Teodorescu and Matyjaszewski (1999) originally concluded that the solution ATRP of (meth)acrylamides is not controlled. This view was supported by Rademacher and co-workers (2000) who studied the ATRP of DMA. Teodorescu and Matyjaszewski offered several reasons for the lack of control in the copper mediated ATRP of (meth)acrylamides after studying the polymerisation of DMA, N-tert-butylacrylamide and perhaps most relevant for this study, HPMA 2 (see also section 2.5 for HPMA results). It was suggested that plausible reasons for the lack of control could be due to (1) the inactivation of catalyst by polymer complexation, (2) a strong bond between bromine and the terminal monomer unit in the polymer and (3) the nucleophilic displacement of terminal bromine by the penultimate amide group either by the nucleophilic attack of the carbonyl oxygen (route a, Scheme 3.4) or the nitrogen atom of the amide group (route b, Scheme 3.4). However, no evidence to support nucleophilic displacement was provided.
Scheme 3.4. Possible nucleophilic displacement of terminal halogen in the ATRP of acrylamides (taken from Teodorescu & Matyjaszewski, 1999).

Since then Teodorescu and Matyjaszewski (2000) have described the controlled polymerisation of DMA at room temperature using a methyl-2-chloropropionate (MCP)/CuCl/tris(2-dimethylaminoethyl)amine (Me₆TREN, 45) initiating system in toluene. This system overcomes low values of the ATRP equilibrium constants proposed for CuBr and linear amines/substituted and unsubstituted bipyridine catalysts used in earlier studies by using a more powerful catalyst. The catalytic activity in ATRP can be changed with the choice of ligand and increases generally in the order: bipy 43 < multidentate amines (e.g., N,N,N',N',N''-pentamethyldiethylenetriamine 44 (PMDETA)) < tripodal amines (e.g., Me₆TREN 45) < some cyclic amines (1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane 46 (Me₄Cyclam)) (Teodorescu & Matyjaszewski, 2000). Generally, more electron donating ligands better stabilise the higher oxidation state of the metal and accelerate the polymerisation (Patten & Matyjaszewski, 1998).
Chapter 3: Active Ester Homopolymers

The proposed easy displacement of the C-X bond by a nucleophilic amide group at the end of the chain was addressed by using alkyl chlorides rather than bromides, lower temperatures and low polarity solvents (toluene). The carbon-chlorine bond, being stronger than the corresponding carbon-bromine bond, should be less prone to $S_{N}2$ reactions and so should allow ATRP to proceed.

Concurrent with ATRP, other CRP techniques have also demonstrated varying success in the controlled polymerisation of acrylamides. Li and Brittain (1998) using SFRP found that there was no control in their attempted polymerisation of DMA. Using a different SFRP system Benoit and co-workers (1999) have since reported narrow MWD ($M_w/M_n < 1.15$) DMA and control over the experimental $M_n$ by varying the ratio of monomer to initiator. RAFT has also been used to prepare poly(styrene-b-DMA) block copolymers of narrow MWD (Chong et al., 1999).

The apparent lack of progress associated with the CRP of (meth)acrylamide monomers, as indicated in the literature, makes the study of the ATRP of active ester monomers that can be converted to poly(meth)acrylamides all the more desirable. However, a paucity of literature exists for the ATRP of active ester monomers. Only one example for ATRP could be found, and this was a copolymerisation using styrene and $p$-nitrophenol methacrylate (Liu et al., 1999) suggesting that the controlled polymerisation of active ester monomers may also require carefully optimised conditions.
Chapter 3: Active Ester Homopolymers

2 Results and Discussion

2.1 ATRP procedures applied to MOSu 35

![Scheme 3.5. General scheme for the application of copper-mediated ATRP to 35.](image)

In preliminary experiments, the copper mediated ATRP of the solid 35 (mp 102-104 °C) was attempted in THF and acetone using the initiator, 2-hydroxyethyl 2-bromo-2-methylpropanoate 47 at 70 °C (Scheme 3.5). During heating, PMOSu 36 of narrow MWD ($M_n/M_w=1.10-1.19$) precipitated from the reaction solutions (Table 3.1). Polymerisation in acetone reached 95% yield before precipitation (entry 4, Table 3.1), which was an early indication that the polymerisation of 35 may be extremely fast. In addition to the polymer precipitation, it was also observed that the reaction solutions were heterogeneous with respect to the catalyst species. The bipy ligand 43 was used in acetone, after PMDETA 44 was used in the THF polymerisations, to try to improve the CuBr solubility. Poor solubility of the catalyst species can affect control over MW and MWD due to a less effective activation/deactivation equilibrium, and since $M_n$ values were higher than theoretical $M_n$ values it was necessary to try a solvent where both the polymer and catalyst species were soluble. This was considered to be a better long-term strategy for preparing polymeric precursors 36 in a controlled polymerisation over a wide MW range. It was also noted that THF is not considered
an ideal solvent for ATRP due to a propensity for high levels of chain transfer to occur (Müller, 1999).

### Table 3.1. Polymerisations of 35 performed in THF or acetone at 70 °C.

<table>
<thead>
<tr>
<th>No.</th>
<th>solvent</th>
<th>Ratio*</th>
<th>Target Mₙ</th>
<th>% Yield</th>
<th>$M_{n,\text{theo}}$</th>
<th>$M_n$</th>
<th>$M_n/M_{n,\text{theo}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75 wt% THF</td>
<td>100:1:1:1.2</td>
<td>18,300</td>
<td>20</td>
<td>3,660</td>
<td>14,800</td>
<td>1.10</td>
</tr>
<tr>
<td>2</td>
<td>78 wt% THF</td>
<td>200:1:1:1.2</td>
<td>36,600</td>
<td>40</td>
<td>14,640</td>
<td>1,800</td>
<td>1.12</td>
</tr>
<tr>
<td>3</td>
<td>78 wt% THF</td>
<td>100:1:0.3:1.2</td>
<td>18,300</td>
<td>5</td>
<td>915</td>
<td>13,100</td>
<td>1.09</td>
</tr>
<tr>
<td>4</td>
<td>83 wt% acetone</td>
<td>55:1:0.5:1</td>
<td>10,065</td>
<td>95</td>
<td>9,562</td>
<td>20,000</td>
<td>1.19</td>
</tr>
<tr>
<td>5</td>
<td>87 wt% acetone</td>
<td>100:1:0.5:1</td>
<td>18,300</td>
<td>33</td>
<td>6,039</td>
<td>15,000</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Solvent wt% = % of combined solvent and monomer mass; $* = [\text{MOSu}]_0/[47]_0/[\text{Cu(II)}Br]_0:[\text{ligand}]_0$; ligand = PMDETA 44 for entries 1-3 and bipy 43 for entries 4-5; $[\text{MOSu}]_0 = 5.5 \text{ mmol}$; Reaction time = 4 - 16.5 h; Target $M_n = [\text{MOSu}]_0/[47]_0 \times 183 \text{ g mol}^{-1}$ - this equation is used throughout this study; $M_{n,\text{theo}} = \text{Theoretical } M_n = \text{Target } M_n \times \%\text{yield}$ - this equation is used throughout this study.

While 35 is soluble in most organic solvents, 36 is only soluble in highly polar solvents such as DMF and DMSO (Ferruti et al., 1972). Both the monomer and polymer are insoluble in water. Although ATRP is reported to be sensitive to DMF concentration through the competitive chelation of copper species with the solvent (Pascual, 1999; Ziegler et al., 1999; this point will be elaborated later) several polymerisations were conducted in DMF and also DMSO to maintain solution homogeneity (Table 3.2). The temperature of the polymerisation was increased to 110-130 °C to ensure the solution remained homogeneous. In these exploratory reactions the polymer yield was used as a measure of successful polymerisation because this implied a homogeneous reaction and high monomer conversion.
Table 3.2. Effect of highly polar organic solvent concentration on the yield of 36.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>No.</th>
<th>wt% solvent</th>
<th>Ratio*</th>
<th>Time / h</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF</td>
<td>1</td>
<td>67</td>
<td>100:1:1:1.2</td>
<td>20</td>
<td>trace</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>38</td>
<td>55:1:1:2</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>23</td>
<td>55:1:1:2</td>
<td>3</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9</td>
<td>100:1:1:3</td>
<td>10</td>
<td>insoluble</td>
</tr>
<tr>
<td>DMSO</td>
<td>5</td>
<td>60</td>
<td>55:1:1:2</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>50</td>
<td>100:1:1:2</td>
<td>20</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>43</td>
<td>55:1:1:2</td>
<td>2</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>43</td>
<td>100:1:1:2</td>
<td>2</td>
<td>99</td>
</tr>
</tbody>
</table>

* = [MOSu]₆[47]₆[CuBr]₆[bipy]₆; [MOSu]₆ = 5.5 mmol; T =130 °C.

The reactions were quenched by rapid cooling and dilution with further solvent. The diluted reaction solutions were then added to stirred acetone to precipitate polymer (experimental section 3.3, Chapter 7). The polymerisations in DMF and DMSO were both shown to be sensitive to the ratio of solvent to monomer. In 67 wt% DMF, only a trace quantity of polymer 36 was isolated, but reducing the concentration to 39 wt% resulted in 50% yield of polymer. At a DMF concentration of 25 wt%, the yield of 36 was 80% (entries 1-4, Table 3.2). In DMSO at 60 wt%, a 40% yield of 36 was obtained which increased to 52% and 90% when the concentration of DMSO was reduced to 50 wt% and 43 wt% respectively (entries 5-8, Table 3.2).

Dilution of the reaction with solvent is a known method of slowing down the propagation rate in ATRP (Matyjaszewski, 1997). Hence, lower yields are due to a lower propagation rate. Ziegler and co-workers (1999) also suggested that dilution of
the reaction with a highly polar solvent can lead to a loss of control in ATRP. Several reasons were proposed; co-ordinating solvents such as DMF may displace ligands from the catalysing species and may also saturate the co-ordination sphere around the Cu(I) species, decreasing the activity of the catalysing species. Furthermore, polar solvents may promote side reactions such as outer sphere electron transfer and nucleophilic substitution or elimination of the halogen by the co-ordinating ligands. One benefit of using an appropriate amount of a polar solvent such as DMF is that it can lead to an increase in the solubility of both Cu(I) and Cu(II) species which can improve control due to a more effective activation/deactivation equilibrium (Matyjaszewski et al., 1998).

The results obtained for the polymerisation of 35 suggested that the catalyst species used was more sensitive to DMF than DMSO since 43 wt% DMSO resulted in 90% yield, whereas 39 wt% DMF resulted in only 50% yield of polymer. Further homogeneous reactions were conducted at 43 wt% DMSO to give 36 over a range of molecular weights by varying the monomer to initiator ratio (Table 3.3; entries 1-5).

<table>
<thead>
<tr>
<th>No.</th>
<th>[35]₀:[47]₀</th>
<th>T°C</th>
<th>Target Mₙ</th>
<th>% Yield</th>
<th>Mₙ, theo</th>
<th>Mₙ</th>
<th>Mₙ/Mₙ₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10:1</td>
<td>100</td>
<td>1,830</td>
<td>85</td>
<td>1,560</td>
<td>12,500</td>
<td>1.17</td>
</tr>
<tr>
<td>2</td>
<td>20:1</td>
<td>80</td>
<td>3,660</td>
<td>92</td>
<td>3,370</td>
<td>16,800</td>
<td>1.15</td>
</tr>
<tr>
<td>3</td>
<td>50:1</td>
<td>100</td>
<td>9,150</td>
<td>89</td>
<td>8,140</td>
<td>22,700</td>
<td>1.20</td>
</tr>
<tr>
<td>4</td>
<td>100:1</td>
<td>100</td>
<td>18,300</td>
<td>96</td>
<td>17,570</td>
<td>29,000</td>
<td>1.14</td>
</tr>
<tr>
<td>5</td>
<td>150:1</td>
<td>110</td>
<td>27,450</td>
<td>80</td>
<td>21,960</td>
<td>40,700</td>
<td>1.13</td>
</tr>
<tr>
<td>6*</td>
<td>100:1</td>
<td>100</td>
<td>18,300</td>
<td>49</td>
<td>8,970</td>
<td>23,330</td>
<td>1.15</td>
</tr>
</tbody>
</table>

[47]₀:[CuBr]₀:[Bipy]₀ = 1:1:2; [35]₀ = 13.7 mmol; solvent = DMSO 43 wt%; (*) Reaction stopped after 2.5 min by dilution with DMSO and rapid cooling.
The reactions were quenched after 10-15 min by rapid cooling and dilution with further DMSO (experimental section 3.3, Chapter 7). Precipitation in acetone of the diluted reaction solutions afforded narrow MWD polymers (for an example GPC chromatogram see Appendix IV) in good isolated yields (80-96%). The polymerisations occurred remarkably fast, even after 2.5 min the yield of 36 was nearly 50% (Table 3.3, entry 6). This is in contrast to typical ATRP reaction times such as that obtained by Ziegler et al. (1998) who found 78% conversion of methyl acrylate to poly(methyl acrylate) occurred only after 13 h at 70 °C in 5% DMF. Fast, controlled ATRP polymerisation has been observed in aqueous media. For example, Armes and co-workers (Wang et al., 2000; Wang & Armes, 2000) have reported conversion often exceeding 95% within 20-30 min using aqueous solutions at 20 °C with various hydrophilic monomers such as sodium 4-vinylbenzoate.

The apparent rapid polymerisation of 35 may be undesirable for controlled polymerisation (including initiator efficiency) since such a fast reaction implies a high concentration of radicals. A low concentration of radicals is more beneficial in order to minimise bimolecular termination and ensure a controlled polymerisation (Matyjaszewski, 1997). The values of $M_n$ obtained for 36 in DMSO and with the earlier reactions in THF, acetone and DMF were consistently higher than the theoretical $M_n$ values. This indicated that (1) the PMMA GPC standards may have a significantly different hydrodynamic volume compared with 36 and/or (2) there may be a significant level of side reaction (termination) in the initiation step or early stages of propagation. To obtain a better indication of the real $M_n$, a sample of 36 was hydrolysed to poly(methacrylic acid Na salt) (PMAA Na salt, 62, see Scheme 5.2 and section 2.1 in Chapter 5 for synthesis) and GPC performed with PMAA Na salt standards.
With a theoretical $M_n$ of 15,250 g·mol$^{-1}$, the apparent $M_n$ by GPC of the polymer prior to hydrolysis using PMMA standards was 24,800 g·mol$^{-1}$ ($M_w/M_n = 1.20$). After hydrolysis, aqueous GPC analysis (phosphate buffered saline solution adjusted to pH 8.5) gave $M_n = 22,000$ g·mol$^{-1}$ ($M_w/M_n = 1.28$), which is still higher than the theoretical $M_n$. Thus, PMMA is not an ideal GPC standard for 36, and moreover, this result implies that a significant side reaction (bimolecular termination) occurs in the early stages of the polymerisation of 35 in DMSO. Due to the fast reaction times, side reactions may presumably be due to the generation of high concentrations of radicals in the reaction. Further evidence to support a side reaction(s) comes from the colour of the reaction solutions. The brown starting solutions of all the polymerisations of 35 conducted in DMSO using the bipy ligand, began to turn green in colour within 1 min of heating, quickly becoming permanently green for the rest of the reaction. This indicated oxidation of the catalyst from Cu(I) to Cu(II), since CuBr$_2$ and bipy dissolve in DMSO to give a green solution. A significant and irreversible build-up of Cu(II) can only occur through side reactions, i.e., termination of growing radicals, and perhaps an inefficient deactivation process.

2.2 Optimisation of the polymerisation of MOSu 35

2.2.1 Reduction of Cu(I) concentration and the addition of Cu(II)

To try to decrease the stationary concentration of radicals by reducing the rate of propagation, the concentration of activating Cu(I) added to the polymerisation of 35 was reduced to one tenth that used previously (entries 1 & 2, Table 3.4). The result was still a high yielding polymerisation (85%) but there was an increase in both $M_n$ and $M_w/M_n$, suggesting some loss of control. It was plausible that the loss of control could be due to an insufficient concentration of Cu(II) to produce a suitable activation/deactivation equilibrium. To investigate this, a series of polymerisations
were performed with the addition of Cu(II)Br$_2$ (Table 3.4). The introduction of deactivating Cu(II) species to the reaction is a method for reducing the rate of propagation. Hence, termination is decreased because of a reduction in the concentration of radicals, and control over MW and MWD is improved (Patten & Matyjaszewski, 1998).

When Cu(II) was added in a stoichiometric or excess quantity to the concentration of Cu(I), a reduction in both the MWD and $M_n$ was observed (entries 3, 4 & 5, Table 3.4) indicating the polymerisation became better controlled. Increasing the deactivator concentration to twice that of the Cu(I) present led to only a 10% yield after 24 h (entry 4, Table 3.4) implying that the concentration of Cu(II) became so high that propagation was drastically reduced. The concentration of Cu(II) required to observe these effects are much larger than the concentration of Cu(II) used in the literature to see better control (e.g., 10 mol% Cu(II)Br$_2$ added relative to Cu(I)Br (Matyjaszewski et al., 1998)) again indicating that deactivation is extremely inefficient in the polymerisation of 35.

Table 3.4. Addition of Cu(II) deactivator to the ATRP of MOSu 35 at 100 °C.

<table>
<thead>
<tr>
<th>No.</th>
<th>Ratio*</th>
<th>[Cu(II)Br]</th>
<th>Time/ h</th>
<th>% Yield</th>
<th>$M_{n, \text{theo}}$</th>
<th>$M_n$</th>
<th>$M_n/M_\text{w}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50:1:1:2</td>
<td>-</td>
<td>0.25</td>
<td>89</td>
<td>8,140</td>
<td>22,700</td>
<td>1.20</td>
</tr>
<tr>
<td>2</td>
<td>50:1:0.1:0.2</td>
<td>-</td>
<td>0.25</td>
<td>85</td>
<td>7,780</td>
<td>37,100</td>
<td>1.35</td>
</tr>
<tr>
<td>3</td>
<td>50:1:0.1:0.4</td>
<td>0.10</td>
<td>0.25</td>
<td>98</td>
<td>8,970</td>
<td>33,600</td>
<td>1.27</td>
</tr>
<tr>
<td>4</td>
<td>50:1:0.1:0.5</td>
<td>0.15</td>
<td>21</td>
<td>72</td>
<td>6,590</td>
<td>26,000</td>
<td>1.14</td>
</tr>
<tr>
<td>5</td>
<td>50:1:0.1:0.6</td>
<td>0.20</td>
<td>24</td>
<td>10</td>
<td>915</td>
<td>18,100</td>
<td>1.17</td>
</tr>
</tbody>
</table>

*Ratio = [35]$_0$:[47]$_0$:[CuBr]$_0$:[bipy]$_0$; [35] = 13.7 mmol; solvent = DMSO 43 wt%; Target $M_n = 9150$ g mol$^{-1}$. 

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Despite differences in the polymerisation of MOSu 35 with the addition of Cu(II), the values for $M_n$ were still higher than the theoretical $M_n$ values. The speed of these polymerisations and the higher than expected values obtained for $M_n$ indicates that the kinetics of the polymerisation do not approximate very well to a typical ATRP based on an active/dormant equilibrium. The results obtained for the addition of deactivator species and the narrow MWD obtained however, suggests that there is some level of mediation by the copper catalysing species is occurring. A way to assess copper mediation is to obtain kinetic data for the polymerisation.

### 2.2.2 Analysis of quenched polymerisation samples of MOSu 35 for kinetic profile

The speed of the polymerisations observed for 35 in DMSO presents practical problems in studying kinetic aspects of the reaction. Typical ATRP kinetic studies are performed using a single reaction with samples taken at different time points. One problem for the polymerisation of 35 is to stop the polymerisation at defined time points when the reaction appears to be so fast (min range). One possibility was to perform a polymerisation in an NMR tube for $^1$H NMR analysis every 30 s, but the polymerisation was considered too fast for this to be successful and the NMR spectrometer could not be used for the analysis of samples heated above 60 °C.

To establish some kinetic observations for the polymerisation of 35, five separate and equivalent reactions were performed and quenched after different time periods (0.5, 1, 2, 3 & 5 min) by dilution with DMSO and rapid cooling (see experimental section 3.3, Chapter 7). These reactions are based on the assumption that each will exhibit identical kinetic profiles.
Table 3.5. Results of five separate quenched polymerisation reactions using 35.

<table>
<thead>
<tr>
<th>No.</th>
<th>Time quenched/ min</th>
<th>% monomer present by $^1$H NMR*</th>
<th>% Yield</th>
<th>$M_n$</th>
<th>$M_w/M_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>62</td>
<td>21</td>
<td>15,600</td>
<td>1.26</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>14</td>
<td>48</td>
<td>16,400</td>
<td>1.34</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0</td>
<td>74</td>
<td>17,900</td>
<td>1.35</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>0</td>
<td>78</td>
<td>18,700</td>
<td>1.33</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0</td>
<td>79</td>
<td>18,800</td>
<td>1.35</td>
</tr>
</tbody>
</table>

$[35]_o:[47]_o:[Cu(II)Br]_o:[bipy]_o = 50:1:1:2, T = 100 °C, Target $M_n = 9150$, solvent = DMSO 43 wt%, *calculated from proton integral values of 1,4-dichlorobenzene ($\delta$ 7.43) added as a standard and monomer vinylic proton integrals ($\delta$ 6.30 & 6.05)

Analysis by $^1$H NMR of the quenched reaction solutions (with a known volume of 1,4-dichlorobenzene dissolved in DMSO added as a standard) indicated 38% of the monomer had undergone reaction in the sample quenched after 0.5 min (entry 1, Table 3.5). This compares with a 21% yield of polymer obtained by precipitation of this sample into acetone. For the sample quenched after 1 min, NMR analysis indicated 86% of monomer had undergone reaction, yet only a 48% yield of polymer was isolated by precipitation (entry 2, Table 3.5). The 2, 3 and 5 min samples under NMR analysis did not show evidence of any monomer vinylic protons and the yields of polymer obtained were very similar (74, 78 and 79% respectively, entries 3, 4 & 5, Table 3.5).

The results in Table 3.5 indicate that more monomer was consumed than can be accounted for by the yields of polymer obtained. This therefore implies that side reactions had occurred and consequently it can be speculated that this occurred in the earlier stages of the polymerisation, i.e, initiation and early propagation, forming low MW compounds such as oligomers by biomolecular termination that are sufficiently
soluble not to be precipitated during the isolation of the polymers. If this happened, then it would be expected to find such low MW compounds in the waste filtrate from the isolation of the polymers. The GPC columns available were unable to resolve a sample of the waste filtrate to indicate whether such low MW compounds were present. MS analysis however, did show the presence of compounds between approximately 150 and 700 in MW, providing evidence for significant levels of termination in the earlier stages of the reaction.

A feature of conventional radical polymerisation is that high MW polymer is formed immediately the reaction begins and the average chain length shows little variation throughout the course of the polymerisation (Cowie, 1991). Significantly, $M_n$ for the 0.5 min sample reached 15,600 g·mol$^{-1}$, very high for such a short reaction time and very close to the maximum $M_n$ obtained of 18,800 g·mol$^{-1}$ for the 5 min sample. This result may preclude 'pure' ATRP and indicate that the polymerisation may proceed by more than one process, e.g., with both conventional free-radical polymerisation.

2.2.3 Reduction in temperature and use of more strongly co-ordinating ligands.

A further set of reactions were conducted at the much reduced temperature of 25 °C (Table 3.6) in an attempt to slow down the rate of propagation, decreasing the stationary concentration of radicals and aiming to establish an ATRP active/dormant equilibrium.

Using the bipy ligand, polymerisations at increasing DMSO dilutions, which were necessary to ensure homogeneous solutions at the lower temperature, gave lower yields than those obtained at 100 °C and higher polydispersities indicating loss of control (entries 1, 2 & 3, Table 3.6). This is consistent with previous experiments (Table 3.2) and the explanations given by Ziegler et al. (1999), as stated earlier, on the possible effects of high concentrations of polar solvents.
Table 3.6. Polymerisation of 35 at 25 °C with different ligands.

<table>
<thead>
<tr>
<th>No.</th>
<th>Ligand</th>
<th>Time / h</th>
<th>DMSO wt%</th>
<th>% Yield</th>
<th>( M_n )</th>
<th>( M_w/M_n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>bipy</td>
<td>16.5</td>
<td>50</td>
<td>50</td>
<td>18000</td>
<td>1.60</td>
</tr>
<tr>
<td>2</td>
<td>bipy</td>
<td>5</td>
<td>67</td>
<td>52</td>
<td>16600</td>
<td>1.70</td>
</tr>
<tr>
<td>3</td>
<td>bipy</td>
<td>2</td>
<td>80</td>
<td>24</td>
<td>24200</td>
<td>1.38</td>
</tr>
<tr>
<td>4</td>
<td>PMDETA</td>
<td>15</td>
<td>80</td>
<td>34</td>
<td>14000</td>
<td>1.92</td>
</tr>
<tr>
<td>5</td>
<td>Me₆TREN</td>
<td>19</td>
<td>80</td>
<td>30</td>
<td>25000</td>
<td>1.62</td>
</tr>
<tr>
<td>6</td>
<td>Me₆TREN</td>
<td>15</td>
<td>58</td>
<td>20</td>
<td>24200</td>
<td>1.87</td>
</tr>
</tbody>
</table>

Conditions: \([35]_o;[47]_o;[CuBr]_o = 50:1:1; [bipy]_o = 2[CuBr]_o; [PMDETA]_o = [Me₆TREN]_o = [CuBr]_o; [35]_o = 2.7 mmol; Target \( M_n = 9150 \)

Since Cu complexes give very strongly coloured solutions, support for the influence of DMSO on the catalysing species may be indicated by inspection of the colour of the starting solutions. At 43 wt% DMSO (Table 3.1) the starting solutions were brown in colour. As the DMSO concentration was increased (entries 1, 2 & 3, Table 3.6) the solution became greener (67 wt%) until completely green (80 wt%). This suggests a displacement of the ligand, because CuBr without ligand dissolves in DMSO to give a green solution. When the more strongly complexing PMDETA and Me₆TREN ligands (Teodorescu & Matyjaszewski, 2000) were used for the polymerisation of 35, vividly coloured solutions of blue and green respectively were obtained and these respective colours remained throughout heating. This implies that these ligands co-ordinate more strongly with Cu(I) compared with bipy and hence competitive co-ordination with the solvent is reduced/minimised. However, only low yields of polymer were obtained (< 52%) with high polydispersities \( (M_w/M_n = 1.38-1.92) \) in these reactions, indicating poor control (entries 4, 5 & 6 Table 3.6). This would appear consistent with more powerful catalysing system using these ligands, since it has already been shown with bipy that deactivation may be inefficient. With
these more strongly complexing ligands the polymerisation kinetics may approximate to that of a conventional redox initiated free radical polymerisation, i.e., with no active/dormant equilibrium of ATRP (Matyjaszewski, 1998).

2.2.4 Use of copper(I) chloride

In another attempt to slow down the rate of propagation CuBr was replaced with CuCl (Table 3.7). A bromine-terminated initiator/CuCl combination can work well in ATRP because once a radical is generated from the initiator and monomer has added, the radical will readily react with CuCl$_2$ or CuClBr in order to form the more stable C-Cl bond, thus reducing the stationary radical concentration (Matyjaszewski et al., 1998). Compared to an identical CuBr reaction (entry 1, Table 3.7) the use of CuCl led to only slight improvements in $M_n$ and apparent initiator efficiency (entry 2, Table 3.7), suggesting the polymerisation of 35 is kinetically similar using either CuCl or CuBr.

<table>
<thead>
<tr>
<th>No.</th>
<th>Catalyst</th>
<th>Ratio*</th>
<th>Time/ h</th>
<th>% Yield</th>
<th>$M_n$</th>
<th>$M_d/M_n$</th>
<th>$f_{app}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CuBr</td>
<td>50:1:0.1:0.2</td>
<td>0.25</td>
<td>85</td>
<td>37100</td>
<td>1.35</td>
<td>0.21</td>
</tr>
<tr>
<td>2</td>
<td>CuCl</td>
<td>50:1:0.1:0.2</td>
<td>0.25</td>
<td>88</td>
<td>29300</td>
<td>1.37</td>
<td>0.28</td>
</tr>
</tbody>
</table>

*Ratio = [35]:[47]:[CuBr]:[bipy]; [35] = 13.7 mmol; Solvent = DMSO (43 wt%); Target $M_n = 9150$

2.2.5 Use of different ATRP initiators

![Diagram of 47, 48, 49, and 50](image)

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In addition to 47, three other initiators were examined for the polymerisation of MOSu 35 (Table 3.8). Although 2-hydroxylethyl 2-bromopropanoate 48 initiated the polymerisation (entry 2, Table 3.8) the polydispersity was higher ($M_w/M_n = 1.43$) and the yield moderate (34%) compared with 47. The differences between 47 and 48 observed in the two polymerisations can be explained in terms of the stability of the initiator radical, 47 forming a more stable, less reactive, tertiary radical compared to the secondary radical for 48, hence leading to a significant reduction in side reactions. This possible explanation can also be extended to the use of (1-bromo-ethyl)benzene 49, which did not result in precipitation of a polymer when the reaction solution was added to acetone.

Table 3.8. ATRP procedures applied to 35 using different initiators at 100 °C.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Initiator</th>
<th>Ratio</th>
<th>% Yield</th>
<th>$M_n$</th>
<th>$M_w/M_n$</th>
<th>$f_{app}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>47</td>
<td>50:1:0:1:0.2</td>
<td>85</td>
<td>37,100</td>
<td>1.35</td>
<td>0.21</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>50:1:0:1:0.2</td>
<td>34</td>
<td>40,400</td>
<td>1.43</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>50:1:1:2</td>
<td>No ppt*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>50:1:1:2</td>
<td>No ppt*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(i) Ratio = [35]$_o$:[Initiator]$_o$:[Cu(I)Br]$_o$:[bipy]$_o$; (ii) Example result using 47; [35] = 13.7 mmol; Solvent = DMSO 43 wt%; Target $M_n = 9150$ g mol$^{-1}$; Reaction time = 0.25 - 24 h.

The polymerisation of 35 was also attempted using p-toluenesulfonylchloride 50. It is proposed in the literature that the p-toluenesulfonyl radical of 50 formed in ATRP can only add to monomer and initiate polymerisation (Wang et al., 1997) since p-toluenesulfonyl radicals do not terminate biomolecularly because the formation of $\alpha,\alpha$-disulfones is relatively slow (da Silva Correa & Waters, 1968; Wang et al., 1997). However, no polymer was isolated using 50 after precipitation of the reaction solution into acetone. Since 50 is also reported to be a good ATRP initiator for
methacrylates because of easy activation to form a high concentration of radicals (Wang et al., 1997) it may be the case therefore, that initiation readily occurred but a high concentration of radicals may have resulted in bimolecular termination and hence formation of low MW acetone-soluble oligomers of 35. For successful ATRP, Matyjaszewski (1997; 1998) has stated that the initiator efficiency should be maximised by using an initiator whose structure is similar to that of the active chain end. Initiator 47 was chosen originally, not for the structural similarities to 35, but for the hydrophilic nature, potentially more suitable for a biological environment, which would be conferred to one end of the polymer chain.

2.3 Consideration of other factors for higher than expected MW's in the polymerisation of MOSu 35

Ersin-Acar and co-workers (2000) reported that contamination of ATRP reaction mixtures with air can cause initiation at high temperatures (120 °C, reaction times on the time-scale of hours), possibly due to oxidation reactions generating initiating species. This conclusion was reached after studying the copper- and nickel-mediated polymerisations of phenylethyl methacrylate without an initiator in the presence and absence of air. The work also highlighted the fact that septa sealed reaction vessels may not be sufficient to prevent air contamination unless kept under a positive pressure of nitrogen (and presumably other inert gases) or if the septa are reinforced with tape or Parafilm.

It can be speculated that the presence of oxygen may also be a cause of deviation from controlled ATRP of 35 described in this study, i.e., $M_n$ values that are unpredictable from the ratio of monomer to initiator. The ATRP procedure employed for all the polymerisations of 35 (except those in THF, see below) used a septum sealed reaction vessel in combination with approximately 15 min of argon purging to the reaction mixtures (see experimental section 3, Chapter 7). The fast reaction times
of the polymerisations compared to the study by Ersin-Acar and co-workers, together with the differences in the outcome of the polymerisation of 35 using different initiators suggest that initiation due to oxygen does not occur during the polymerisation time-scale. Nevertheless, oxygen may be involved in termination reactions of growing radicals at an early stage, which may lead to a higher than expected MW of the precipitated polymer.

The behaviour of oxygen with the catalysing species can be very complex (Ersin-Acar et al., 2000). Observations of colour changes suggested that there may be a build up of deactivating Cu(II) during the polymerisations of 35 in DMSO solution (section 2.1) yet high conversions (yields) were still obtained. Thus, it can be hypothesised that oxidation of Cu(I) to Cu(II) involving oxygen may possibly lead to Cu(II) species that either do not participate, or participate inefficiently in the deactivation cycle, preventing adequate control of the polymerisation.

In summary, evidence from the literature suggests that the presence of oxygen may be a possible source of deviation (i.e., unpredictable MW) from conventional controlled ATRP in the polymerisation of 35. However, for the polymerisations performed in THF (Table 3.1) the reaction solutions were prepared in an oxygen-free (< 5 ppm) glovebox with reagents that had been carefully de-oxygenated, yet $M_n$ values were still higher than theoretical values, indicating oxygen may not be the sole reason for unpredictable $M_n$ values. It is possible that in these THF reactions, the heterogeneity of the catalysing species may be a separate reason for the unpredictable MW values, i.e., a different effect than that observed in the polymerisations conducted in DMSO, DMF and acetone.

For future work it may be more appropriate to use the freeze-thaw method of oxygen removal from the reaction solution to ensure that initiation or other described reactions due to the presence of oxygen cannot occur.
2.4 Removal of copper species and atomic absorption spectroscopy

The efficient removal of the catalysing species in ATRP is a potential limiting factor for many applications because metal contamination can influence the mechanical properties of the polymer, hinder the characterisation and cause discolouration of the polymer. With respect to the consequences of the medicinal use of polymers contaminated with copper, copper is an essential element present in all organisms and all bodily tissues in humans. Hence, there is a daily requirement of about 2 mg for adult humans with a biological half-life of about 4 weeks (Aaseth & Norseth, 1986). Chronic copper poisoning has not been described in normal human beings; systemic effects, especially hemolysis, liver and kidney damage have been reported but only after the ingestion of large amounts of copper salts (Aaseth & Norseth, 1986; Siltig, 1981).

The removal of copper from polymers prepared by ATRP is often achieved either by passing a diluted reaction mixture through an aluminium oxide column, by precipitation, ion-exchange resins and sometimes by dialysis (Matyjaszewski, 1997). In the experiments described here, the polymer 36 was isolated as a white powdery solid by first precipitating a reaction mixture diluted with DMSO into vigorously stirred acetone followed by filtration (see experimental section 3.3). The acetone filtrate was a deep green in colour indicating that the majority of the copper content was removed from the polymer. Atomic absorption spectroscopy (AAS) analysis indicated the copper content to be 0.153 ppm (average of three readings) at a polymer concentration of 28.0 mg·ml⁻¹ in DMF using CuSO₄ standards. This sample of 36 had been isolated from one precipitation into acetone. After a further precipitation from DMSO solution into acetone the copper concentration fell to 0.067 ppm, determined at the same polymer concentration in DMF.

A final purification step when preparing soluble macromolecular therapeutics before biological evaluation is to dialyse against water. Since any residual copper is of
low MW it is assumed that any remaining copper can be removed during this purification step which has to be performed regardless of the method of preparation.

2.5 Pilot reaction to assess the potential for block copolymer formation using MOSu 35

Although not the direct aim of this study, the use of block copolymers consisting of a reactive ester block and a biocompatible, hydrophilic block would be a useful addition as polymeric precursors because it could be used to define the area of drug location in the polymer-drug conjugate molecule. Understanding the solution conformation of polymer-drug conjugates and the effect on the biological efficacy (e.g., cellular uptake, degradation by enzymes) is little understood. Block copolymers may be one way to prepare conjugate candidates with varied solution conformations to further probe structure-activity relationships. Additionally, if the hydrophobic drug molecules (or hydrophobic groups) are located in a controlled location along the polymer backbone, as is possible with a block copolymer, then there is the potential for micelle formation (Chapter 1, Section 7). For these reasons it was of interest to see if the ATRP procedures applied to the homopolymerisation of 35 could also be extended to the preparation of block copolymers.

From the homopolymerisation experiments already described, it can be envisaged that using 35 to produce block copolymers by ATRP procedures would be hindered by several problems. The solubility of the monomer and more importantly the polymer may limit the reaction solvent to the highly polar DMSO, which is not ideal for ATRP, especially if the active ester block is to be prepared first. Conversion of all or part of an active ester homopolymer block to a macro-initiator soluble in solvents more suitable for ATRP (by reaction with a suitable amine) may be
problematic or not possible because of the possibility of nucleophilic substitution of the halogen terminal end group of the homopolymer with the amine reactant (Coessens & Matyjaszewski, 1999\textsuperscript{a} & 1999\textsuperscript{b}), although this was not investigated. This also assumes that the PMOSu 36 homopolymer chains are terminated with a bromine atom after the polymerisation. A better method may be to prepare the non-PMOSu block first, i.e., a macro-initiator in order to perform the polymerisation in a solvent where competitive chelation is not so prevalent.

An advantage of using a more appropriate ATRP solvent may be to reduce the rate of propagation and the stationary concentration of radicals, which may in turn lead to better control through a more effective ATRP equilibrium. The use of a macro-initiator may also suffer from the apparently poor initiator efficiency as observed in the homopolymerisation of 35. If this occurred then the uninitiated chains would have to be separated from the initiated chains, for example, by differences in solubility, using (semi)preparative GPC or High Performance Liquid Chromatography (HPLC)).

Attempts were made to prepare a PHPMA macro-initiator 51 by the ATRP of HPMA 2 (e.g, Scheme 3.6). Using different initiators, ligands and solvent none of the conditions provided controlled polymerisation (Table 3.9). For the polymerisations in water, tetraethyleneglycol 2-bromo-2-methylpropanoate 52 was used as an initiator because it was water-soluble unlike 47. No attempt was made to investigate reasons for this lack of control.

However, it was discovered that using a similar initiating system to that used by Teodorescu and Matyjaszewski (2000, entry 8, Table 3.9), who obtained 18.5% conversion of 2 in ethanol ($M_w/M_n = 1.29$, $M_n = 4480$, theoretical $M_n = 1850$, PMMA standards, room temperature), no polymer was isolated from the reaction solution by precipitation.
**Scheme 3.6.** Example attempt at preparing HPMA macro-initiator 51 (entry 6, Table 3.9).

![Diagram of Scheme 3.6](image)

**Table 3.9.** Summary of results for the attempted ATRP of HPMA 2.

<table>
<thead>
<tr>
<th>Ratio (wt%)</th>
<th>Solvent</th>
<th>Initiator</th>
<th>ligand</th>
<th>T/°C</th>
<th>Yield</th>
<th>(M_n)</th>
<th>(M_w/M_n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100:1:1:1.2</td>
<td>THF (88)</td>
<td>47</td>
<td>PMDETA</td>
<td>70</td>
<td>trace(^a)</td>
<td>1,500</td>
</tr>
<tr>
<td>2</td>
<td>200:1:1:1.2</td>
<td>THF (88)</td>
<td>47</td>
<td>PMDETA</td>
<td>70</td>
<td>trace(^a)</td>
<td>2,800</td>
</tr>
<tr>
<td>3</td>
<td>100:1:1:1.2</td>
<td>EtAc (50)</td>
<td>47</td>
<td>PMDETA</td>
<td>80</td>
<td>0(^b)</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>100:1:1:3</td>
<td>MeOH (75)</td>
<td>47</td>
<td>bipy</td>
<td>70</td>
<td>trace(^a)</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>100:1:1:3</td>
<td>H(_2)O (60)</td>
<td>52</td>
<td>bipy</td>
<td>80</td>
<td>trace(^a)</td>
<td>11,800</td>
</tr>
<tr>
<td>6</td>
<td>100:1:1:1.2</td>
<td>H(_2)O (75)</td>
<td>52</td>
<td>Me(_3)TREN</td>
<td>23</td>
<td>30(^b)</td>
<td>31,700</td>
</tr>
<tr>
<td>7</td>
<td>100:1:1:1.2</td>
<td>H(_2)O (55)</td>
<td>52</td>
<td>Me(_3)TREN</td>
<td>70</td>
<td>3(^b)</td>
<td>49,900</td>
</tr>
<tr>
<td>8</td>
<td>50:1:1:1</td>
<td>EtAc (88)</td>
<td>MCP</td>
<td>Me(_3)TREN</td>
<td>40</td>
<td>0(^b)</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>50:1:1:2</td>
<td>EtAc (88)</td>
<td>MCP</td>
<td>Me(_3)TREN</td>
<td>40</td>
<td>0(^b)</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^{a}\) \(i = [2]_\text{c} \cdot [\text{Initiator}]_\text{c} \cdot [\text{Cu(I)Br}]_\text{c} \cdot [\text{ligand}]_\text{c}\); \(^{b}\) For reaction number 8, CuCl was used instead of CuBr; \([2]_\text{c} = 3.5 - 7.0\) mmol; \(^{a}\) sample prepared by passing reaction solution through a neutral aluminium oxide column and lyophilisation of the resulting solution; \(^{b}\) sample prepared by precipitating reaction solution (or methanol solution) into acetone.
The synthesis of an active ester block copolymer was also attempted using a PEG macro-initiator 55 for ATRP (Jankova et al., 1998; Wang & Armes, 2000). PEG makes an excellent choice for a constituent of a block copolymer since it is widely used for the conjugation of bioactive molecules but can be limited by a lack of conjugating sites (see also section 5, Chapter I). In this study a PEG macro-initiator was prepared using a similar method to that of Jankova et al. (1998) by allowing monomethoxy-PEG 53 to react with 2-bromoisobutyryl bromide 54 in the presence of pyridine (Scheme 3.7) (see experimental section 3.3, Chapter 7). The macro-initiator 55 was purified by dialysis, and in an alternative method, by precipitation. Both methods gave products satisfactory by $^1$H NMR and consistent with the literature, an example is shown in Figure 3.1.

Scheme 3.7. Synthesis and use of a monomethoxy-PEG macro-initiator 55.
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Figure 3.1. $^1$H NMR (CDCl$_3$) of PEG macro-initiator 55.

Figure 3.2. Gel permeation chromatogram obtained for product of the polymerisation using PEG macro-initiator 55 and MOSu 35.
A pilot reaction was performed using 55 (MW 2000), PMDETA ligand and Cu(I)Br in methanol at 50 °C (see experimental section 3, Chapter 7). After 7 h of heating, the reaction solution did not appear to increase in viscosity, but was precipitated into diethyl ether to give 43% yield of a polymeric product. GPC analysis (Figure 3.2) indicated the presence of poly(MOSu-b-ethylene glycol) 56 and uninitiated 55. This work has been subsequently developed by a colleague, Dr Neli Koseva (Koseva, 2001). Dr Koseva has shown that after performing the reaction in ethylene carbonate at 80 °C the copolymer 56 can be separated from the uninitiated PEG 55 and copper species by passing the reaction mixture through a neutral aluminium oxide column and precipitating the eluent into acetone. This polymer was found to have a $M_n$ and $M_w/M_n$ of 32,500 and 1.24 respectively (GPC, DMF, PMMA standards). This work is continuing in order to establish optimal conditions for the polymerisation but has already demonstrated that the preparation of active ester diblock copolymers using 35 and a PEG macro-initiator 55 is feasible.
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2.6 Extension of ATRP procedures to N-acryloxysuccinimide 57 (AOSu) and N-ethycryloxysuccinimide 60 (EOSu)

2.6.1 AOSu 57

The DMSO reactions conditions applied to the polymerisation of 35 (Table 3.7) were examined for the polymerisation of 57 (Scheme 3.7) with the aim of preparing narrow MWD polyacrylamides. Similar to PMOSu 36, poly(N-acryloxysuccinimide) 58 also exhibits poor solubility except in DMF and DMSO

(Ferruti et al., 1972).

Scheme 3.8. Example conditions attempted to prepare 58.

Disappointingly, only extremely small yields of 58 precipitated into acetone from the reaction solution (entries 1, 2 & 3 Table 3.10). A key difference between acrylates and methacrylates in conventional free radical polymerisation is the propagating radicals of methacrylates are generally more stable and propagate more slowly than the corresponding acrylate. This can have a pronounced effect on ATRP (Beers et al., 1999). The polymerisations described for MOSu 35 indicated an extremely fast propagation rate because of the short reaction times, therefore, it was envisaged that the propagating radical of 57 may potentially be subject to a extremely high level of chain transfer and termination side reactions. The polymerisation was
repeated in less polar solvents and reduced temperature to attempt to decrease the rate of propagation (entries 4, 5 & 6, Table 3.9) but again only low yields were obtained.

Table 3.10. ATRP procedures applied to AOSu 57.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Ratio¹</th>
<th>Time/ h</th>
<th>T/°C</th>
<th>Yield</th>
<th>⁹Mₙ</th>
<th>⁹Mₙ/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 50 wt% DMSO</td>
<td>50:1:0.1:0.2</td>
<td>1</td>
<td>100</td>
<td>no precipitate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 52 wt% DMSO</td>
<td>50:1:0.2:0.4</td>
<td>19</td>
<td>25</td>
<td>7</td>
<td>9,900</td>
<td>1.64</td>
</tr>
<tr>
<td>3 33 wt% DMSO</td>
<td>50:1:0.2:0.4</td>
<td>18</td>
<td>25</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 83 wt% acetone</td>
<td>50:1:1:2</td>
<td>18</td>
<td>25</td>
<td>trace²</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 72 wt% toluene</td>
<td>50:1:1:2</td>
<td>20</td>
<td>70</td>
<td>no precipitate</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(i) = [57]₀:[47]₀:[Cu(I)Br]₀[biPy]₀; (ii) precipitated during the reaction; [57]₀ = 5.9 mmol.

2.6.2 Preparation and attempted polymerisation of EOSu

The polyelectrolyte poly(2-ethylacrylic acid) 59 (below) can be used to design lipid bilayer membranes that release their contents in response to changes in environmental parameters such as pH and this can utilised for drug delivery (Thomas et al., 1995). MW and copolymer composition are two parameters that can be changed in order to adjust the pH for the onset of membrane reorganisation. In recognition of this, the synthesis of narrow MWD 59 has recently been reported using the anionic polymerisation of benzyl 2-ethylacrylate followed by hydrolysis to the acid (Kim & Tirrell, 1999).

\[
\begin{align*}
\text{CH}_2 & - \text{C} - \text{CO} - \text{OH} \\
\text{CH}_3 & \\
\text{CH}_2 & \\
\end{align*}
\]
To simplify the preparation of 59 and copolymers of 59, EOSu 60 was synthesised in a four-step procedure from diethyl malonate (Scheme 3.9) in order to prepare poly(N-ethylcryloxysuccinimide) 61 by ATRP for use as a polymeric precursor.

![Scheme 3.9. Synthesis of EOSu 60 from diethyl malonate (experimental section 3.1, Chapter 7).](image)

Monomer 60 is a white crystalline solid that is not described in the literature. An acetone solution of 60 was allowed to react with AIBN free-radical initiator, but surprisingly no polymerisation was observed (entries 1 & 2, Table 3.11). This was also the outcome using benzyol peroxide, another free radical initiator, in bulk and using suspension polymerisation with benzyol peroxide.
Table 3.11. Summary of conditions attempted to polymerise EOSu 60.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Free radical initiator</th>
<th>Temp/°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetone</td>
<td>AIBN</td>
<td>50</td>
</tr>
<tr>
<td>acetone</td>
<td>benzoyl peroxide</td>
<td>60</td>
</tr>
<tr>
<td>none</td>
<td>benzoyl peroxide</td>
<td>100</td>
</tr>
<tr>
<td>water (suspension)</td>
<td>benzoyl peroxide</td>
<td>60</td>
</tr>
</tbody>
</table>

Scheme 3.10. Failure of EOSu 60 to polymerise under conditions listed in Table 3.11.

Both 35 and 57 are readily polymerised by a conventional free radical initiator to give high polydispersity polymers (Table 3.12). The homopolymerisation of 2-ethylacrylic acid 59.5 also proceeded readily using AIBN at 50 °C. Copolymerisation using 50 wt% 59.5 and 50 wt% 60 also did not result in any polymerisation. It is not clear why this monomer should not polymerise but possibly the polymerisation is sterically hindered by the ethyl and succinimidyl substituents. An ATRP experiment was not performed with EOSu 60 on the basis of these conventional free radical polymerisation results.

Table 3.12. Conventional free radical polymerisation of MOSu 35 & AOSu 57.

<table>
<thead>
<tr>
<th>No.</th>
<th>Monomer</th>
<th>$M_n$</th>
<th>$M_v/M_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>68,200</td>
<td>2.02</td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>50,800</td>
<td>2.03</td>
</tr>
</tbody>
</table>

Conditions: AIBN 7.5 wt%, 50 °C, acetone solution.
Chapter 3: Active Ester Homopolymers

3 Conclusions

Narrow MWD homopolymers of MOSu 35 were prepared with suitable MW’s for drug conjugation by the application of Cu mediated ATRP procedures. Different MW’s were obtained by varying the ratio of monomer to initiator. At the time of writing this thesis, the polymerisation of 35 was the only example of the homopolymerisation of a (meth)acrylic acid derived active ester by ATRP procedures described in the literature. However, evidence supporting an extremely fast polymerisation and a high level of irreversible termination during initiation and the early stages of propagation was found for 35, resulting in higher than expected values for $M_n$. It was proposed therefore that the polymerisation of 35 may also involve conventional redox-initiated free radical polymerisation kinetics in addition to the ATRP process. Homogeneous conditions for a high yielding polymerisation were found to be 43 wt% DMSO at $> 80$ °C. These reactions were carried out on a 1.8 to 6 g scale. Narrow MWD PMOSu 36 was also achieved in acetone and THF solutions, with the polymer precipitating during heating at lower MW’s ($M_n = 1,800$ to 20,000 g·mol$^{-1}$) than obtained in the homogenous DMSO reactions. It was also shown that an active ester-based block copolymer, poly(MOSu-b-ethylene glycol) 56, could successfully be prepared by using a PEG macro-initiator 55 synthesised by the functionalisation of monomethoxy-PEG 53.

Attempts to directly transfer the ATRP conditions for 35 to AOSu 57 were unsuccessful. Additionally, the novel monomer EOSu 60 was prepared but it was shown not to polymerise even by conventional free radical polymerisation.
Chapter 4

**Conjugation Chemistry and the Preparation of Polymer-Drug Conjugates**

1 Introduction

Critical to the success of the homopolymer precursor strategy is the conversion of the precursor PMOSu 36 to the required end product. The aim of this study was to understand the conjugation chemistry (polymer analogous reactions) of 36, i.e., reactions I and II in Scheme 3.1, Chapter 3. If the conjugation chemistry is not well understood and competitive side reactions are not minimised, then the value of the polymeric precursor is diminished.

1.1 Issues of PMOSu 36 derivatisation

Polymer bound functional groups in general show significantly different reaction rates compared to low MW analogues (Odian, 1991; Strohrieg1, 1993). Polymer molecules are generally present in solution as random coil conformations. The concentration of functional groups is high within the polymer coils and zero outside (Odian, 1991). The concentration of the small molecule reactant inside the polymer coil relative to the concentration outside determines whether the reaction is at a higher, lower or comparable rate to the corresponding low MW analogue reaction. Attraction or repulsion experienced by the small molecule reactant, or the use of poor solvents can all affect the rate and extent of reaction. High degrees of substitution can be achieved if both precursor polymer and the transformed polymer are soluble.
Neighbouring group effects are another consideration and describe the direct effect on the reactivity of a functional group in a polymer by an adjacent neighbouring group (Arshady, 1994; Odian, 1991). Neighbouring group effects are influenced by the type of adjacent functionality, the type of reaction and the stereochemistry involved.

A disadvantage with the chemical modification of a polymer is that side reactions can lead to structural defects (e.g., carboxylic acid groups from the hydrolysis of methacrylate repeat units) that may not be possible to remove. If it is possible to characterise the side products then the reaction conditions can be optimised to avoid or limit the unwanted defects. This is a key factor in this study. These issues are further described, along with other determinants of polymer reactions during section 2 as they become relevant to the discussion of the results.

Polymer 36 shows three bands in the carbonyl (C=O) region under IR analysis (for full spectrum see Appendix V). The two bands at 1779 and 1734 cm\(^{-1}\) are due to the symmetric and anti-symmetric stretch of the imide group respectively (Figure 4.1), while the third band at 1807 cm\(^{-1}\) is due to the ester carbonyl (Batz \textit{et al.}, 1972; McKittrick & Katon, 1990; Ringsdorf \textit{et al.}, 1972).

![Symmetric and Anti-Symmetric Vibrations of the Imide Moiety](image)

**Figure 4.1.** Symmetric and anti-symmetric vibrations of the imide moiety of PMOSu 36 (McKittrick & Katon, 1990).
In fact, these three bands are very prominent features of the IR spectrum of \( \text{36} \). On conversion to methacrylamide polymers it can be expected that these bands will be replaced by an amide carbonyl band at a distinctly different wavenumber (approximately \( 1650 \text{ cm}^{-1} \)). Since carbonyl bands strongly absorb IR radiation this enables easier detection, especially at low concentration. Therefore IR spectroscopy may provide the opportunity to follow the aminolysis of the precursor polymer and aid characterisation of the final product.

In this study, Attenuated total reflectance (ATR) FT-IR spectroscopy was primarily used to characterise and follow the extent of reaction. With ATR, the IR beam is reflected through a high refractive index IR transmitting prism. A portion of the radiation extends beyond the surface of the prism and for samples in intimate contact with the prism a spectrum can be obtained (Omnic, 1998). This is a very quick (no sample preparation required) and quantitative method (providing there is excellent contact with the mirror, i.e., for liquids and solutions) for analysing solids and liquids, including reaction solutions. Quantitative analysis can be performed using ATR FT-IR since the effective path length (depth of penetration x number of internal reflections in the ATR mirror) is constant. This is due to a mathematical ATR correction that allows for the different depths of penetration of different wavelengths of IR radiation. Emphasis is placed on FT-IR since this is more convenient method of analysis compared to NMR, where polymer-drug conjugates can be extremely difficult to interpret due to complex spectra (Pincirolri et al., 1997).
2 Results and Discussion

2.1 Preparation of PHPMA 1

The reaction of 1-amino-2-propanol 10 with 36 (Scheme 4.1) was attempted under varying conditions of amine stoichiometry ($x = n$ to $100n$), concentration (100 mg of 36 in 0.4 to 3.0 ml solvent), temperature (0 to 60 °C), and solvent (DMSO or DMF). It was observed that the conditions used were extremely important in obtaining IR and NMR spectra indistinguishable with conventionally prepared 1 (i.e., prepared from the conventional free radical polymerisation of HPMA 2). For reactions performed at room temperature, after 15 min the reactions went to, or neared completion, providing evidence that 36 has strong reactivity towards primary amines due to their high nucleophilicity. Conventional PHPMA 1 exhibits two characteristic bands in the IR at 1665 cm$^{-1}$ and 1539 cm$^{-1}$ corresponding to an amide carbonyl band stretch (amide I) and C-N-H in-plane bend (often referred to as amide II band and also involving C-N stretch) respectively (spectrum A, Figure 4.2). The majority of the conjugation reactions resulted in products that exhibited a shoulder band to amide I at around 1700 to 1710 cm$^{-1}$ in the IR (e.g., spectrum B, Figure 4.2). This was often the

Scheme 4.1. Preparation of PHPMA 1 from PMOSu 36.
only difference in the IR compared with conventional 1. Additionally, in a small number of samples where the amine reactant stoichiometry was equal to the number of moles of active ester groups, an additional peak of generally small intensity was observed around 1772 cm$^{-1}$ (e.g., spectrum C, Figure 4.2, the sample with largest intensity peak at 1772 cm$^{-1}$). Since these additional bands are in the carbonyl region of the IR, it suggested that a side reaction at the active ester carbonyl was occurring.

**Figure 4.2.** FT-IR spectra of PHPMA 1 samples. Spectrum A is conventional 1, while spectra B and C are example 1 samples obtained from the reaction of 10 with precursor 36 that show evidence of side reaction(s).
In $^1$H NMR analysis of the polymer products, there was in many cases a strong signal at approximately $\delta 2.3$ (in DMSO-$d_6$) that varied in concentration depending on the sample. The chemical shift suggested that the signal may be due to additional methylene protons that are not present with conventional 1. Evidence to explain these differences in the IR and NMR spectra between precursor-prepared and conventional 1 in terms of competitive hydrolysis and imide formation is described below in sections 2.3 and 2.4 respectively. However, with respect to the preparation of homopolymer 1 from 36, using dilute solutions and an excess of amine 10 (above 2 equiv.) gave products that were most consistent by IR and $^1$H NMR (Figure 4.3) analysis with conventional 1. These observations can be explained by the increased solvation of 36 at higher dilution, which allows the low MW amine reactant 10 to penetrate more easily into the polymer coil to react. Using a large excess of 10 increases the probability of the desired reaction occurring over side reactions. Between the two solvents used, DMSO and DMF, the outcomes of aminolysis of 36 with 10 were comparable.

Using DMSO solutions of model compounds at 25 °C, Coessens and Matyjaszewski (1999) have reported that the halogen end groups of polymers prepared by ATRP are easily displaced by mixing with 1.1 end group equiv. of primary amines. These are similar conditions used to prepare PHPMA 1 from 36. Although no reactions to assess this were performed, it is therefore possible that the reaction of 36 with primary amines may also displace any bromine end groups present, preventing polymers prepared from 36 (e.g., 1) being used as macro-initiators for ATRP for the preparation of block copolymers.
Figure 4.3. \(^1\)H NMR analysis of PHPMA 1 derived from PMOSu 36 (in DMSO-\(d_6\)).

GPC analysis (Figure 4.4) of precursor-prepared 1 showed a narrow MWD polymer (\(M_w = 22,000 \text{ g mol}^{-1}\), \(M_w/M_n = 1.3\), PEG standards, PBS eluent). This compares with conventional 1, also shown in Figure 4.4, which exhibited a much broader MWD (\(M_w = 35,000 \text{ g mol}^{-1}\), \(M_w/M_n = 2.9\), PEG standards, PBS eluent). The narrow MWD for precursor-prepared 1 indicates that no cross-linking between polymer chains occurred during the conjugation of 1-amino-2-propanol 10 due to the hydroxyl groups of conjugated or unconjugated 10 entering the reaction as nucleophiles (and so therefore not a likely cause of the extra bands observed in the IR in some of the conjugation reactions using 10).
Figure 4.4. Gel Permeation chromatograms showing the narrow MWD PHPMA 1 (labelled A) obtained from the reaction of PMOSu 36 with 1-amino-2-propanol 10 compared with conventional 1 (labelled B).

To further investigate the potential problem of competitive hydroxyl group reaction, the conjugation of a primary alcohol was examined by heating PMOSu 36 with methanol. After 5 h at 50 °C there was no IR evidence of any reaction although no base, known to catalyse polymer reactions (Arshady, 1994), or acylating agent was added (e.g., 4-(dimethylamino)pyridine). This was a further indication that the hydroxyl groups of 10 would not be involved in side reactions under these relatively mild reaction conditions. However, this does not take account of neighbouring group effects that may increase the rate of conjugation of the hydroxyl group of alcohols due to the effect of neighbouring amide groups in the conjugation of 10.
2.2 Cytotoxicity of PHPMA 1 prepared from PMOSu 36

The cytotoxicity of a sample of 1 derived from 36 as described in section 2.1 and purified by dialysis (see experimental section 4.5, Chapter 7) was compared with conventional 1. Using B16F10 cells over 72 h (Figure 4.5, dextran and poly-L-lysine are included as negative and positive controls respectively) the precursor derived 1 was shown to be non-toxic up to 5 mg·ml⁻¹, which was comparable with the conventional 1 tested.

![Graph showing cytotoxicity of PHPMA 1 prepared via PMOSu 36 and conventional 1 using B16F10 cells over 72 h (mean ± SD).](image)

**Figure 4.5.** Cytotoxicity of PHPMA 1 prepared via PMOSu 36 and conventional 1 using B16F10 cells over 72 h (mean ± SD).

2.3 Competitive hydrolysis

To investigate the possibility of PMOSu 36 undergoing competitive hydrolysis during aminolysis resulting in the formation of undesired methacrylic acid repeat units on the polymer, several experiments were performed. Firstly, a sample of 36 was
hydrolysed using aqueous NaOH to the sodium salt of PMAA 62 (see Scheme 5.2 and section 2.1 for synthesis, Chapter 5). In the IR, this compound gave a CO$_2^-$ stretch band at ~1559 cm$^{-1}$. The protonated form of this polymer was obtained by the addition of HCl and the solid product gave a carbonyl stretch at the higher frequency of 1703 cm$^{-1}$. This suggested that the presence of protonated carboxylic acid repeat units may be responsible for the shoulder band observed at around 1700-1710 cm$^{-1}$ seen to varying degrees in the conjugation reactions of 36 with 1-amino-2-propanol 10 (as described in section 2.1) due to competitive hydrolysis.

To further investigate competitive hydrolysis, a sample of 36 in DMSO was allowed to react with aqueous 10 to promote hydrolysis (experimental section 4.1, Chapter 7). IR analysis of the product showed a prominent band at 1710 cm$^{-1}$ providing evidence for competitive hydrolysis (spectrum A, Figure 4.6). This sample was then dissolved in dilute NaOH. An IR spectrum of the solid isolated from the NaOH solution showed the near disappearance of the band at 1710 cm$^{-1}$ and an increase at 1550 cm$^{-1}$ (spectrum B, Figure 4.6). This is consistent with the presence of MAA Na salt repeat units in the polymer, which would be expected if carboxylic acid groups in the polymer were treated with NaOH. The sample was then dissolved in HCl to re-protonate the suspected carboxylate groups. After isolation of the polymer, the band at ~1710 cm$^{-1}$ had returned in the IR spectrum (spectrum C, Figure 4.6) further supporting the hypothesis of competitive hydrolysis.
Analysis of the sample by $^1$H NMR also indicated hydrolysis, since the backbone methylene and methyl protons had larger integrals than would be expected for just HPMA homopolymer 1. The ratio of HPMA to MAA repeat units was calculated to be approximately 1.5:1 respectively from the NMR integral values, i.e., 40% hydrolysis had occurred. The extent of hydrolysis in a sample of PHPMA 1 prepared from PMOSu 36 in section 2.1, i.e., without the addition of water to the reaction mixture, was calculated to be approximately 4%.

To further assess the hydrolytic stability of 36 in solution, an anhydrous DMSO solution of 36 was heated with triethylamine (0.5 equiv.) at 60 °C. After 1 h, the IR spectra of the starting and heated samples were identical, indicating no hydrolysis had occurred. However, after 2 h a 13% decrease in peak height measured at 1735 cm$^{-1}$ was observed. After 16 h at 60 °C the decrease became 22% indicating that the polymer will undergo hydrolysis with heating in the presence of base and trace water from the solvent. It was also observed that compared to freshly prepared 36, samples of 36 that had been stored under ambient conditions for several months had a slight shoulder band in the IR at around 1710 cm$^{-1}$ that indicated hydrolysis had occurred (carbonyl band of carboxylic acid groups, Figure 4.7).

Figure 4.7. FT-IR spectroscopy comparison of fresh (blue spectrum) and stored (red spectrum) samples of PMOSu 36.
With $^1$H and $^{13}$C NMR there was no distinction between the stored and freshly prepared 36, as there was no signal at approximately $\delta$ 12.28 (broad) relating to COOH (value obtained from analysis of hydrolysed 36 in DMSO-$d_6$) in $^1$H NMR analysis and no evidence of the carbonyl carbon of the acid (approx. $\delta$ 180) in $^{13}$C analysis. However, elemental combustion analysis (Table 4.1) revealed discrepancies between theoretical and experimental values; not only in the stored samples of 36 but also the fresh samples, suggesting the polymer samples tested may have undergone some very slight hydrolysis during the polymerisation (at 70 to 100 °C) and/or isolation and purification.

<table>
<thead>
<tr>
<th></th>
<th>% C</th>
<th>% H</th>
<th>% N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theory based on C$_4$H$_8$NO$_4$ repeat unit</td>
<td>52.46</td>
<td>4.95</td>
<td>7.65</td>
</tr>
<tr>
<td>Found</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh sample*</td>
<td>50.82</td>
<td>5.18</td>
<td>7.50</td>
</tr>
<tr>
<td>Stored sample</td>
<td>47.52</td>
<td>5.38</td>
<td>6.64</td>
</tr>
</tbody>
</table>

**Table 4.1.** Elemental combustion analysis of PMOSu 36 samples; (*) average of five different samples.

Competitive hydrolysis cannot occur in the absence of water from the reaction. However, the hygroscopic nature of DMSO and DMF makes water exclusion difficult to achieve in practice. An examination of different solvents (perhaps mixed solvent) systems that are less hygroscopic may be a solution to this. Alternatively, a longer-term solution may be to prepare copolymer precursors to confer solubility in other less polar organic solvents. As concluded earlier, however, the use of a large excess of nucleophile in the preparation of homopolymers (e.g., 100 equiv. excess) is one way to minimisation/eliminate the shoulder band at ~1710 cm$^{-1}$ in the solid product.
2.4 Competitive imide formation

Competitive cyclic imide and anhydride formation (Scheme 4.2) are well known side reactions of reactive polymers (Arshardy, 1994; Strohriegl, 1993). A mechanism of anhydride formation for 36 in the preparation of poly(methacrylamides) is due to the hydrolysis of an active ester group followed by reaction with the neighbouring active ester (Scheme 4.2, A).

![Scheme 4.2](image)

**Scheme 4.2.** Anhydride (A) and imide (B) formation between neighbouring groups.

Since hydrolysis can theoretically be prevented by the vigorous exclusion of water or substantially reduced or elimination by the use of a large excess of nucleophilic reactant, anhydride formation can therefore also be eliminated/minimised. If cyclic anhydride groups were present it can be expected that carbonyl bands in the regions 1870 to 1845 cm$^{-1}$ (symmetric stretch) and 1800 to 1775 cm$^{-1}$ (anti-symmetric stretch) would be observed in the IR (Vie et al., 1991). This is not consistent with the bands seen at 1772 cm$^{-1}$ and around 1710 cm$^{-1}$ (shoulder) in the reactions to prepare HPMA homopolymer 1 in section 2.1 (see spectrum C, Figure 4.2).
However, cyclic imide bands are consistent with these wavenumbers since they typically occur at a lower frequency, 1800 to 1735 cm\(^{-1}\) (symmetric stretch) and 1750 to 1680 cm\(^{-1}\) (anti-symmetric stretch) (Vie \textit{et al.}, 1991). The sample of I prepared from 36 where the band at 1772 cm\(^{-1}\) was most prominent also exhibited a large signal at \(\delta\) 2.3 in a \(^1\)H NMR spectrum (labelled \(h\) in Figure 4.8) which was not present in conventional PHPMA I spectrum. This suggests that this NMR signal is due to a structural moiety also responsible for the additional IR band at 1772 cm\(^{-1}\).

![Figure 4.8. \(^1\)H NMR spectrum of PHPMA I derived from PMOSu 36 that from IR analysis (spectrum C, Figure 4.2) may possess imide impurity in the backbone similar to the simplified structure shown.](image)

In addition to the imide formation formed by the reaction of immediately adjacent amide and active ester repeat units (Scheme 4.2, B) there is also the possibility of cross-linking between non-neighbouring functional groups in a polymer or between separate polymer chains leading to cross-linking and a broadening of the MWD. To probe this, an experiment was performed using a low MW active ester
model compound, N-propionyloxysuccinimide 63 with n-propylamine 64 (Scheme 4.3, see also experimental section 4.3, Chapter 7).

Scheme 4.3. Investigation of amide and imide formation using low MW succinimidyloxymethyl ester model 63.

Under similar conditions (i.e., concentration and temperature) employed for conjugation reactions involving PMOSu 36, no evidence of the imide 65 was obtained, indicating that competitive imide formation for 36 may be solely a polymer effect due to the close proximity of adjacent neighbouring functional groups and the likely formation of energetically favoured six-membered rings (Strohriegl, 1993). However, this reaction may not be representative of a polymer reaction which is characterised by very high concentrations of functional (reactive groups) inside the polymer coil, held in close proximity by the polymer backbone. Therefore, the structure proposed in Figure 4.8 may be the simplest structure possible with the actual structure containing many types of imide moieties (e.g., cross-linking imide bonds).

The formation of imide groups in the backbone is a more significant concern when conjugating drug-linker molecules to precursor 36 (reaction 1 in Scheme 3.1, Chapter 3) since neighbouring amide and active ester functional groups will be present in the polymer (product 38, Scheme 3.1) until the remaining active ester groups are quenched with amines to confer aqueous solubility to the conjugate. The next section describes reactions involving less than equivalent reactive group stoichiometries of simple primary amines, and the problem of imide formation is further addressed.

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2.5 Reaction of PMOSu 36 with fractional equivalents of amine

To understand the reaction of 36 with fractional equivalents of an amine, which is necessary for polymer-drug conjugate preparation, a set of 4 reactions were first performed using 0.25, 0.50, 0.75 and 1.00 equiv. of 1-amino-2-propanol 10 (experimental section 4.1 Chapter 7). In each reaction, 10 was added neat to a DMSO solution of 36 under stirring. DMSO, unlike DMF, does not show any bands in the carbonyl region of the IR spectrum, which may interfere with any IR analysis of the reaction solution. Using relatively concentrated reaction solutions (50 mg of 36 in 400 µl DMSO) ensured the imide carbonyl bands of 36 remained prominent for any IR analysis of the reaction solution. IR analysis of the starting solution (i.e., without added amine) showed the imide carbonyl anti-symmetric stretch of 36 to be at 1735 cm⁻¹ in DMSO (for example spectrum see Figure 4.2 below, red spectrum). The reaction mixtures were then heated at 50 °C for 30 min. The IR spectra (overlaid on each other in Figure 4.9) obtained from the reaction solutions after this time showed very good agreement between the stoichiometries of 10 added and the percentage decrease in the band at 1735 cm⁻¹ (listed in Table 4.2). In ATR-FT-IR, both height and area measurements of IR bands can be used for quantitative analysis between samples in absorbance mode providing there is excellent contact with the mirror (Omnic, 1998; refer also to the introduction section of Chapter 4). Band heights were used throughout this study because height measurements were less affected by the overlap of adjacent neighbouring bands. Evidently the inclusion of a standard in the samples for IR spectroscopy may be a more desirable method to enable quantitative differences in the spectra to be measured since this eliminates problems due to differences in the contact of the reaction solvent with the ATR mirror. However, the inclusion of a standard may have led to precipitation of the polymer from the reaction solution since precipitation was sometimes observed when a large amount of amine reactant was used. One solution to this problem was to use a band attributable to the
DMSO reaction solvent as an internal standard, which would remain constant throughout the reaction. To this end, a band at 1436 cm\(^{-1}\) was chosen and the values obtained (also listed in Table 4.2) compared to the values obtained using just the decrease in the height of the carbonyl band at 1735 cm\(^{-1}\) for the conjugation of 10. These two sets of values agreed to within 4%, indicating that the contact of the reaction sample with the ATR mirror was sufficiently reproducible so as to negate the need for an internal standard. Similar results for the apparent quantitative decrease in band height upon conjugation were also obtained for glycine methyl ester 66 (Table 4.2), which was added as an HCl salt with an equal molar equivalent of triethylamine. In this case, it was not possible to compare the values against the band at 1436 cm\(^{-1}\) because overlap with neighbouring bands caused inconsistent height values. The conjugation of 66 was evidence that peptidyl linkers that may be required for drug conjugation could be successfully conjugated to polymer 36.
**Figure 4.9.** Overlaid FT-IR spectra showing the DMSO reaction solutions of precursor 36 after the addition of different stoichiometries of 1-amino-2-propanol 10

<table>
<thead>
<tr>
<th>equiv. of amine added</th>
<th>% decrease in peak height measured at 1735 cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-amino-2-propanol 10</td>
</tr>
<tr>
<td></td>
<td>direct</td>
</tr>
<tr>
<td>0.25</td>
<td>23</td>
</tr>
<tr>
<td>0.50</td>
<td>49</td>
</tr>
<tr>
<td>0.75</td>
<td>73</td>
</tr>
</tbody>
</table>

**Table 4.2.** % decrease in height of IR band at 1735 cm⁻¹ after addition of different stoichiometries of amines to PMOSu 36. (*) Normalised against an IR band at 1436 cm⁻¹ attributable to the DMSO solvent.
The polymer products obtained in the reactions of 10 with 36 were subsequently used for the biological study described in Chapter 5, where NMR data is presented.

2.5.1 Non-reproducible conjugation

While it appeared that the quantitative reaction of primary amines was feasible using 36 (at least by IR analysis) it became clear as more reactions were performed that these quantitative reactions were sometimes not always reproducible and appeared to be dependent on the reaction time. Sometimes, less than expected decreases were observed in the band at 1735 cm\(^{-1}\) and other times greater than expected decreases were observed. The demonstrated ability to use the decrease of the band at 1735 cm\(^{-1}\) in this way is due to the anti-symmetric imide carbonyl stretch of \(N\)-hydroxysuccinimide 61 (NHS), which is liberated during conjugation, being located at 1712 cm\(^{-1}\) and not 1735 cm\(^{-1}\) (Figure 4.10, red spectra).

\[ \text{61} \]

The addition of a base (triethylamine) to a DMSO solution of 61 indicated that an acid-base reaction had occurred (Paquet & Bergeron, 1982) because of a decrease in the height of the band at 1712 cm\(^{-1}\) and the appearance of a band at \(~1684\) cm\(^{-1}\) in the IR (Part A, Figure 4.10) that did not increase significantly with the addition of further triethylamine. This result is consistent with a band at 1712 cm\(^{-1}\) appearing during conjugation that did not increase proportionally with the decrease in the polymer bound 61 at 1735 cm\(^{-1}\), i.e., since 61 can be present in both free (1712 cm\(^{-1}\)) and salt form (1684 cm\(^{-1}\)). This result indicated that any free amine reactant present in
the conjugation reaction solutions may form a salt with the NHS 61 being generated, which may interfere with further conjugation.

![Figure 4.10. Overlaid IR spectra showing the addition of different stoichiometries (0.25, 0.5, 0.75, 1, 2 and 3 mol. equiv.) of triethylamine (A) and 1-amino-2-propanol (B) to a DMSO solution of NHS 61 (red spectra).]

When 1-amino-2-propanol 10 was added to a solution of NHS 61 instead of triethylamine (Part B, Figure 4.10) a distinct difference in the IR spectrum was observed. Similar to observations on the addition of triethylamine to 61, a band was evident at ~1680 cm\(^{-1}\). However, on addition of increasing stoichiometries of 10, two bands were seen to form at 1671 cm\(^{-1}\) and 1649 cm\(^{-1}\). Although, possibly, this can be attributed to the presence of a primary amine salt (since the anti-symmetric and symmetric regions of -N'H\(_2\) deformation are 1625-1560 cm\(^{-1}\) and 1550-1500 cm\(^{-1}\) respectively (Lin Vein et al., 1991)) the intensity and wavenumber appear more characteristic of an amide-containing molecule derived from NHS 61. In addition to the formation of stable NHS salts 69 (Paquet & Bergeron, 1982), amines have been shown to react readily with 61 to form succinic acid derivatives (67 & 68 in Scheme 4.4, Ranadive & Samant, 1995).
Scheme 4.4. Possible reactions of primary and secondary amines with NHS 61 (adapted from Ranadive & Samant, 1995).

In the study by Ranadive and Samant the reaction of an equivalent amount of a primary amine with 61 formed monoamide of N-hydroxysuccinamic acid 68, while an excess of a primary amine formed diamide of succinic acid 67. These products 67 and 68 were formed readily at room temperature and in high yields (>75%). Ranadive and Samant also found that with secondary amines and with bulky t-butylamine only the corresponding salts (e.g., 69) were formed on mixing with solutions of 61, regenerating 61 on acidification. The potential reaction of primary amines with 61 has implications for drug conjugation to PMOSu 36 (as described by Scheme 3.1 in Chapter 3) due to the possibility that this side reaction may prevent quantitative reaction of the linker-drug molecule. To investigate this, the tertiary amine triethylamine (0.5 equiv.) was added to a DMSO-\(d_6\) solution of 61 (1 equiv.) and a \(^1\)H NMR spectrum obtained. The spectrum was consistent with the two starting reagents.
When this experiment was repeated with 1-amino-2-propanol 10 (1 equiv.) instead of triethylamine, the NMR spectrum showed multiple signals not consistent with the two reagents, thus indicating reaction(s) had occurred (for NMR signals see experimental section 4, Chapter 7). Analysis of the reaction solution of 61 with 10 by mass spectroscopy indicated the presence of a monoamide of N-hydroxysuccinamic acid, N-hydroxy-N’-(2-hydroxy-propyl)succinamide 70 (MS (EI): m/z 189 [M - H]).

\[
\begin{align*}
\text{H} & \quad \text{O} \\
\text{N} & \quad \text{O} \\
\text{C} & \quad \text{N} \\
\text{C} & \quad \text{O} \\
\text{C} & \quad \text{N} \\
\text{H} & \quad \text{O} \\
\end{align*}
\]

In a further reaction, triethylamine (0.5 equiv.) was first added to a DMSO solution of NHS 61 (1.0 equiv.) followed by 1-amino-2-propanol 10 (0.25 equiv.). IR analysis of the reaction solution suggested that the presence of triethylamine prevented the reaction of 10 with 61 since the spectrum was consistent with Figure 4.10 (part B). Analysis by \(^1\text{H} \text{NMR} \) was also consistent with the three reagents being present, again indicating that the reaction of 10 and 61 had been prevented. These observations suggest that a practical way to conjugate linker-drug molecules to PMOSu 36 would be in the presence of a base such as triethylamine.

While a less than expected decrease in the IR band at 1735 cm\(^{-1} \) on reaction between an amine and 36 may be explained by the reaction of the nucleophile with the NHS 61 being liberated, the reactions where greater than expected decreases are observed cannot be so easily interpreted. Rationally, this suggests that imide or hydrolysis is occurring, as described in sections 2.3 and 2.4. To probe this, the simple monofunctional amine, \( n \)-propylamine 64 (0.5 equiv.) was added to a DMSO solution of 36 and triethylamine (1.0 equiv.) under vigorous stirring (see experimental section 4.2, Chapter 7). After stirring the reaction solution for 50 min at room temperature a decrease of 49% in the IR band at 1735 cm\(^{-1} \) was observed compared to the starting solution. The formation of amide I and II bands at 1650 cm\(^{-1} \) and 1550 cm\(^{-1} \).
respectively was also observed, all suggesting the near quantitative conjugation of 64 (Figure 4.11, light blue spectrum).

![Figure 4.11. Overlaid IR spectra showing a solution of PMOSu 36 at different time points after the addition of n-propylamine 64 (0.5 equiv.).]

After stirring the reaction for a further 17 h the decrease at 1735 cm\(^{-1}\) became 66\% (purple spectrum) but with no further increase in the amide I and II bands. The reaction solution was then heated for 2.5 h at 50 °C and the decrease became 77\% (dark blue spectrum), again with no further increase in amide I or II bands. This indicated that the apparent additional reaction of the active ester groups that occurred was not due to the further conjugation of the amine 64. Accompanying these decreases at 1735 cm\(^{-1}\) was the appearance of a band at \(\sim1711\) cm\(^{-1}\). Previously this band has been attributed to free NHS 61 and/or the carbonyl groups of protonated acid moieties present on the polymer resulting from hydrolysis (section 2.3). However, these two possibilities are unlikely to be so prevalent due to the presence of an excess of triethylamine (leading to salt formation in both cases, the products of which exhibit bands at different wavenumbers). It is proposed therefore, that this band may in part be due to the presence of imide groups (anti-symmetric carbonyl stretch).
A product from this \textit{n-}propylamine reaction with PMOSu \textbf{36} was isolated by precipitation of the reaction solution into acetone:diethyl ether (\textasciitilde 20:80 v/v). Analysis by \textsuperscript{1}H NMR (Figure 4.12) showed a complex structure producing many signals, which would be expected due to the possible reactions and side reactions of conjugation, i.e., \textit{n-}propylamine conjugation, imide formation, hydrolysis and the presence of unreacted ester groups. However, there is a signal present at \(\delta\) 2.3 (labelled \textbf{x} in Figure 4.12) which is consistent with earlier observations for the methylene protons expected for imide formation (compare with Figure 4.8). This signal cannot be explained by any of the other reaction products outlined above: \textit{n-}propylamine conjugation (CONHCH\textsubscript{2} \(\delta\) 2.9; NHCH\textsubscript{2}CH\textsubscript{2} \(\delta\) 1.4), unreacted ester groups (\(CH\textsubscript{2}CH\textsubscript{2}\delta\) 2.8).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.12}
\caption{\textsuperscript{1}H NMR (in DMSO-\(d_6\)) of the solid product formed after allowing \textit{n-}propylamine \textbf{64} (0.50 equiv.) to react with PMOSu \textbf{36}.}
\end{figure}
2.6 Preparation of copolymers - the preparation of polymer-drug conjugate models

In the literature, p-nitroanilide has been used as a model drug for polymer conjugation because of the similar characteristics, such as size and hydrophobicity to a typical drug, and the usefulness of the chromophore in following enzymatic release studies (Duncan et al., 1984; Subr et al., 1986). Furthermore, in the pre-clinical development of HPMA copolymers glycyl-glycine (Gly-Gly) linkers were used as controls because they did not undergo enzymatic hydrolysis. Therefore, H-Gly-Gly-β-napthylamide (H-Gly-Gly-NA) was elected as a representative drug-linker model for polymer conjugation using PMOSu 36 (Scheme 4.5).

H-Gly-Gly-NA-HBr 72 (0.1 equiv.) was allowed to react with 36 in DMSO in the presence of excess triethylamine at 50 °C for 15 min (experimental section 4.1, Chapter 7). A 10 % decrease in the peak height at 1735 cm⁻¹ was observed (Figure 4.13 below) compared with starting solution, accompanied by the slight appearance of amide I and II bands at 1667 cm⁻¹ and 1550 cm⁻¹ respectively. The addition of a second amine, 1-amino-2-propanol 10 (10 equiv.) followed by heating at 50 °C for 30 min resulted in the total disappearance of the 1735 cm⁻¹ band and the appearance of more intense amide I and II bands at 1662 cm⁻¹ and 1550 cm⁻¹ respectively. Analysis by ¹H NMR indicated that the linker-drug model had successfully conjugated to the polymer (see experimental section 4.1, Chapter 7). The model HPMA copolymer 73 produced was the first example of a polymer-drug conjugate derived from 36. The reaction was also repeated with 0.25 and 0.50 equivalents of 72 with similar outcomes.
Scheme 4.5. Preparation of HPMA copolymer-Gly-Gly-β-napthylamide conjugate 73
- a polymer-drug conjugate model.

Figure 4.13. Overlaid IR spectra displaying PMOSu on addition of 0.1 equiv. of H-Gly-Gly-NA 72, then excess 1-amino-2-propanol 10.
Chapter 4: Conjugation Chemistry

The apparent quantitative conjugation represents an extremely efficient use of both oligopeptide linker and drug molecules. As an example of the type of molecules required to utilise this precursor 36 in the preparation of polymer-drug conjugates containing oligopeptide linkers, it was found that Gly-Phe-Leu-Gly (and a activated derivative for drug conjugation) could be readily prepared by solution phase and solid phase procedures. These procedures are described in Appendix VI.

Consistent with earlier studies into the problem of competitive side reactions, when the conjugation of 0.1 equiv. of H-Gly-Gly-NA was repeated but with extended heating (30 min to 1 h at 50 °C and standing at room temperature overnight) further reaction was evident. After three attempts, an average of 22% (± 3%) decrease was observed, essentially double the amount of added amine reactant and therefore suggesting imide formation. This result supports the argument that if primary amines are to be conjugated to PMOSu 36 in fractional stoichiometries then the reaction time must be minimised before the addition of the second amine so as to prevent/limit side reaction. Also, it is not clear from the experiments performed whether heating is necessary or whether this may favour side products such as stable six-membered imide rings.

2.7 Conjugation studies with other functionality

The formation of imide structures in the polymer backbone from secondary amine reactants is not possible. Consequently, the use of secondary amines may be a simple way to eliminate imide impurities in the final polymer-drug conjugate. In Chapter 2, the synthesis of secondary amine oxazolidines was described (e.g., 16 in Scheme 2.8, Chapter 2). It can be speculated that if these oxazolidines can be prepared with sufficient purity and then conjugated to 36 (although steric inhibition may be a problem, see Chapter 2), this would result in products that, after treatment with acid, would lead to degradation and the preparation of PHPMA 1 without imide impurity.
For the preparation of polymer-drug conjugates based on HPMA, the use of oxazolidines in this way is not feasible for the conjugation of the linker-drug molecule, although it is a plausible strategy for the solubilising pendent chain conjugation providing HPMA copolymers are required. To eliminate the possibility of imide formation for the conjugation of the linker-drug compound, it may be possible to use a linker that possesses secondary amine functionality for conjugation to prevent imide formation. The conjugation of a secondary amine with 36 was examined using benzylmethylamine 71 (experimental section 4, Chapter 7).

Using 0.5 equiv. of 71 in the presence of triethylamine (0.75 equiv.), the rate of conjugation was slow compared to the conjugation of a primary amine (i.e., 10) because after 2 h at 60 °C only 24% conjugation had occurred (measured using the decrease in peak height at 1735 cm$^{-1}$ and conjugation was confirmed by the presence of a band at 1640 cm$^{-1}$ attributable to the carbonyl stretch of a tertiary amide). Using a secondary amine throughout the conjugation, i.e., for linker-drug molecules and for solubilising amines (reactions I and II, Scheme 3.1, Chapter 3) may not be suitable because this may unfavourable affect the aqueous solubility of the resulting polymers. However, for the conjugation of the solubilising amine in the second step (reaction II, Scheme 3.1), it may be sufficient just to use a large excess of amine to minimise/prevent imide formation as described in section 2.1.
It was of interest to see if the synthesis of PMMA was possible from 36 because PMMA can be used to obtain the real MW of 36 using GPC, since PMMA standards are readily available. Strohriegl (1993) used sodium methoxide 76 to prepare PMMA (IR C=O, 1727 cm\(^{-1}\)) from a poly(methacryloyl chloride) homopolymer. In an analogous fashion it was found that, on allowing 36 to react with 76, the polymer hydrolysed to give PMAA (IR C=O, 1703 cm\(^{-1}\)) (experimental section 4, Chapter 7). It was also found that the aryl amine, aniline 78, would not react with 36 after heating at 50 °C for 2 h. This result indicates that aromatic amines present in drug molecules may not necessarily competitively conjugate to 36 under the typical conditions employed for this study. Other conditions that may be more favourable for aryl amine reaction with 36, such as the use of acylating agents or the use of aryl amines with activating substituents, were not attempted.

**Scheme 4.6.** Reaction of PMOSu 36 with sodium methoxide 76 and benzylamine 78.
3 Conclusions

The active ester polymer PMOSu 36 can be used as a precursor to prepare polymer drug conjugates. As for many polymer-analogous reactions, competitive side reactions can occur and this study has highlighted that competitive hydrolysis and imide formation can be a potential problem in the aminolysis of 36. However, the preliminary reactions described have shown that the desired reaction(s) can be maximised by optimising the reaction conditions. The aminolysis of 36 could be followed using ATR FT-IR spectroscopy. However, the concentrated reaction solutions needed for IR analysis of the reaction solutions may not be ideal because of an increased propensity for side reactions (i.e., imide and hydrolysis) to occur.

It was found that the best way to prepare methacrylamide homo- and copolymers from 36 was to use dilute solutions and excess amine reactant where appropriate (e.g., above 10 equiv.). In this way, side reactions are eliminated/minimised. For the preparation of copolymers (conjugates) by sequential amine addition, the presence of a base (e.g., triethylamine) in the reaction may be necessary to prevent irreversible reaction of the amine reactant with the NHS 61 liberated from the polymer. It was proposed that secondary amines could be used for the conjugation of linker-drug molecules to 36 since imide formation is not possible using secondary amines. The second step of adding an amine to confer aqueous solubility may best be accomplished by the reaction of a large excess of amine reactant. Using a dilute reaction solution in the presence of triethylamine it was possible to prepare HPMA copolymer-napthylamide conjugate models with tailored drug loading that were consistent with the proposed structure by NMR analysis.
Chapter 5

*Preparation of Carboxylate Copolymers for Cytokine and Chemokine Release Study*

1 Introduction

The aim of this study was to synthesise PMAA Na salt homo- and co-polymers with incremental changes in the anionic charge density (number of carboxylate repeat units) using the homopolymer precursor strategy developed in Chapters 3 and 4, and to use these polymers to investigate the effect of these polyanions on cytokine and chemokine release from peritoneal macrophages. The biological effect of polyanions is known to be very dependent on structural features such as MW, charge density and tacticity (Kaplan et al., 1985). In conjugating different stoichiometries of 1-amino-2-propanol 10 to PMOSu 36 (reaction I, Scheme 5.1) followed by hydrolysis of the remaining active repeat units (reaction II, Scheme 5.1) a family of MAA Na salt-co-HPMA polymers (based on structure 81) can be prepared with varying but defined anionic charge density. The HPMA co-repeat units produced through the conjugation of 10 serve as biologically inert pendant chains.

\[\text{Scheme 5.1. Preparation of MAA Na salt copolymers based on structure 81.}\]
1.1 Immunomodulatory effects of polymers

Polymers have a broad range of biological activity (see also section 7, Chapter 1) including immunomodulatory effects and the stimulation of macrophages (Kaplan et al., 1985). Macrophages play an important role in the immune system as antigen presenting cells. Macrophages are the first cells to recognise invading foreign bodies and produce a variety of cytokines and chemokines (Sorimachi et al., 1999). Cytokines are soluble regulatory proteins that are released by cells of the immune system (lymphocytes) and act as intercellular mediators in the generation of an immune response. Macrophages have been shown to be stimulated by polymers to produce cytokines, e.g., tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) which play an important role in immune response and in inflammatory reactions (Otterlei et al., 1991). Chemokines are a subset of the cytokines, with specific functions related to chemoattraction of immune cells (chemotaxis). There are two main families of chemokines classified on the position of conserved cysteine residues: Alpha chemokines contain an amino acid between two cysteine residues; beta chemokines do not contain an intervening amino acid. Certain β-chemokines (e.g., macrophage inflammatory protein-1-alpha (MIP-1α), macrophage inflammatory protein-1-beta (MIP-1β), and regulated-upon-activation normal T expressed and secreted (RANTES)) have been shown to affect the activity of HIV-1 (human immunodeficiency virus-1) since certain chemokine receptors (e.g., CCR-5, CXCR-4) are necessary for entry by HIV-1 into cells (Cocchi et al., 1995). Hence, the control of chemokine release could have potential for protecting macrophages from infection by HIV-1. In summary, the regulation of cytokine and chemokine response and the ability to alter an immune response in a particular and predetermined manner has great significance in the treatment of tumours, organ transplants, auto-immune and immune deficiency diseases (Baird & Kaplan, 1980).
2 Results and Discussion

2.1 Synthesis of carboxylate polymers from PMOSu 36

2.1.1 PMAA Na salt 62

![Scheme 5.2. Preparation of PMAA Na salt 62 from 36.](image)

The carboxylate homopolymer 62 was prepared by heating 36 in DMSO with aqueous NaOH (Scheme 5.2, experimental section 5.1, Chapter 7). After addition of the NaOH solution to the reaction at room temperature, some precipitation of the polymer was observed and the vessel immediately became warm. With stirring, a homogenous solution quickly formed, indicating that a reaction was occurring. The resulting solution was heated at 70 °C for 24 h. With carboxylic acids in the salt form, the C=O and C-O bonds are replaced by two equivalent, or nearly equivalent, C—O 'bond and a half' bonds (Lin-Vien et al., 1991). These CO$_2^-$ bonds interact out-of-phase and in-phase giving rise to two bands in the IR. The asymmetric CO$_2^-$ stretch band is usually observed between 1650-1540 cm$^{-1}$, while the symmetric band is usually seen between 1450-1360 cm$^{-1}$. After purification by dialysis, the white solid polymer obtained from lyophilisation was consistent with commercial 62 by FT-IR analysis (Figure 5.1). Also present in the IR spectrum, was a broad band attributable to OH stretching at about 3350 cm$^{-1}$, bands at 1680 cm$^{-1}$ and 1547 cm$^{-1}$ attributable to C=O stretching and asymmetric CO$_2$ stretching respectively, and a band at 1255 cm$^{-1}$ attributable to C-O stretching, all of which indicated the presence of both protonated
and salt forms of the acid repeat units. No evidence of succinimidyl imide carbonyl bands at 1735 cm$^{-1}$ were present, indicating the complete reaction of each active ester group of the precursor 36. For the sample of 36 before hydrolysis $M_n = 24,800$ g·mol$^{-1}$ and $M_w/M_n = 1.20$ (PMMA standards, DMF with 0.1% LiCl eluent). After hydrolysis, for the prepared 62, $M_n = 22,000$ g·mol$^{-1}$ and $M_w/M_n = 1.28$ (PMAA Na salt standards, PBS eluent).

![Figure 5.1. FT-IR comparison of (A) commercial PMAA Na salt 62 and (B) 62 prepared by hydrolysis of PMOSu 36.](image)

**2.1.2 P(MAA Na salt-co-HPMA)'s**

Three separate reactions were initially performed using 0.25, 0.50 and 0.75 equivalents of 1-amino-2-propanol 10 (experimental section 5.1, Chapter 7). These reactions were performed by adding 10 neat to a stirred solution of 36 (0.3 g) in DMSO (1 ml). After heating the reaction mixtures for 2 h at 50 °C, the anti-symmetric imide carbonyl stretch at 1735 cm$^{-1}$ in the IR exhibited respective decreases of 23, 49 and 73% for the 0.25, 0.50 and 0.75 equivalents of 10 added compared to their
respective starting solutions (see Figure 4.9, Table 4.2 and text in Chapter 4 for FT-IR spectra and further discussion) suggesting that near-quantitative conjugation of amine had occurred. The reaction solutions were then mixed with aqueous NaOH at room temperature for 5 h to hydrolyse the remaining active ester groups. The polymer products were purified by dialysis of diluted reaction solutions against water and isolated by lyophilisation. The prepared carboxylate polymers are henceforth referred to as 82, 83 and 84 as detailed in Table 5.1.

<table>
<thead>
<tr>
<th>equiv. of 10 added to reaction solution</th>
<th>product number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>82</td>
</tr>
<tr>
<td>0.50</td>
<td>83</td>
</tr>
<tr>
<td>0.75</td>
<td>84</td>
</tr>
</tbody>
</table>

**Table 5.1.** Labelling of the prepared copolymers.

From the conjugation reactions performed in Chapter 4, it was observed that extended heating of the reaction solution can lead to an increased propensity for imide formation and hydrolysis to occur. Competitive hydrolysis would not be a concern in these reactions since hydrolysis of the reactive groups is required to prepare the desired copolymers. FT-IR analysis of the solid compounds obtained after lyophilisation revealed that all the active ester groups had undergone reaction and confirmed the presence of carboxylate groups (asymmetric CO$_2^-$ stretch 1568 cm$^{-1}$) and the presence of amide groups (C=O 1647 cm$^{-1}$). The FT-IR spectra also indicated the presence of imide group impurity in the backbone due to the presence of bands at about 1765 cm$^{-1}$ and 1694 cm$^{-1}$ in each of the samples (see Figure 5.2 for example spectrum of 84). The copolymer products 82, 83 and 84 obtained in these reactions were all non-toxic against peritoneal macrophages up to the highest concentration analysed (2000 μg·ml$^{-1}$, experimental section 5.2, Chapter 7) and were subsequently used in the biological study described below in section 2.2. The characterisation of
these copolymers is further discussed after the presentation of the biological data where it is relevant to the biological results described.

![FT-IR spectrum of solid product 84](image)

**Figure 5.2.** FT-IR of solid product 84 obtained from reaction of 36 with 0.75 equiv. of 10 followed by hydrolysis with aqueous NaOH.

2.2 *MIP-1β and TNF-α release by peritoneal cells cultured with the prepared carboxylate (co)polymers 62, 82, 83 and 84*

The following biological experiments were performed by Dr. Elisabetta Gianasi. A limitation on the interpretation of biological data collected using primary cells would be the presence of any endotoxin impurities (a heat-resistant toxin, specifically a lipopolysaccharide, found in the cell walls of certain pathogenic bacteria) in the prepared polymers due to the ability of endotoxin to release chemokines/cytokines. Therefore, the polymers were purified to remove endotoxin and subsequently shown to contain less than 1 EU·ml⁻¹. This value is acceptable for clinical use.
Peritoneal cells were obtained from patients on CAPD (continuous ambulatory peritoneal dialysis) and cultured with media and with each of the polymers. The culture supernatants were harvested after 36 h of incubation, and MIP-1β and TNF-α levels measured by EIA (ELISA [enzyme-linked immunosorbent assay] inhibition assay). The results show that MIP-1β was released during incubation of the macrophages with the prepared polymers (Figure 5.3) and appeared to indicate that the level of MIP-1β release was dependent on the level of carboxylate groups present in the polymers. The higher the level of anionic charge the greater the release of MIP-1β. Compared to the copolymers 82, 83 and 84 the release of MIP-1β was significantly higher (P < 0.001) in the presence of PMAA Na salt 62, where all the repeat units in the polymer possessed an anionic charge. However, as shown in Figure 5.4, the release of TNF-α was only partially influenced by the presence of carboxylate groups. The release of MIP-1β from peritoneal macrophages cultured with 62 (Figure 5.5) was shown to be concentration dependent. No release was seen at 10 and 100 µg·ml⁻¹, while the release was significantly increased (P < 0.01) at 500 µg·ml⁻¹. The release of MIP-1β at 500 µg·ml⁻¹ (2484 ± 285 pg·ml⁻¹) was similar to the value obtained at 2000 µg·ml⁻¹ (2376 ± 160 pg·ml⁻¹). Therefore 500 µg·ml⁻¹ was identified as a lower concentration for activity.

The apparent trend relating MIP-1β release with anionic charge density led to the preparation of an additional copolymer 85, which was synthesised using 0.10 equiv. of 1-amino-2-propanol 10. This copolymer 85 was prepared in a different manner to the previous copolymers (82, 83, and 84) in that the conjugation was performed in the presence of triethylamine to prevent side reaction of the primary amine with the NHS liberated (see Chapter 4). Also, a less concentrated reaction solution was used to prepare 85 than previously used to prepare 82 to 84 (0.35 g PMOSu 36 dissolved in 3 ml DMSO compared with 0.3 g 36 dissolved in 1 ml DMSO) and the time between addition of 10 and hydrolysis was reduced to 15 min with no heating (experimental section 5.1, Chapter 7). These conditions were found to lead to a reduced propensity for imide formation in Chapter 4.
Figure 5.3. Release of MIP-1β from peritoneal macrophages incubated for 36 h with precursor derived 62, 82, 83, and 84 at 2000 μg·ml⁻¹ (n = 6, P < 0.0001).

Figure 5.4. Release of TNF-α from peritoneal macrophages incubated for 36 h with precursor derived 62, 82, 83, and 84 at 2000 μg·ml⁻¹ (n = 3, n = 8, P < 0.0001).
**Figure 5.5.** Release of MIP-1β from peritoneal macrophages incubated for 36 h with precursor derived 62 \((n = 6, P < 0.0001)\).

**Figure 5.6.** Release of MIP-1β from peritoneal macrophages incubated for 36 h with precursor derived 62, 84 and 85 at 500 μg·ml⁻¹ \((n = 12, P < 0.0001)\).
When peritoneal macrophages were incubated with 85, the release of MIP-1β was higher than in the case of 82 prepared using 0.25 equiv. of 10 (Figure 5.6). In other words, copolymer 85 which was synthesised to possess anionic charge at a level in-between 82 and 62, did indeed exhibit a release of MIP-1β greater than 82 but lower than 62. However, due to 85 possessing a four-fold higher level of endotoxin than acceptable, the release data for this copolymer may have been compromised. Additionally, due to the method of preparation of 85 (and 82 to 84) the sample will evidently contain a distribution of copolymers with varying levels of the inert co-pendent chain. This is because it is impossible to guarantee that each polymer chain of the precursor sample 36 will react with an equal number of molecules of 10. Depending on the broadness of this distribution some polymer chains in the sample may contain very few of the HPMA repeat units and therefore may structurally resemble 62 more than the desired copolymer. This is a limitation of polymer analogous reactions.

Significantly, the level of MIP-1β release observed for 62 was not seen with commercial samples of 62 of a range of MW’s ($M_n = 1.3, 22.1$ and 129 kg·mol$^{-1}$), tested as controls, which exhibited MIP-1β release comparable with copolymers 82 to 85 (Gianasi et al., 2001). This experiment was performed at the same time as 62 from 36 and suggests that are structural differences between these two samples of 62, which are responsible for the observed differences in release. Since the $M_n$ of the precursor-prepared 62 was comparable to one of the commercial samples of 62 (i.e., $M_n \sim 22,000$ g·mol$^{-1}$) an effect due to the influence of MW in this study can be eliminated. A comparison of the NMR spectra of these two polymers (i.e., 36 prepared and commercial 62) indicated that they were structurally very alike and may share similar tacticity because of the resemblance in the methyl signals. The $^1$H NMR spectrum for 62 prepared from PMOSu 36 is shown in Figure 5.7 and commercial 62 is shown in Figure 5.8 (for $^{13}$C NMR signals see experimental section 5.1 in Chapter 7). The assignment of the $^{13}$C NMR peak to the polymer tacticity is a difficult task (Plate et al., 1995).
Figure 5.7. $^1$H NMR spectrum of PMAA Na salt 62 derived from PMOSu 36 (X = signals not consistent with proposed structure).

Figure 5.8. $^1$H NMR spectrum of a commercial sample of PMAA Na salt 62.
It is also noted that there are several weak signals in the sample of 62 derived from 36 that cannot be explained by the proposed structure, reagents or side products. These signals are marked x in Figure 5.7. Since the IR comparison of commercial and precursor-prepared 62 (Figure 5.2 in section 2.1.1) was very similar also, it is not clear why such a difference in MIP-1β release was observed.

For compounds 82 to 84, NMR analysis was not consistent with the pure HPMA-co-MAA Na salt polymers desired, since additional signals were present at δ 2.5 (in D₂O, see Figure 5.9 label x for example). In Chapter 4, it was proposed that imide formation in the backbone of the polymer may result in an additional signal in ¹H NMR analysis at δ 2.3 in DMSO-d₆.

Figure 5.9. ¹H NMR spectrum of copolymer 84 prepared from PMOSu 36 (in D₂O) (x = possible methylene protons from imide ring – see text).

If this work was to be developed further, then it would be necessary to investigate the MIP-1β release for samples of 62 prepared from 36 of different MW's.
The signals observed in all copolymers 82 and 84 at around δ 2.3 were not as sharp as observed for the signals seen in the imide studies of Chapter 4 (e.g., see Figure 4.8). Following quantitative reaction of 0.5 equiv. of a primary amine with an active ester homopolymer, in the extreme case where there is an alternating amide-active ester repeat unit, there is the potential for all of the remaining 0.5 equiv. of active ester groups to be involved in imide formation. While such an alternating repeat pattern is unlikely, it illustrates that imide formation can be a serious problem with respect to the preparation of copolymers from an active ester homopolymer if the reaction conditions are not optimised.

If the broad signals at δ 2.3 are assumed to be part of the backbone methylene protons of the required HPMA-co-MAA Na salt copolymers, and not due to imide formation, then using NMR to calculate the percentage of the two repeat units in 82 to 85 revealed that the percentage of HPMA repeat units was actually significantly less than expected (Table 5.1). This is despite IR evidence of the reaction solutions suggesting that the conjugation of 1-amino-2-propanol 10 was very high (Figure 4.9, Chapter 4). In these reactions, no base was added to prevent side reaction of the amine with the NHS generated (as described in section 2.5.1, Chapter 4) which could explain a lower level of conjugation than expected.

<table>
<thead>
<tr>
<th>product</th>
<th>equivs. of 1-amino-2-propanol added to reaction solution</th>
<th>% HPMA repeat units in copolymer by $^1$H NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
<td>0.75</td>
<td>29</td>
</tr>
<tr>
<td>83</td>
<td>0.50</td>
<td>23</td>
</tr>
<tr>
<td>82</td>
<td>0.25</td>
<td>16</td>
</tr>
<tr>
<td>85</td>
<td>0.10</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 5.2. % HPMA repeat units in copolymers 82, 83, 84 and 85 by $^1$H NMR.
It is also possible that the addition of aqueous NaOH to the reaction mixture after the conjugation of 10 caused not only hydrolysis of the active ester groups present, but may have also caused some of the amide bonds of the HPMA repeat units to be hydrolysed. To probe this, conventional free radical prepared PHPMA 1 was mixed with aqueous NaOH under the same conditions used for the preparation of the compounds for 24 h. No hydrolysis was observed by FT-IR or $^1$H NMR analysis. However, for methacrylate-type polymers, hydrolysis of ester groups in a copolymer can proceed not only due to the action of external OH ions but also due to the action of adjacent ionised carboxyl groups that attack the carbonyl group of the ester (Scheme 5.3; Zimmering et al., 1957; Bender & Neveu, 1958; Plate et al., 1995).

Scheme 5.3. A mechanism of hydrolysis for methacrylate polymers containing carboxylate groups repeat units.

It can be speculated therefore that the rate of hydrolysis for the HPMA repeat units in copolymers 82 to 84 may be significantly faster than for PHPMA 1, so that hydrolysis does occur on treatment with NaOH during the time-scale of the reaction. For compound 85, the FT-IR spectra of the sample isolated after dialysis and lyophilisation did not show a band at around 1770 cm$^{-1}$. This suggests no imide impurity, unlike products 82 to 84. However, this may be misleading due to the low
concentration of amine 10 added and the weak intensity of the proposed symmetric stretch imide band. No FT-IR data was obtained during the reaction because of the dilute nature of the reaction solutions. In addition to NMR analysis, other ways to ascertain the quantity of carboxylate groups in the copolymers 82 to 85 include acid-base titration and combustion analysis (Marconi et al., 2001). Meanwhile, despite the fact that the copolymers 82 to 84 appear not to be the pure derivatised products desired due to their method of preparation, the release study results are of significant interest.

Since the release of the chemokine MIP-1β was increased with respect to the control (media) this suggests a stimulation of the immune system due to the biological functions of this chemokine. Chemokines act locally and are not toxic to the body. Cytokines however are systemic and can exhibit activity far away from the site where they are produced. Cytokines are very toxic to the body and a high release can lead to death. Therefore, the release of cytokines should be maintained at physiological values or comparable to the media (as in the case of TNF-α). These results are biologically significant because PMAA Na salt 62 prepared from 36 may have potential to act as an adjuvant (any substance which enhances the immune-stimulating properties of an antigen or the pharmacological effect of a drug) for the immune system promoting the release of chemokines but not affecting (or only slightly) the release of cytokines. The results presented form part of a larger study of cytokine and chemokine release by carboxylate-containing polymers in collaboration with Dr. Sunil Shaunak of Imperial College, University of London (Gianasi et al., 2001).
3 Conclusions

PMAA Na salt 62 and PMAA Na salt copolymers 82 to 85 were prepared from PMOSu 36. These polymers, which all share the same MW characteristics of the precursor and therefore would be extremely difficult to prepare by any other method, caused different levels of cytokine release when incubated with peritoneal macrophages. The results indicate that the release was dependent on the level of anionic charge present in the polymers, with 62 causing a significantly higher release than the compounds with fewer carboxylate groups since MW variations between the prepared polymers can be eliminated. This result was not observed with a commercial sample of 62, indicating that there were structural differences between the commercial and precursor-prepared 62. However, any structural differences, e.g., tacticity, were not obvious from FT-IR and NMR data. The level of MIP-1β release observed for these prepared polymers is significant for the design of new adjuvants.

Similar to the conclusions expressed in Chapter 4, FT-IR and NMR analysis of the prepared polymers, 82 to 85, show that imide formation can be a problem when conjugating less than equivalent stoichiometries of amine to PMOSu 36. However, imide formation can be minimised using dilute reaction solutions and short reaction times before quenching the remaining active ester groups.
Chapter 6

General Discussion

The aim of this thesis was to develop polymeric precursors that assist the development of polymer-drug conjugates through better structural definition and versatile preparation. To this end, a homopolymer precursor, PMOSu 36, was developed that required (1) the implementation of ATRP procedures to polymerise the active ester monomer, MOSu 35, with narrow MWD and (2) an examination of the conjugation chemistry of 36 to ensure the suitability of this homopolymer as a precursor to methacrylamide copolymers. While the methodology of using 36 as a precursor to polymer-drug conjugates was shown to be feasible by the preparation of a narrow MWD HPMA copolymer-napthylamide model conjugate (section 2.6 in Chapter 4), there were several caveats relating to both the polymerisation and conjugation chemistry.

1) Polymerisation Considerations

In ATRP when the conditions are optimised the polymerisation results in polymers with MW's that are predictable and with MWD's that are narrow. With regard to the polymerisation chemistry for 36, the MWD's were narrow and the MW could be varied by the concentration of initiator, but the MW's were not predictable from the monomer to initiator ratio (Chapter 4). This may not necessarily be a concern since the aim of this thesis was to prepare polymers to be used as precursors for polymer-drug conjugate preparation; therefore the MW's obtained (< 50,000 g·mol⁻¹) are suitable for this purpose. Furthermore, the attempted kinetic experiments using five separate reactions (section 2.2.2, Chapter 3) suggested that reactions performed under identical conditions may lead to products with broadly the same MW
characteristics (Table 3.5 entries 3, 4 & 5, Chapter 3). Hence, from this point of view, the MW’s may become reasonably predictable providing identical reaction conditions between batches are used.

One of the limitations of using 35 as a monomer is the limited number of solvents that the resulting polymer 36 is soluble in, which can hinder the polymerisation and conjugation chemistry (see section 2 Chapter 3 and section 1, Chapter 4). The preliminary ATRP reactions of 35 which were performed in acetone and THF (Section 2.1, Chapter 3), produced narrow MWD (typically the polydispersities, $M_n/M_w = 1.1$) after the polymer precipitated. The homogeneous polymerisations conducted in DMSO were often characterised by a slightly broader MWD of the resulting polymers (i.e., $M_n/M_w$ sometimes above 1.2). This broadening is presumably due to the higher conversion, increased viscosity and a reduction in the monomer concentration, leading to termination reactions becoming more relevant. Since $M_n$ values were generally lower in the acetone and THF reactions, ranging from 1,800 to 20,000 g·mol$^{-1}$, these precipitation polymerisations are a useful way of preparing 36 with low $M_n$ and narrow MWD. It can be envisaged that such low MW polymer could be employed in polymer-protein conjugation (compare with the low MW SMA copolymer for example, used to prepare SMANCS in section 5.4, Chapter 1).

![Statistical copolymer](image1.png) ![Block copolymer](image2.png) ![Gradient copolymer](image3.png)

**Figure 6.1.** Pictorial representation of the composition of statistical, block and gradient copolymers (black and white circles represent two different monomers).
One way to overcome the poor solubility may be to attempt to prepare active ester-containing copolymers (Figure 6.1) as opposed to an active ester homopolymer, so as to confer better solubility in other solvents through the choice of the comonomer. Another advantage of copolymers, as mentioned in section 2.4 in Chapter 3, is the potential to define the area of drug location. The effect of drug placement along the polymer molecule on the biological performance of a polymer-drug conjugate is not well understood. Drug placement is very likely to affect the solution properties such as hydrodynamic volume and therefore affect the pharmacokinetic profile of the conjugate. There are also issues with regard to the accessibility of the linker to the active sites of enzymes where peptidic linkers are used. Therefore the preparation of block copolymers and also perhaps gradient copolymers, which take advantage of the preferential polymerisation of one monomer in a two monomer system resulting in a composition gradient, would be of particular interest. Gradient copolymers have been reported using ATRP by Matyjaszewski and co-workers (Matyjaszewski et al., 2000). Such copolymers help to define the conjugate structure at a higher level than is possible for a homopolymer precursor.

It may be the case that MOSu 35 is not the optimal active ester monomer to use in terms of polymerisation and conjugation chemistry. Although halogen substituted active aryl ester polymers (e.g., derived from monomer 86) may be soluble in a greater range of organic solvents than 36 (Arshady, 1994), the corresponding monomers may presumably be unsuitable for ATRP because of the possibility that the halogen atoms may atom transfer, creating additional initiation centres.
Many other active ester monomers, e.g., 87 (Bodanszky, 1993) may be unsuitable for controlled ATRP because they may competitively chelate the transition metal species due to the presence of nitrogen atoms (although the polymerisation of nitrogen containing MOSu 35 still proceeded).

Also, ATRP is only one of many CRP techniques available that can exhibit control (see section 1, Chapters 2 & 3). It can be speculated therefore, that ATRP may not be the only (or ideal) way of preparing active ester polymers such as 36. Colleagues in the Biomedical Polymers Group have attempted using other CRP methods to polymerise 35. These studies have indicated that SFRP of 35 using the Vladimir nitroxide initiator (Benoit, 1999) was not controlled (Gac-Breton, 2000). Along with the data obtained in the polymerisation of 35 by ATRP procedures in this study, this combined data suggests that the controlled polymerisation of methacryloyl active ester monomers might only be achieved under very specific, optimised conditions. Importantly, the polymer PMOSu 36 could be isolated from the copper species present in the reaction mixture by the simple process of precipitation into acetone and washing with fresh acetone to give a white polymer with very low residual copper content (section 2.4, Chapter 3). This may not be the case for other monomers/polymers.

(2) Conjugation Considerations

The conjugation reactions performed were designed to increase understanding of the problems that are likely to faced in using PMOSu 36 as a polymer-drug conjugate precursor and therefore the experimental conditions for these reactions may not be fully optimised. However, these reactions highlighted many experimental issues such as methods of characterisation of side products. In order to ensure that the imide carbonyl bands of 36 could still be seen in the FT-IR analysis of conjugation reaction solutions, those solutions had to be sufficiently concentrated (e.g., 100 mg 36 in 0.4 ml DMSO) but concentrated reaction solutions may lead to increased propensity
for side reaction, i.e., imide formation and hydrolysis (sections 2.3 & 2.4 respectively in Chapter 4). For the GPC analysis of 36, the addition of LiCl (0.1%) to the DMF eluent was needed to prevent aggregation of the polymer. It is possible that if aggregation also occurs in the conjugation reactions performed in DMF (and also DMSO) then the conjugation chemistry may be affected, i.e., that the accessibility of the reactive groups of the polymer to the amine reactant may not be uniform, with some groups being less accessible than others (e.g., inside the aggregate). No conjugation studies were performed in the presence of LiCl (or other salt) to investigate the possible effects of aggregation. For the first amine reactant, this heterogeneity in accessibility may have a profound effect on the distribution of functional groups along the polymer backbone in the preparation of conjugates compared to conjugation reactions where aggregation does not occur. A lack of accessibility of amine reactant may also lead to increased side reactions such as imide formation and competitive hydrolysis.

With regard to side reactions, it is not known whether a trace level of carboxylate impurity in a polymer drug conjugate would elicit a biological effect. Polyanions are known to have a broad range of biological activity (Chapter 6), such as macrophage activation, and this activity is widely believed to be dependent on factors such as MW and charge density since moderately charged polysaccharides found on cell surfaces do not elicit a macrophage response (Davison et al., 1990). This suggests that conjugates containing very few carboxylate groups may not elicit any biological response due to the presence of anionic charge because of their very low charge density. Furthermore, HPMA-based conjugates prepared by a typical copolymer precursor (Scheme 1.1) can also suffer with carboxylate impurities due to competitive hydrolysis of active groups as described in Chapter 1, yet these conjugates which have entered clinical evaluation do not appear to cause biological responses due to this type of impurity. Imide formation in the backbone is also unlikely to elicit a biological effect, but does cause heterogeneity between separate polymer-drug conjugate
molecules. Other researchers have also found side reactions occurring using reactive polymers. Strohriegl (1993) found at least 95% of the desired substitution for a series of polymethacrylates derived from broad MWD poly(methacryloyl chloride), where the reactions were carried out in anhydrous THF. Therefore, the complete 100% transformation of a reactive polymer without side reaction may be a limitation of a precursor strategy no matter what monomer/polymer is used.

An important feature of PMOSu 36 is that it can be easily hydrolysed to PMAA Na salt 62 using NaOH (Chapter 5). Hence, a better determination of the real molecular weight of the homopolymer by GPC using PMAA Na standards can be achieved and this provides a means to determine the degree of polymerisation of the precursor and any therapeutic polymer (e.g., polymer-drug conjugate) derived from it. This is essential for the assessment of structure-biological activity relationships.

Increasingly, the biological rationale for using polymers in medicine is becoming better understood and creating demand for more structurally optimised physiologically soluble polymers. In the context of improved synthetic methods for the preparation of polymethacrylamide-drug conjugates, a narrow MWD precursor 36 has been developed that can address these demands and enable the preparation of biologically relevant polymers possessing incremental changes in structure and narrow MWD. The speed of preparation, efficient use of reagents, and the ability to alter (structure and concentration) the bioactive molecule, linker and solubilising pendent chain represents a significant advantage and improvement to conjugate preparation compared to a HPMA copolymer strategy as depicted in Scheme 1.1 (Chapter 1). These factors also open up the possibility of a combinatorial approach to conjugate preparation, i.e, parallel synthesis of libraries of structurally related polymers for structure-property correlation (e.g., one drug loading at one DP with different solubilising pendent chains). Furthermore, analytical strategies to determine structural impurities due to unwanted side reactions, which is essential for the characterisation of any medicine, have been established (Chapter 4). Therefore, a precursor has been developed that meets the initial aim of this thesis.
Chapter 7

Materials and Methods

1 Materials and General Methods

All chemicals were reagent grade unless stated otherwise and were purchased from either the Aldrich, Fluka, Lancaster, Avocado, Acros or Sigma chemical companies. CuBr (Aldrich, 98%) was further purified by mixing with water:acetic acid (50:50 v/v) for 24 h followed by washing with methanol and drying in vacuo. Anhydrous solvents were purchased in anhydrous form except for THF, which was freshly distilled from sodium/benzophenone, and DCM, which was freshly distilled from calcium hydride. Where water is used this means de-ionised water. $^1$H and $^{13}$C NMR spectra were recorded on Bruker AM 250 FT-NMR spectrometer running at 250 MHz and 62.5 MHz respectively. All NMR experiments were performed by Dr M. Zloh and Mr W. Baldeo of the NMR service at the School of Pharmacy (SOP), University of London. Chemical shifts are reported relative to TMS standard. Multiplicities are indicated as follows: s (singlet), d (doublet), t (triplet), dd (doublet of doublets), q (quartet), m (multiplet), td (triplet of doublets), qn (quintet), tt (triplet of triplets) and br (broad signal). The chemical shifts of diastereotopic protons are listed together. AAS was performed with the assistance of Mr D. Marley using Perkin Elmer A Analyst 100 equipment and CuSO$_4$.5H$_2$O standards. Elemental analysis was performed by Dr L. Randall of the microanalysis service at SOP. Mass spectroscopy was performed by Mr M. Domin of the mass spectroscopy service at SOP. Thin layer chromatography was performed on precoated plates (Merck TLC aluminium sheets, silica 60 F$_{254}$). The spots were visualised using phosphomolybdic acid, iodine or UV light. Melting points were determined using Stuart Scientific SMP1 equipment and are...
uncorrected. Chromatography refers to the procedure developed by Clark Still (Clark Still et al., 1978) and was carried out using silica gel (Merck Kieselgel 60, mesh size 0.040-0.063 mm). The Gel Permeation Chromatography (GPC) system comprised Waters Styragel HR4 and HR3 (7.3 x 300 mm) columns coupled to a Gibson 133 refractive index detector. DMF with 0.1% LiCl was used as the eluent with a flow rate of 0.5 ml-min\(^{-1}\) at 50 °C. For water-soluble polymers, Waters Ultrahydrogel 250 and 1000 (7.8 x 300 mm) columns coupled to a Gibson 133 refractive index detector and phosphate buffered saline (PBS, Oxion UK) eluent were used at 37 °C with a flow rate of 1 ml-min\(^{-1}\). For the GPC measurement of PMAA Na salt (section 5) the PBS eluent was adjusted to pH 8.5 using 0.1 M aqueous NaOH. Both GPC systems were operated with Polymer Laboratories software. GPC standards were purchased from Polymer Standards Service, Mainz, Germany. ATR FT-IR spectroscopy was performed using a Nicolet Avatar 360 spectrometer fitted with a germanium crystal and an Avatar OMNI-sampler smart accessory and operated with OMNIC v. 5.0 software. Spectra were obtained using 32 scans at a resolution of 4 cm\(^{-1}\) in absorbance mode.

2 Work Described in Chapter 2

2.1 Monomer synthesis

Synthesis of N-(2-hydroxypropyl)methacrylamide (HPMA 2): To a 100 ml three-necked flask fitted with two dropping funnels was added 1-amino-2-propanol (10.0 g, 0.13 mol), 2 M aqueous NaOH (66 ml) and a magnetic stir bar. The resulting solution was cooled to less than 5 °C and the two dropping funnels charged separately with methacryloyl chloride (13.9 g, 0.13 mol) and 2 M aqueous NaOH (66 ml). The contents of the funnels were then simultaneously added dropwise to the 1-amino-2-
propanol solution under stirring while maintaining the temperature below 5 °C. After complete addition the product was extracted from the aqueous phase using EtOAc (3 x 150 ml). The organic phases were combined and washed with 0.1 M NaHCO₃ (2 x 100 ml) and then with water (100 ml) followed by drying with MgSO₄. The mixture was filtered and the solvent removed from the filtrate using a rotary evaporator to leave a crude white product, which was recrystallised from acetone to give 2 as white crystals (13.0 g, 68% yield): m.p. 66-67 °C; ¹H NMR (DMSO-d₆) δ 1.00 (d, 3H, CH₃CH), 1.84 (s, 3H, CH₃C(CH₂)CO), 3.05-3.02 (m, 2H, CH₂), 3.69-3.66 (m, 1H, CH), 4.65 (s, 1H, OH), 5.30 (s, 1H, CH₂ vinylic), 5.64 (s, 1H, CH₂ vinylic), 7.76 (br s, 1H, NH); ¹³C (DMSO-d₆) 18.6, 21.1, 46.8, 65.1, 118.7, 140.3, 187.8; analysis calculated for C₇H₁₃NO₂ (found): C, 58.72 (58.90); H, 9.15 (9.24); N, 9.78 (9.72).

2.2.1 Monomer protection chemistry

Di-tert-butylsilane protected HPMA 14: To a 10 ml round-bottom flask charged with HPMA (0.5 g, 3.5 mmol), 1-hydroxybenzotriazole (0.05 g, 0.35 mmol), acetonitrile (10 ml) and a magnetic stir bar was added di-tert-butyl-dichlorosilane (0.82 g, 3.85 mmol) and triethylamine (1.77 g, 17.5 mmol) and the resulting solution was stirred at 60 °C for 30 min. Chloroform (200 ml) was then added to the reaction mixture in a separating funnel and the resulting solution washed with 0.1 M aqueous NaHCO₃ (3 x 75 ml) and then once with water (75 ml). The organic phase was then dried with MgSO₄, filtered and the solvent removed using a rotary evaporator to give a yellow oil product. The mass of product was not recorded due to the very low yield. ¹H NMR analysis of the oil product was consistent with HPMA 2 and not the desired compound 14 (see synthesis of HPMA 2 for NMR signals).
Chapter 7: Experimental

Synthesis of N,O-isopropylidene-N-(2-hydroxypropyl)methacrylamide 18: To a stirred mixture of potassium carbonate (21 g, 0.16 mol) and anhydrous DCM (150 ml) in a 250 ml round-bottom flask was added 1-amino-2-propanol (10 g, 0.13 mol) and acetone (7.5 g, 0.13 mol). After 16 h of stirring the mixture was filtered and the solvent removed from the filtrate on a rotary evaporator to leave a crude colourless liquid product. NMR analysis of this crude product showed 2 compounds, 2,2,5-trimethyl-oxazoline 16 and the Schiff base 17 in the ratio 2:1 respectively. NMR signals for 16 were: \( ^1H \) NMR (CDCl\(_3\)) \( 1.16 (d, 3H, CH(CH_3)) \), \( 1.30 (s, 3H, C(CH_3)_2) \), \( 1.33 (s, 3H, CH(CH_3)) \), \( 2.65 (q, 1H, O/CH) \), \( 3.22 (q, 1H, CH_2) \), \( 3.97 (qn, 1H, CH) \). The crude liquid product was then allowed to react with methacryloyl chloride (13.9 g, 0.13 mol) under Schotten-Baumann conditions assuming 100% yield of 16 (see synthesis of HPMA 2). This afforded a white crystalline product 18 that was purified by vacuum distillation, collecting the fraction at 65 °C (~ 2 mm-Hg) (15.0 g, 61% yield): m.p. < 30 °C; \( ^1H \) NMR (CDCl\(_3\)) \( \delta 1.31 (d, 3H, CH(CH_3)) \), \( 1.62 (s, 3H, C(CH_3)_2) \), \( 1.66 (s, 3H, C(CH_3)_2) \), \( 1.95 (s, 3H, CH(CH_3)C=CH_2) \), \( 3.16 (t, 1H, CH_2) \), \( 3.72 (dd, 1H, CH) \), \( 4.19 (m, 1H, CH) \), \( 5.09 (s, 1H, CH_2\text{vinylic}) \), \( 5.19 (s, 1H, CH_2\text{vinylic}) \); \( ^13C \) NMR (CDCl\(_3\)) \( \delta 14.8, 16.6, 21.0, 23.0, 51.8, 67.1, 91.9, 112.1, 139.3, 165.5 \); analysis calculated for C\(_{16}H_{17}NO_2\) (found): C, 65.53 (65.30); H, 9.37 (9.60); N, 7.64 (7.44); MS (FAB) m/z 184 ([M + 1]+).

Synthesis of N,O-isopropylidene-N-(2-hydroxypropyl)acrylamide 28: In an analogous manner to the synthesis of monomer 18 above, acryloyl chloride (12.1 g, 0.13 mol) was allowed to react with 2,2,5-trimethyl-oxazoline 16 (assumed 0.13 mol as for the synthesis of 16), which afforded a white crystalline product 28 that was purified by vacuum distillation, collecting the fraction at 60 °C (~ 2 mm-Hg) (12.6 g, 55% yield): m.p. < 30 °C; \( ^1H \) NMR (CDCl\(_3\)) \( \delta 1.36 (d, 3H, CH(CH_3)) \), \( 1.62 (s, 3H, C(CH_3)_2) \), \( 1.69 (s, 3H, C(CH_3)_2) \), \( 3.21 (t, 1H, NCH_2) \), \( 3.81 (t, 1H, NCH_2) \), \( 4.25-4.32 (m, 1H, CHCH_3) \),
5.67-5.69 (m, 1H, CH₂ vinylic), 6.37 (s, 1H, CHC=O), 6.37-6.39 (m, 1H, CH₂ vinylic); analysis calculated for C₉H₁₅NO₂ (found): C, 63.88 (63.89); H, 8.93 (9.11); N, 8.28 (8.36); MS (FAB) m/z 170 ([M + 1]⁺).

**Synthesis of N,O-ethylidene-N-(2-hydroxypropyl)methacrylamide 23.** Three methods were attempted:

**Method 1. Synthesis of 2,5-dimethyl-oxazolidine followed by reaction with methacryloyl chloride:** In an analogous manner to the preparation of 2,2,5-trimethyl-oxazoline 16 in the synthesis of 18, the reaction of 1-amino-2-propanol (10.0 g, 0.13 mol) with acetaldehyde (5.7 g, 0.13 mol) was performed to prepare 2,5-dimethyl-oxazolidine and the crude liquid product allowed to react with methacryloyl chloride (13.9 g, 0.13 mol) under Schotten-Baumann conditions as for the synthesis of 18 (assuming 100% yield of 2,5-dimethyl-oxazolidine) to give a liquid product 23 (4.2 g, 19% yield): NMR showed that the crude product was not pure but the NMR signals of the desired product 23 were as follows: ¹H NMR (CDCl₃) δ 1.17 (d) 1.23 (d) (3H, CH₃CHCH₂), 1.32 (d) 1.38 (d) (3H, CH₃CHN), 1.87 (s, 3H, CH₃C(CH₃)C=O), 3.00 (br m) 3.20 (br m) (1H, CH₂CH), 3.60 (br m) 3.70 (br m) (1H, CH₂CH), 4.34 (q, 1H, CHCH₂), 5.23 (s) 5.29 (s) 5.36 (s) 5.41 (s) (2H, CH₂ vinylic) 5.32 (q) 5.50 (q) (1H, CH₂N); ¹³C (CDCl₃) 17.4, 18.5, 19.4, 19.7, 20.0, 20.4, 52.1, 53.7, 72.0, 73.4, 85.6, 86.5, 116.7, 117.7, 141.3, 169.6; MS (FAB) m/z 170 ([M + 1]⁺).

**Method 2. Reaction of acetaldehyde with HPMA 2:** To a stirred anhydrous DCM (50 ml) solution of 2 (5 g, 35 mmol) and camphorsulphonic acid (0.41 g, 2 mmol) in a 100 ml round-bottom flask was added acetaldehyde (16.8 g, 380 mmol) and the resulting solution stirred for 48 h. Additional DCM (30 ml) was then added and the total
organic phase was washed with 1 M aqueous NaOH (2 x 20 ml) and water (1 x 40 ml). The organic phase was then dried with MgSO₄, filtered and the solvent removed from the filtrate. The colourless liquid remaining was distilled under vacuum collecting the fraction at 65 °C (~ 2 mm·Hg) to give a colourless liquid product (3.3 g, 56% yield). Analysis by ¹H NMR showed the product was impure. For NMR signals of the desired product 23 only, see Method 1. Analogous reactions with acetone gave a very low yield (< 15 %).

Method 3. Acetaldehyde dimethyl acetal 26 reaction with HPMA 2: First procedure in the presence of boron trifluoride etherate (Bouffard et al., 1980): To a stirred anhydrous DCM (5 ml) solution of 2 (0.50 g, 3.5 mmol) and 26 (0.32 g, 3.5 mmol) in a 10 ml round-bottom flask was added boron trifluoride etherate (0.05 g, 0.4 mmol). The resulting solution was stirred at room temperature (RT) for 2 h and then washed with water (2 x 4 ml), dried with MgSO₄, filtered and the solvent removed from the filtrate to leave a slightly yellow liquid product (0.05 g, 8% yield): ¹H NMR analysis indicated a mixture of products but predominantly 2. For ¹H NMR signals of 23 see Method 1. Second procedure in the presence of pTSA: Similar conditions used as for the reaction involving boron trifluoride etherate but using pTSA monohydrate (20 mg, 0.1 mmol) resulting again in a yellow liquid product (0.09 g, 15% yield): ¹H NMR analysis indicated a mixture of products but predominantly HPMA 2.

Synthesis of N,O-methylidene-N-(2-hydroxypropyl) methacrylamide 24: Two of the methods (1 and 3 above) used for the synthesis of 23 were also used to prepare 24 on the same scale. Method 1 was altered to enable the azeotropic removal of water as follows: A 100 ml round-bottom flask was charged with 1-amino-2-propanol (5.0 g, 67 mmol), toluene (50 ml) and a magnetic stir bar and the resulting solution cooled using an ice bath. Formaldehyde (5.4 ml of 37% w/v aqueous solution, 67 mmol) was
then added slowly to the 1-amino-2-propanol solution under stirring. After complete
addition and 30 min of stirring, the flask was then fitted with a Dean and Stark trap
(with condenser) and the reaction solution heated under reflux for 2 h. After heating
the toluene was removed from the reaction solution using a rotary evaporator to leave
approximately 3.0 ml of an orange liquid, which was then allowed to react with
methacryloyl chloride (3.6 g, 34 mmol) under Schotten-Baumann conditions (see
synthesis of HPMA 2). The crude product obtained was distilled under vacuum
collecting the fraction at 62 °C (~ 2 mm-Hg) to give a colourless liquid product (1.0 g,
10% yield). The ^1H NMR analysis showed that the product was not pure. The signals
for the desired product 24 were: ^1H NMR (CD$_3$OD) δ 1.34 (br s, 3H, CH$_2$CH) 1.94 (s,
3H, CH$_2$CH$_2$C=O), 3.08 (br s) 3.16 (br s) (1H, CH$_2$CH), 3.67 (br s) 3.80 (br s) (1H,
CH$_2$CH) 4.23 (br s, 1H, CH$_2$CH$_2$CH$_2$), 4.89 (s, 1H, CH$_2$ vinylic), 5.15 (s, 1H, CH$_2$ vinylic)
5.24 (s) 5.34 (s) (1H, CH$_2$O); MS (FAB): m/z 156 ([M + 1]+). This
method was also repeated using paraformaldehyde (2 g, 67 mmol) in the presence of p-
TSA (0.13 g, 0.7 mmol) but the yield of the crude product isolated after the
methacryloyl chloride reaction was extremely low (0.31g, 3%) and the NMR analysis
showed a considerable amount of impurities. For the analogous reaction to Method 3
above using 2 (0.2 g, 1.4 mmol) and dimethoxymethane (1.06 g, 280 mmol) in the
presence of p-TSA (0.02 g, 0.8 mmol).

**Attempted Synthesis of 5-methyl-2-phenyl-oxazolidine 20 - Actual Synthesis of N-(2-
hydroxypropyl)benzylideneamine 21:** Using the same procedure as for the synthesis of
2,2,5-trimethyl-oxazoline 16, 1-amino-2-propanol (10 g, 0.13 mol) was allowed to
react with benzaldehyde (17.0 g, 0.16 mol) to give a white solid product 21 that was
recrystallised from EtOAc (18.7 g, 86% yield): ^1H NMR (CD$_3$OD) δ 1.23 (d, 3H,
CH$_3$), 3.53 (dd, 1H, CH$_3$), 3.70 (dd, 1H, CH$_3$), 4.05 (m, 1H, CHCH$_3$), 7.44 (d, 3H,
ring), 7.75 (d, 2H, ring), 8.32 (s, 1H, N=CH); FT-IR (ATR, cm⁻¹) 3216 (OH), 1646 (C=N).

### 2.2 Polymerisation chemistry

**Suspension polymerisation procedure with N,O-isopropylidene-N-(2-hydroxypropyl)methacrylamide 18:** In a 15 ml pressure tube with a magnetic stir bar added, benzoyl peroxide (25 mg, 0.1 mmol) was dissolved in protected HPMA (0.5 g, 2.7 mmol) and this solution was then added to water (5 ml) whereupon a suspension of monomer droplets formed. The mixture was then purged with nitrogen for 15 min. The reaction tube was then sealed with a screw-cap lid and placed in an oil bath at 60 °C for 12 h. The reaction mixture did not become more viscous during heating and there were no visible signs of polymer. No evidence of polymerisation was obtained by precipitation of the reaction solution into hexane.

**Redox emulsion polymerisation procedure with N,O-isopropylidene-N-(2-hydroxypropyl)methacrylamide 18:** To a 15 ml pressure tube was added, protected HPMA (0.5 g, 2.7 mmol), potassium persulphate (0.15 ml of 0.68% w/v aqueous solution), sodium stearate (0.47 ml of 3.56% w/v aqueous solution), water (0.6 g) and a magnetic stir bar. The tube was then sealed with a screw-cap lid and placed in an oil bath with stirring at 80 °C for 4 h. The reaction mixture did not appear to change during heating and no evidence of polymerisation was obtained by precipitation.

**AIBN initiated polymerisation of N,O-isopropylidene-N-(2-hydroxypropyl)acrylamide 28 - Synthesis of poly(N,O-isopropylidene-N-(2-hydroxypropyl)acrylamide) 29:** A 15 ml pressure tube was charged with monomer 28 (0.75 g, 4.4 mmol), AIBN (60 mg,
7.5% of monomer mass), acetone (4 ml) and a magnetic stir bar. The resulting homogeneous solution was purged with argon for 15 min. The tube was then sealed and heated at 50 °C for 3 h under stirring. The resulting colourless solution was then added dropwise to water to precipitate a white solid product 29 that was isolated by filtration and dried in vacuo (0.50 g, 69% yield): 1H NMR (DMSO-d6) δ 1.01, 1.20 (br s, 3H, CH₃CHO), 1.40 (br s, 3H, C(CH₃)₂), 1.45 (br s, 3H, C(CH₃)₂), 1.66 (br, 2H, CH₂CHC=O), 2.25 (br s, 1H, CHC=O), 3.01 (br s, 1H, NCH₂), 3.65 (br s, 1H, NCH₂), 4.19 (br s, 1H, CH₃CH); FT-IR (ATR) 1643 cm⁻¹ C=O stretch (amide I); Analysis calculated for C₉H₁₅NO₂ (found): C, 63.88 (63.72); H, 8.93 (8.94); N, 8.28 (8.16); GPC (DMF, PMMA standards) $M_w = 83,500$ g·mol⁻¹, $M_n = 37,300$ g·mol⁻¹, $M_w/M_n = 2.24$.

Anionic polymerisation experiment using N,O-isopropylidene-N-(2-hydroxypropyl)-methacrylamide 18 (performed in Prof. Müller's laboratories at the University of Mainz): The monomer was distilled from CaH₂ immediately prior to the polymerisation. The preparation of the initiator and monomer solutions was performed in a glove box, containing oxygen at less than 5 ppm. All glassware had been previously cleaned by overnight heating in an oven at 500 °C. Polymerisation procedure: To a THF initiator solution (5 ml THF, anhydrous) in an ampoule was added LiCl (dried in vacuo at 300 °C for 48 h) dissolved in THF (5 ml, anhydrous) and additional THF (30 ml). The ampoule was then sealed using a rotaflow device. Into another ampoule, monomer 18 (2.20 g, 12 mmol) and n-octane (1 ml, anhydrous, for use as a standard for gas chromatography analysis of monomer consumption) and THF (5 ml) were added and the ampoule sealed. The ampoules were then fitted to a reaction flask which was attached to a vacuum line and a nitrogen supply, and the flask was then subsequently heated under vacuum using a hot air gun in order to dry it. The initiator solution was added to the reaction flask under nitrogen and cooled to -
78 °C using a propan-2-ol/dry ice bath. The monomer solution was then added quickly to the initiator solution under rapid stirring. Samples were taken after 5, 10, and 20 min using a positive pressure of nitrogen. Samples were quenched by collection into a beaker of methanol. The reaction was finally stopped after 30 min by quenching with methanol. Gas chromatography of the quenched samples showed a constant ratio of n-octane standard to monomer indicating no polymerisation had taken place. The reaction was repeated several times with different samples of monomer with the same result.

**Synthesis of semi-telechelic poly(N,O-isopropylidene-N-(2-hydroxypropyl)-acrylamide) 31**: A 15 ml pressure tube was charged with monomer 28 (0.50 g, 4.4 mmol), AIBN (25 mg, 5% of monomer mass), 2-mercaptoethylamine hydrochloride (11 mg, 0.1 mmol), methanol (5 ml) and a magnetic stir bar and the resulting solution was purged with argon for 15 min and sealed. The pressure tube was then placed in oil bath at 50 °C for 24 h with stirring. The resulting colourless solution was then added dropwise to 0.1 M aqueous NaHCO₃ to precipitate a white solid that was isolated by filtration. The isolated solid was dissolved in diethyl ether (5 ml) and this organic phase was dried with anhydrous potassium carbonate, filtered and the solvent removed to leave a white solid product 31 that was dried in vacuo (0.17 g, 34.7% yield): $^1$H NMR (DMSO-$d_6$) $\delta$ 1.01, 1.20 (br s, 3H, $CH_3$CHO), 1.40 (br s, 3H, C($CH_3$)$_2$), 1.45 (br s, 3H, C($CH_3$)$_2$), 1.66 (br, 2H, $CH_2$CHC=O), 2.25 (br s, 1H, $CHC=O$), 3.01 (br s, 1H, NCH$_2$), 3.65 (br s, 1H, NCH$_2$), 4.19 (br s, 1H, $CH_3$CH); GPC (DMF, PMMA standards) $M_n = 5,300$ g·mol$^{-1}$, $M_w/M_n = 1.5$. A repeat reaction using 21 mg of 2-mercaptoethylamine hydrochloride (0.2 mmol) gave $M_n = 2,500$ g·mol$^{-1}$, $M_w/M_n = 1.60$. 

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2.3 Deprotection chemistry

Deprotection of \(N,O\)-isopropylidene-\(N\)-(2-hydroxypropyl)methacrylamide 18: Monomer 18 was melted by gently heating and mixed under stirring with dilute HCl for approximately 10 min. During this time a white solid formed that upon isolation and drying gave a \(^1\)H NMR spectrum identical to that of unprotected HPMA 2 (For NMR data see synthesis of HPMA 2).

Deprotection of poly\((N,O\)-isopropylidene-\(N\)-(2-hydroxypropyl)acrylamide) 29: To 100 mg of 29 in a 10 ml round-bottom flask was added THF (4 ml) and water (1 ml) and a drop of TFA. The resulting solution was allowed to stir for 48 h using a magnetic stir bar. The reaction solution was then diluted with further water (approximately 30 ml) and then dialysed using regenerated cellulose membrane (SpectraPor, MW cut off (MWCO) 1000) against water to give a white solid product, PHPA 30 (77 mg, 92% yield): FT-IR (ATR) 3308 cm\(^{-1}\) OH stretch, 1647 cm\(^{-1}\) C=O stretch (amide I), 1550 cm\(^{-1}\) -C(O)NH- (amide II); \(^1\)H NMR (DMSO-\(d_6\)) 1.01 (br s, 3H, \(CH_3\)), 1.17-2.21 (br m, 2H, \(CH_2\)CHC=O), 2.68-3.27 (br m) (2H, \(CH_2\)CHOH) + (1H, \(CHC=O\)), 3.65 (br s, 1H, \(CHOH\)), 4.90 (br s, 1H, \(OH\)), 7.75 (br s, 1H, \(NH\)).

3 Work Described in Chapter 3

2.3 Monomer synthesis

Synthesis of MOSu 35: To a 250 ml three-necked flask fitted with two dropping funnels and a nitrogen inlet was added NHS (27.5 g, 0.24 mmol) and anhydrous DCM (110 ml) under a nitrogen atmosphere. The resulting mixture was cooled to less than 5 °C and the dropping funnels charged separately with methacryloyl chloride (25.0 g, 0.24 mmol dissolved in 50 ml DCM) and triethylamine (24.2 g, 0.24 mmol dissolved...
in 50 ml DCM). The contents of the two funnels were then simultaneously added dropwise to the stirring NHS mixture slowly to maintain the temperature below 5 °C. After complete addition the reaction mixture was further stirred for 1 h and then washed with water (x 1), 0.1 M aqueous NaHCO₃ (x 2) and a saturated aqueous NaCl solution (x 1). The organic phase was then dried with MgSO₄, filtered and the solvent removed from the filtrate using a rotary evaporator to leave a crude white solid product that was then recrystallised from EtOAc:hexane (90:10 v/v) to give 35 as white crystals (33.2 g, 76% yield): m.p. 102-104 °C; ¹H NMR (DMSO-d₆) δ 1.99 (s, 3H, CH₃), 2.83 (s, 4H, CH₂CH₂), 6.08 (s, 1H, CH₂ vinylic), 6.33 (s, 1H, CH₂ vinylic); ¹³C NMR (DMSO-d₆) δ 18.1, 25.6, 130.2, 131.9, 162.1, 169.1; analysis calculated for C₉H₇NO₄ (found): C, 52.46 (52.36); H, 4.95 (4.98); N, 7.65 (7.63).

**Synthesis of AOSu 57:** Using the same method as for the synthesis of MOSu 35, monomer 57 was prepared using acryloyl chloride (21.6 g, 0.24 mol) as a white crystalline solid (26.3 g, 65% yield); m.p. 68-69 °C; ¹H NMR (CDCl₃) δ 2.84 (s, 4H, CH₂CH₂), 6.15 (d, 1H, CH₂ vinylic), 6.30 (dd, 1H, CHCO), 6.67 (d, 1H, CH₂ vinylic); ¹³C NMR (CDCl₃) δ 25.6, 123.0, 136.0, 161.0, 169.0; analysis calculated for C₇H₇O₄N (found): C, 49.71 (49.64); H, 4.17 (4.29); N, 8.28 (8.35).

**Synthesis of EOSu 60:** Monomer 60 was prepared in a four-step procedure from diethyl malonate (Steps 1 to 3 Ferritto and Tirrell, 1992).

1 2-Carboethoxybutyric acid. To a 1 l round-bottom flask charged with 700 ml of 1 M KOH in ethanol and a magnetic stir bar was added diethyl malonate (100 g, 0.53 mol) and the resulting solution was allowed to stir for 20 h. The white precipitate that had formed was then isolated by filtration and the filtrate concentrated using a rotary evaporator. The colourless oil was added to the
precipitate and the mixture dissolved in a minimum amount of water and acidified with dilute aqueous HCl to pH 2. Upon acidification, an oil separated from the solution which was taken up into diethyl ether. The aqueous layer was further extracted with ether (3 x 200 ml) and the ether extracts combined, dried with MgSO$_4$ and filtered. Removal of the diethyl ether from the filtrate gave a colourless oil (85 g, 99% yield): $^1$H NMR (CDCl$_3$) $\delta$ 0.95 (t, 3H, $CH_3CH_2CH$), 1.30 (t, 3H, $CH_3CH_2O$), 1.93 (td, 2H, $CH_2CH$), 3.32 (t, 1H, $CH$), 4.16 (q, 2H, $CH_2O$), 8.83 (br s, 1H, COO$\cdot$).

Ethyl-2-ethylacrylate. To cooled (approximately -5 °C) crude 2-carboxethyloxybutyric acid (84.9 g, 0.53 mol) in a 250 ml round-bottom flask fitted with a condenser was added diethylamine (55 ml, 0.53 mol) and a magnetic stir bar. A dropping funnel was placed on top of the condenser and charged with a formalin solution (37% w/w aqueous formaldehyde solution, 43.5 g, 0.54 mol), which was slowly allowed to drip into the cooled reaction solution allowing the solution to warm to RT. After stirring for 24 h the solution was warmed to 60 °C and further stirred for 7 h. The two-layer mixture formed was then cooled to 0 °C and concentrated H$_2$SO$_4$ added very carefully, resulting in a violent reaction and evolution of gas. The addition of acid was continued until no further reaction was visible after which a product was extracted from the resulting mixture into diethyl ether (3 x 200 ml). The combined ether extracts were then dried with MgSO$_4$, filtered and the ether removed to leave a colourless oil product (36 g, 53% yield): $^1$H NMR (CDCl$_3$) $\delta$ 1.18 (t, 3H, $CH_3CH_2C$), 1.30 (t, 3H, $CH_3CH_2O$), 2.32 (t, 2H, $CH_3CH_2C$=), 4.21 (q, 2H, OCH$_2$), 5.51 (s, 1H, $CH_2$ vinylic), 6.13 (s, 1H, $CH_2$ vinylic).

2-Ethylacrylic acid 59.5. To crude ethyl 2-ethylacrylate (36 g, 0.28 mol) and a magnetic stir bar in a 1 L round-bottom flask fitted with a condenser was added 630 ml of 1 M aqueous KOH and the resulting mixture refluxed for 24 h
until the mixture became homogeneous. After cooling the solution to RT the solution was acidified with dilute aqueous HCl to pH 2 resulting in separation of an oil which was then extracted into diethyl ether (4 x 200 ml). The ether extracts were then combined, dried with MgSO₄, filtered and the ether removed from the filtrate on a rotary evaporator to leave a slightly yellow oil 59.5 (24 g, 88% yield): ¹H NMR (CDCl₃) δ 1.08 (t, 3H, CH₃), 2.32 (q, 2H, CH₃CH₂), 5.64 (s, 1H, CH₂ vinylic), 6.28 (s, 1H, CH₂ vinylic), 9.8 (br s, 1H, COOH).

4 EOSu 60: To a cooled chloroform solution (50 ml, anhydrous) of crude 2-ethylacrylic acid (10 g, 0.1 mol) and NHS (11.5 g, 0.1 mol) in a septum sealed 100 ml round-bottom flask with magnetic stir bar was slowly added under stirring and an argon stream, 1,3-dicyclohexylcarbodiimide (DCC, 22.7 g, 0.1 mol). Taking care not to allow the contents to become hot during the addition of the coupling reagent, the complete addition took approximately 30 min. The mixture was further stirred for 3 h and the resulting white precipitate (1,3-dicyclohexylurea) was removed by filtration. The filtrate was diluted with EtOAc (150 ml), filtered again and then washed with water (1 x 50 ml), 1 M aqueous NaHCO₃ (1 x 75 ml) and a saturated aqueous brine solution (100 ml). The organic solution was then dried with MgSO₄, filtered and the solvent removed from the filtrate on a rotary evaporator to leave a white solid product 60 that was recrystallised from EtOAc:hexane (12.1 g 63% yield): m.p. 53-54 °C; ¹H NMR (CDCl₃) δ 1.14 (t, 3H, CH₃), 2.41 (q, 2H, CH₃CH₂), 2.85 (s, 4H, (CH₂CH₂), 5.85 (s, 1H, CH₂ vinylic), 6.44 (s, 1H, CH₂ vinylic); analysis calculated for C₉H₁₁NO₄ (found): C, 54.82 (55.07); H, 5.62 (5.76); N, 7.10 (7.00); MS (FAB) m/z 198 ([M + 1]⁺).
2.4 Synthesis of ATRP initiators and ligands

Synthesis of 2-hydroxyethyl 2-bromo-2-methylpropanoate 47: To a 250 ml round-bottom flask fitted with a nitrogen inlet was added anhydrous DCM (100 ml) and ethylene glycol (27.3 g, 0.44 mol) and the resulting solution cooled to less than 5 °C under a nitrogen atmosphere. Still under nitrogen, to the ethylene glycol was then added 2-bromoisobutyryl bromide (25 g, 0.11 mol, dissolved in 50 ml DCM) and pyridine (8.6 g, 0.11 mol, dissolved in 50 ml DCM) simultaneously from two dropping funnels under vigorous stirring. During the addition the temperature was maintained below 5 °C. After complete addition the mixture was further stirred for 2 h and then washed with water (1 x 50 ml) and 1 M aqueous NaHCO₃ (2 x 50 ml). The organic phase was then dried with MgSO₄, filtered and the solvent removed to leave a colourless oil 47 (21.0 g, 91% yield) which was distilled under reduced pressure, collecting the fraction at 70 °C (~ 2 mm-Hg): ¹H NMR (CDCl₃) δ 1.93 (s, 6H, (CH₃)₂), 2.08 (br s, 1H, OH), 3.85 (t, 2H, CH₂OH), 4.29 (t, 2H, CH₂CO).

Synthesis of 2-hydroxyethyl 2-bromopropanoate 48: Using the same procedure as for the synthesis of 47, ethylene glycol (36.0 g, 232 mmol) was allowed to react with 2-bromopropionyl bromide (10.0 g, 46 mmol) in the presence of pyridine (3.6 g, 46 mmol). The crude product was vacuum distilled, collecting the fraction at 68 °C at approximately 2 mm-Hg to give a colourless liquid product 48 (6.8 g, 74% yield). ¹H NMR (DMSO-d₆) δ 1.72 (d, 3H, CH₃), 3.58 (t, 2H, HOCH₂), 4.06-4.15 (m, 2H, CH₂CO₂), 4.64 (q, 1H, CH(CH₃)), 4.81 (br, 1H, OH).

Synthesis of tetraethyleneglycol 2-bromo-2-methylpropanoate 52: Using the same procedure as for the synthesis of 47, tetra(ethylene glycol) (84.5 g, 0.43 mol) was
allowed to react with 2-bromoisobutyryl bromide (25.0 g, 0.11 mol) in the presence of pyridine (8.6 g, 0.11 mol). A crude colourless liquid product was obtained (27.2 g, 76% yield), a quarter of this product was purified by column chromatography using EtOAc:hexane eluent (5.1 g, 75% yield). ^1H NMR (CD$_3$OD) $\delta$ 1.92 (s, 6H, (CH$_3$)$_2$), 3.55 (t, 2H, CH$^2$), 3.72-3.63 (m, 1OH), 3.74 (t, 2H, CH$^2$), 4.30 (t, 2H, CH$_2$OCO).

Synthesis of PEG macro-initiator 55 (Jankova et al., 1998): To a 250 ml three-neck round-bottom flask equipped with two dropping funnels and a magnetic stirrer was added monomethoxy-PEG (MW 2000, 25.00 g, 12.5 mmol) and anhydrous DCM (100 ml) and the resulting solution cooled to less than 5 °C. The dropping funnels were separately charged with 2-bromoisobutyryl bromide (5.74 g, 25 mmol, dissolved in 50 ml DCM) and pyridine (1.95 g, 25 mmol, dissolved in DCM) and the solutions added dropwise to the PEG solution over the course of 1 h. The resulting mixture was stirred for a further 16 h and then washed with 1 M aqueous NaHCO$_3$ (x 2), and a saturated NaCl solution (x 1). The organic phase was then dried using MgSO$_4$ and filtered. The solvent was removed from the filtrate using a rotary evaporator to leave a white solid product (24.0 g) that was dialysed using regenerated cellulose membrane (SpectraPor, MWCO 1000) against water. After lyophilization of the dialysed solution a white solid product 55 remained (5.0 g): ^1H NMR (CDCl$_3$) $\delta$ 1.90 (s, 6H, (CH$_3$)$_2$CBr), 3.33 (s, 3H, CH$_3$O), 3.74-3.46 (m, (OCH$_2$CH$_2$)$_n$), 4.28 (t, 2H, CH$_2$OCO).

Synthesis of Me$_6$TREN 45 (Ciampolini and Nardi, 1966): To cooled tris(2-aminoethyl)amine (12.5 g, 85 mmol) in a 250 ml round-bottom flask was added formic acid (11.8 g, 9.7 ml, 256 mmol) and formalin (37% w/v aqueous formaldehyde solution, 43.2 ml, 222 mmol). A condenser was then added to the flask and the
reaction mixture was heated under stirring for 7 h at 80 °C. The orange reaction solution was then concentrated using a rotary evaporator and made alkaline by the addition of an appropriate volume of 1 M aqueous NaOH, resulting in the formation of two phases. The top layer was extracted into diethyl ether (120 ml) and the ether was then washed with 1 M aqueous NaHCO₃ (3 x 60 ml), dried with MgSO₄, filtered and the solvent removed to leave a slightly yellow crude liquid product. The crude product was distilled under vacuum, collecting the fraction at 57 °C (~ 2 mm-Hg) to give a colourless liquid 45 (15.8 g, 81% yield): 

\[
\text{^1H NMR (CDCl}_3\text{) } \delta 2.18 \text{ (s, 18H, } 6\text{CH}_3\text{), 2.33 \text{ (t, } 6\text{H, } (C\text{=C}_3\text{N).}}
\]

3.3 ATRP procedures

*General ATRP procedure - Synthesis of PMOSu 36 as an example:* CuBr (31.3 mg, 0.2 mmol), 2-2'-bipyridine (68.3 mg, 0.4 mmol) and MOSu 35 (2.00 g, 10.9 mmol) were added to a 10 ml pear-shaped flask which was then sealed with a septum. Into the flask was then injected DMSO (1.3 g). The resulting brown mixture was gently heated until a solution had formed and then purged with argon for approximately 25 min. An argon purged solution of 47 (46.1 mg, 0.2 mmol) in DMSO (0.2 g) was then injected into the flask and the flask was then heated at 100 °C until the reaction mixture became too viscous for stirring to occur. The polymeric product 36 was isolated by adding 7-8 ml of DMSO to the cooled reaction mixture and then adding the resulting solution dropwise to vigorously stirred acetone (150 ml), causing precipitation of 36. The product 36 was isolated as a white solid after filtration and drying in vacuo (1.78 g, 89% yield): 

\[
\text{^1H NMR (DMSO-d}_6\text{) } \delta 1.38 \text{ (br, } 3\text{H, } CH_3\text{), 2.42 \text{ (br m, } 2\text{H, } CH_2\text{C), 2.78 \text{ (br, } 4\text{H, } CH_2CH_2\text{).}}
\]

\[
\text{^13C NMR (DMSO-d}_6\text{) } \delta 18.0, 25.4, 45.0, 50.0, 169.9; \text{ FT-IR (ATR) } 1807 \text{ cm}^{-1}\text{ CO ester stretch, 1779 cm}^{-1}\text{ symmetric imide CO stretch, 1734 cm}^{-1}\text{ anti-symmetric imide CO stretch; analysis calculated for C}_9\text{H}_9\text{NO}_4.
\]
repeat unit (found): C, 52.46 (50.82); H, 4.95 (5.18); N, 7.65 (7.50); GPC (DMF, PMMA standards) $M_w = 27,200$ g mol$^{-1}$, $M_n = 22,700$ g mol$^{-1}$, $M_w/M_n = 1.20$.

Quenched ATRP reactions with MOSu 35 (section 2.2.2, Chapter 3): Ultra dry DMSO (Aldrich, water < 50 ppm) was used in the following experiments which were based on a 50:1:1:2 - [35]:[47]:[CuBr]:[bipy]. Initiator stock solution: To 47 (230.6 mg, 1.1 mmol) in a 2 ml septum topped vial was added DMSO (1.00 g) and the resulting solution was purged for 45 min with argon. Catalysing species stock solution: To a 25 ml flask fitted with a septum was added CuBr (156.3 mg, 1.1 mmol), bipy (341.3 mg, 2.2 mmol) and DMSO (6.50 g). Standard stock solution: To an argon-purged round-bottom flask fitted with a septum was added 1,4-dichlorobenzene (8.00 g, mol) and DMSO (16.00 ml) and the flask shaken until a homogenous solution had formed. Procedure: To a 10 ml flask was charged 35 (1.00 g, 5.5 mmol) and catalyst stock solution (0.65 g, 590 µl) and a magnetic stirring bar. After sealing the flask with a septum the mixture was gently heated to aid solubilisation of all the reagents and then purged with argon for approximately 20 min. Before the end of purging the purged initiator solution was added by gas-tight syringe followed immediately by the removal of the syringe and purging needles. The flask was then quickly placed in an oil bath at 100 °C for the required duration (0.5 min, 1 min, 2 min, 3 min and 5 min). At the end of the reaction period unpurged DMSO (5.0 ml) was added to cool and quench the reaction and flask was placed in a dry-ice bath to aid cooling. The standard stock solution (1.60 ml) was then also added to the reaction mixture. After ensuring a homogeneous solution, which took between 2 and 10 min in the 2, 3 and 5 min reactions because of the very viscous nature of the reaction solutions, 200 µl of the green solution was mixed with DMSO-$d_6$ (1.0 ml) and a $^1$H NMR spectrum taken within 30 minutes: White polymer products 36 were then isolated by precipitation by adding diluted reaction solutions to vigorously stirred acetone (as for general ATRP...
Chapter 7: Experimental

procedures for 35 above): Yields 0.5 min = 0.21 g (21%), 1 min = 0.48 g (48%), 2 min = 0.74 g (74%), 3 min = 0.78 g (78%), 5 min = 0.79 g (79%); GPC (DMF, PMMA standards) $M_n$ and $M_w/M_n$: 0.5 min = 15,600 and 1.26, 1 min = 16,400 and 1.34, 2 min = 17,900 and 1.35, 3 min = 18,700 and 1.33, 5 min = 18,800 and 1.35.

Preparation of poly(MOSu-b-ethylene glycol) block copolymer 56: A 25 ml round-bottom flask was charged with PEG macro-initiator 55 (0.50 g, 0.3 mmol assuming MW 2000), 35 (0.50 g, 2.7 mmol) and methanol (2.5 ml). The flask was sealed with a septum and the resulting solution was purged with argon for approximately 25 min. In a septum sealed 1.5 ml vial was added CuBr (36 mg, 0.3 mmol), PMDETA (43 mg, 0.3 mmol) and methanol (0.5 ml) and resulting solution was then purged with argon for approximately 25 min. The CuBr solution was then added to the macro-initiator solution under a positive pressure of argon, and the macro-initiator flask was then immediately placed in an oil bath at 70 °C for 7 h. A slightly green product was isolated by precipitating the reaction solution into diethyl ether, filtration and drying in vacuo (0.43 g). No purification of this product was performed (see section 2.4, Chapter 3).

AIBN initiated polymerisation of MOSu 35: A 15 ml pressure tube was charged with 35 (1.50 g, 8 mmol) and AIBN (0.11 g, 7.5% of monomer mass) and acetone (10 ml) and the resulting solution was purged with argon for 15 min and sealed. The sealed pressure tube was then heated at 50 °C for 3 h. After this time, a precipitate was visible that was isolated by filtration, dissolved in DMF and then precipitated into acetone to purify. After isolation by filtration and drying in vacuo, a hard glassy solid product remained - AIBN prepared 36 (0.92 g, 61% yield): $^1$H NMR (DMSO-$d_6$) $\delta$ 1.39 (br, 3H, $CH_3$), 2.43 (br m, 2H, $CH_2C$), 2.78 (br, 4H, $CH_2CH_2$); GPC (DMF, PMMA standards) $M_n$ = 138,000 g·mol$^{-1}$, $M_w$ = 68,200 g·mol$^{-1}$, $M_w/M_n$ = 2.02.
**AIBN initiated polymerisation of AOSu 57:** An analogous method was used as for the polymerisation of 35 above using 0.50 g of 57 to give a white solid product - AIBN prepared PAOSu 58 (0.28 g, 56% yield): \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 2.05 (br, 2H, \(\text{CH}_2\)), 2.80 (br s, 4H, \((\text{CH}_2)_2\)), 3.15 (br s, 1H, \(\text{CH}_2\text{CH}\)); GPC (DMF, PMMA standards) \(M_w = 103,100 \text{ g}\cdot\text{mol}^{-1}\), \(M_n = 50,800 \text{ g}\cdot\text{mol}^{-1}\), \(M_w/M_n = 2.03\).

4 Work Described in Chapter 4

4.1 PMOSu 36 conjugation chemistry

**Synthesis of PHPMA 1 by conjugation of 1-amino-2-propanol 10 to PMOSu 36:** To 36 (0.2 g, 1.1 mmol of reactive groups, \(M_w = 33,200 \text{ g}\cdot\text{mol}^{-1}\), \(M_w/M_n = 1.09\) (GPC, DMF eluent, PMMA standards)) dissolved in anhydrous DMF (3 ml) was added 10 (0.16 ml, 2.1 mmol) dropwise under stirring at 0 °C. The solution was allowed to warm to RT and then heated to 50 °C for 16 h. The reaction mixture was cooled to RT and slowly added to acetone (20 ml) to precipitate a solid product. The product was purified by a second precipitation from a methanol solution into 60:40 (v/v) acetone:diethyl ether to give a water-soluble polymer 1 as a white solid after drying in vacuo (0.14 g, 93% yield assuming 100% conversion): \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 0.99 (br s) 0.74 (br s) \((3H, \text{CH}_3\text{CCH}_2\text{C}=\text{O})\), 1.01 (br s, 3H, \(\text{CH}_3\text{CHOH}\)), 1.70 (br m, 2H, \(\text{CH}_2\text{CCO}\)), 2.87 (br s, 2H, \(\text{NHCH}_2\)), 3.65 (br s, 1H, \(\text{CH}\)), 3.47 (br s, 1H, \(\text{OH}\)), 7.16 (br s, 1H, \(\text{NH}\)); \(^13\)C NMR (DMSO-\(d_6\)) \(\delta\) 21.8, 23.2, 27.6, 49.9-50.3, 52.8, 59.6, 70.0, 182.2; GPC (PBS, PEG standards) \(M_w = 21,700 \text{ g}\cdot\text{mol}^{-1}\), \(M_n = 16,700 \text{ g}\cdot\text{mol}^{-1}\), \(M_w/M_n = 1.3\).
Conjugation of 1-amino-2-propanol 10 to PMOSu 36 in the presence of added water: The same procedure was used as for the conjugation of 1-amino-2-propanol above, using 36 (0.1 g, 0.5 mmol of reactive groups) dissolved in DMSO (1.2 ml) followed by the addition of water (0.2 g, 11.1 mmol) and then 10 (0.1 g, 1.3 mmol). The vial was heated at 60 °C for 18 h and the polymer product isolated by precipitation into acetone followed by filtration and drying in vacuo (0.05 g):

General procedure for the conjugation of fractional equiv. of amines, following the reaction by ATR FT-IR spectroscopy - Conjugation of PMOSu 36 with 0.25 equiv. of glycine methyl ester hydrochloride 66 as a typical conjugation reaction: Polymer 36 (50 mg, 0.27 mmol of reactive groups), 66 (8.6 mg, 0.07 mmol) and a magnetic stir bar were added to a 1.5 ml vial. The vial was sealed with a septum-centred screw cap lid and purged with argon for approximately 2 min. Anhydrous DMSO (0.4 ml) was then injected into the vial under argon and the vial was placed onto a magnetic stirrer. Once a solution had formed, a small sample was removed by syringe under argon for immediate analysis by ATR FT-IR spectroscopy. Triethylamine (14.3 μl, 0.10 mmol) was then added under argon to the vial and the vial was placed in an oil bath at 60 °C for 1 h. After cooling, another sample of the solution was removed from the vial under argon for immediate FT-IR analysis. A similar procedure was also used for the conjugation of fractional equiv. of free amines (i.e., not salts). In those experiments, the amine reactant was added neat to a solution of 36 or 36/triethylamine mixture, of which an IR spectrum had already been obtained.

Synthesis of HPMA copolymer-Gly-Gly-β-napthylamide conjugates 73, a typical procedure: Conjugation of 0.1 equiv. H-Gly-Gly-β-napthylamide hydrobromide 72 followed by quenching with 1-amino-2-propanol 10: Polymer 36 (100 mg, 0.55 mmol of reactive groups), 72 (19 mg, 0.06 mmol) and a magnetic stir bar were added to a
1.5 ml vial. The vial was sealed with a septum-centred screw cap lid and purged with argon for approximately 2 min. Anhydrous DMSO (0.4 ml) was then injected into the vial under argon and the vial was placed onto a magnetic stirrer. Once a solution had formed, a small sample of the solution was removed by syringe under argon for immediate FT-IR spectroscopy. Triethylamine (15.2 μl, 0.11 mmol) was then added under argon to the vial and the vial was placed in an oil bath at 50 °C for 30 min. After cooling, a sample of the solution was removed from the vial under argon for immediate FT-IR spectroscopy. The H-Gly-Gly-NA reaction solution was further heated with 10 (82 mg, 1.1 mmol) which was added dropwise under stirring, at 50 °C for 1.25 h. A product 73 was isolated by precipitation of the DMSO reaction solution into cooled acetone:diethyl ether (50:50 v/v) and further purified by precipitation from methanol into acetone:diethyl ether (50:50 v/v) (30 mg, 34% yield): GPC (PBS) insufficient detection by refractive index detector for analysis; GPC (DMF, RT) aggregation of sample prevented analysis. 1H NMR (CD3OD) δ 1.90-1.50 (br m, b + e + g), 1.50-2.30 (br m, a + f), 2.50 (br s, unassigned – possible cyclic imide methylene protons, see section 2.4, Chapter 4), 2.95-3.25 (br m, c), 3.83 (br s, d), 4.05-4.20 (br m, h + i), 7.35-7.50 (br m, m + n), 7.50-7.95, (br m, j + k + l + o), 8.28 (br s, p).
4.2 Experiments to probe imide formation

**Synthesis of N-propionyloxysuccinimide 63:** The succinimidyld ester 63 was synthesised using propionyl chloride (25 g, 0.27 mol) and NHS (31.1 g, 0.27 mol) in the presence of triethylamine (24.6, 0.27 mol) by the same procedure as for the synthesis of MOSu 35. This afforded white crystals of 63 after recrystallisation from EtOAc:hexane (32.0 g, 69% yield): $^1$H NMR (CDCl$_3$) $\delta$ 2.79 (br, 4H, 2.63-2.58 (q, 2H, CH$_2$CH$_3$), 1.25-1.21 (t, 3H, CH$_3$); $^{13}$C NMR (CDCl$_3$) $\delta$ 8.5, 23.7, 25.3, 169.8, 170.0.

**Reaction of N-propionyloxysuccinimide 63 with n-propylamine 64:** To a 1.5 ml vial was added 63 (100 mg, 0.6 mmol) and a magnetic stir bar. The vial was sealed with a septum-centred screw cap lid and purged with argon for approximately 2 min. Anhydrous DMSO (0.4 ml) was then injected into the vial under argon and the vial was placed onto a magnetic stirrer. Once a solution had formed, 64 (17 mg, 0.3 mmol) was added under argon and the vial was placed in an oil bath at 60 °C for 2 h. Analysis by FT-IR and MS after removal of the reaction solution was consistent with N-propylpropionamide and the starting compound 63.

**Conjugation of 0.5 equiv. of n-propylamine 64 to PMOSu 36 and heating:** To an anhydrous DMSO (0.4 ml) solution of 36 (100 mg, 0.55 mmol of reactive groups) in an argon purged 1.5 ml vial with a septum lid was added triethylamine (55 mg, 0.55 mmol) and an FT-IR spectrum was taken of a sample of the resulting solution. The primary amine 64 (16.2 mg, 0.27 mmol) was then added neat to the solution of 36 under rapid stirring at RT. After 50 min at RT, a FT-IR spectrum of a sample of the reaction solution was taken, which revealed a 49% decrease in the band height at 1735
cm\(^{-1}\) and the formation of amide I and II bands at 1650 cm\(^{-1}\) and 1550 cm\(^{-1}\). The reaction solution was then heated at 50 °C for 2.5 h and another FT-IR spectrum taken. A brown solid product was isolated by precipitation of the reaction solution into hexane:acetone (90:10 v/v) (39 mg); \(^1\)H NMR (see Figure 4.12 and text, Chapter 4).

4.3 Polymerisation chemistry

AIBN initiated polymerisation of HPMA 2 - the synthesis of conventional PHPMA 1 (Kopecek & Bazilova, 1973): To a 15 ml pressure tube was added 2 (0.75 g, 5.2 mmol), AIBN (60 mg, 7.5% of monomer mass) and acetone (4 ml). The resulting solution was purged with nitrogen for several minutes and the tube sealed. After heating the tube in an oil bath at 50 °C for 5 h a white precipitate product had formed which was then isolated by filtration. The crude product was purified by precipitation into acetone from a methanol solution. Filtration and drying in vacuo gave conventional 1 as a white solid product (0.60 g, 80% yield): \(^1\)H NMR (DMSO-\(d_6\) \(\delta\) 1.00 (br s) 0.80 (br s) (3H, CH\(_2\)C), 1.01 (br s, 3H, CH\(_3\)CHOH), 1.65 (br m, 2H, CH\(_2\)C), 2.90 (br s, 2H, NHCH\(_2\)), 3.66 (br s, 1H, CH), 3.45 (br s, 1H, OH), 7.14 (br s, 1H, NH); \(^13\)C NMR (DMSO-\(d_6\) \(\delta\) 16.7, 18.1, 22.5, 44.8-45.2, 47.7, 54.5, 64.9, 177.1; FT-IR (ATR) 3338 cm\(^{-1}\) OH stretch, 1637 cm\(^{-1}\) C=O stretch (amide I), 1530 cm\(^{-1}\) - C(O)NH- (amide II); GPC (PBS, PEG standards) \(M_w = 35,000\) g·mol\(^{-1}\), \(M_n = 12,100\) g·mol\(^{-1}\), \(M_w/M_n = 2.9\).
4.5 Cytotoxicity study of PHPMA 1 derived from PMOSu 36

This cytotoxicity study was performed by Miss Rhondalea MacDonald of the London School of Pharmacy. B16F10 murine melanoma cells were kindly donated by Prof. I. Hart (St. Thomas's Hospital, London, UK). Tissue culture grade DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue and optical grade DMSO were purchased from Sigma. Trypsin, foetal calf serum (FCS) and RPMI 1640, were purchased from Gibco BRL Life Technologies. The growth media was RPMI 1640 containing 10% FCS.

4.5.1 Maintenance of cell lines

All cell culture procedures were performed in a Class II laminar flow cabinet. Cells were routinely maintained in a carbon dioxide (CO₂) culture incubator at 5% v/v CO₂ at 37 °C. All materials used for cell culture were sterile, osmotically balanced and warmed to 37 °C prior to use. B16F10 cells were grown in fresh growth media and were maintained in 75 cm³ tissue culture-treated cantered neck flask with vented (0.2 μm) tops. The cells were split twice weekly in order to maintain them in the exponential phase of growth.

4.5.2 Splitting of cells

The cells were checked every day for confluence or contamination. Upon the cells becoming confluent they were split 1 in 10 by removing the media and washing twice with 10 ml of sterile 0.1 M PBS. Trypsin (1 ml containing ethylenediaminetetraacetic acid (EDTA)) was then added and the flask placed back in the incubator for 5 min. Growth media (9 ml) was added to the trypsinised cells and a 1 ml aliquot of the cell suspension was taken and added to media (9 ml) and placed in a new 75 cm³ flask.
4.5.3 Cell number determination using Trypan blue

Cell viability was routinely assessed for cell suspensions. Trypan blue (0.2% v/v, 20 μl) was added to the cell suspension (20 μl) and, using a Gilson pipette, this mixture was transferred to a haemocytometer slide. The number of blue (dead) cells and colourless (viable) cells were recorded by viewing under a light microscope. The cells, in a known volume (0.1 x 0.1 x 0.1 mm), were recorded and the number of viable cells was calculated per ml.

4.5.4 Assessment of cell viability using the MTT assay

The MTT assay was used to assess polymer cytotoxicity. The basis of this assay is the fact that viable cells are able to metabolise the water-soluble tetrazonium dye MTT, to produce an insoluble formazan salt (Mossmann, 1983). Initially, 96 well microtitre plates were seeded with a suspension of B16F10 murine melanoma cells in growth media containing a density of 1x10^4 cells (100 μl) per well. The cells were then incubated for 24 h followed by the careful removal of the growth media. Sterile controls and polymers dissolved in growth media (100 μl, log10 dilutions) were added (n = 6) to each plate. The plates were then incubated for a further 67 h before addition of MTT (20 μl of 5 mg·ml⁻¹ PBS solution) to each well. After a further 5 h incubation, the growth medium was carefully removed and DMSO (100 μl) cell culture grade was added to each well to dissolve the blue crystals of the tetrazolium dye that had formed. The absorbance at 550 nm was read using a microtitre plate reader and converted to % cell viability using the following formula:

\[
\frac{A_{550} \text{ treated cells}}{A_{550} \text{ control cells}} \times 100 = \text{Percentage cell viability}
\]
5 Work Described in Chapter 5

5.1 Preparation of MAA Na Salt homopolymer 62 & copolymers 82, 83, 84 & 85

Preparation of PMMA Na salt 62 from PMOSu 36: In a 10 ml round-bottom flask, PMOSu 36 (1.00 g, 5.5 mmol, $M_n = 24,800$ g·mol$^{-1}$, $M_w/M_n = 1.20$, DMF eluent, PMMA standards) was dissolved in DMF (5.0 ml) and then 2 M aqueous NaOH (6.00 ml) was added dropwise under stirring, which caused some precipitation of the polymer. The reaction vessel quickly became warm and a homogeneous solution soon followed. The solution was then heated at 70 °C for 24 h after which time further water was added (approximately 50 ml). The diluted solution was then dialysed against water using regenerated cellulose membrane (SpectraPor, MWCO 2000). Lyophilisation of the dialysed solution gave a white solid product 62 (0.31 g, 53% yield assuming 100% hydrolysis). $^1$H NMR (D$_2$O) δ 1.15 and 1.27 (br s, 3H, CH$_3$) 1.68, 2.18 (br m, 2H, CH$_2$), in addition to the proposed structure, two other signals of smaller intensity were also present 2.66 (br s, 0.1H, unassigned) 3.47 (br s, 0.07H, unassigned); $^{13}$C NMR (D$_2$O) δ 15.0, 16.8, 43.1, 43.5, 51.2-51.6, 52.8, 179.5, 180.8. GPC (PBS eluent, PMAA Na salt standards) $M_n = 22,000$ g·mol$^{-1}$ and $M_w/M_n = 1.28$.

Preparation of carboxylate copolymers 82, 83 and 84 from PMOSu 36: For each copolymer, precursor 36 (0.30 g, 1.6 mmol) was added to a 1.5 ml vial, which was then sealed with a septum centered screw-cap lid and purged with argon for approximately 2 min. Anhydrous DMSO (1.0 ml) was injected into the vial resulting in a homogeneous solution after stirring. To this solution was then added under stirring, 1-amino-2-propanol (0.25, 0.50, 0.75 reactive group equiv. in separate reactions to prepare copolymers 82, 83 and 84 respectively) and the vial then added to
an oil bath at 50 °C for between 15 min and 2 h. After allowing the vial to cool to RT an 1 M NaOH (4.25 ml) was then added to each of the reaction solutions to hydrolyse the remaining active ester groups. After allowing the solutions to stir for 5 h at RT, water was then added to each (approx. 50 ml) and the diluted solutions were subsequently dialysed against water using regenerated cellulose membrane (SpectraPor, MWCO 2000). Lyophilisation of the dialysed solution gave white solid products 82, 83 and 84 with respective yields 0.19 g, 0.21 g and 0.24 g: ¹H NMR (D₂O) product 82 δ 0.82-1.60 (br m, 24.5H, b + g + e), 1.60-2.40 (br m, 15.6H, a + f), 2.50 (br s, 2.6H, unassigned – possible cyclic imide methylene protons, see section 2.2, Chapter 5), 3.15-3.65 (br m, 2.7H, c), 3.90-4.35 (br m, 1H, d); product 83 δ 0.80-1.60 (br m, 19H, b + g + e), 1.60-2.40 (br m, 7.6H, a + f), 2.50 (br s, 1.3H, unassigned – possible cyclic imide methylene protons, see section 2.2, Chapter 5), 3.10-3.65 (br m, 2.1H, c), 3.90-4.30 (br m, 1H, d); product 84 (see also Figure 5.10, Chapter 5) δ 0.79-1.60 (br m, 14H, b + g + e), 1.60-2.40 (br m, 4.8H, a + f), 2.50 (br s, 0.9H, unassigned – possible cyclic imide methylene protons, see section 2.2, Chapter 5), 3.05-3.65 (br m, 1.9H, c), 3.90-4.30 (br m, 1H, d).

![Simplified structure](image)
Preparation of carboxylate copolymer 85: PMOSu 36 (350 mg, 1.9 mmol reactive group equiv.), a magnetic stir bar and anhydrous DSMO (2 ml) were added to a 5 ml vial and the resulting mixture stirred until a homogenous solution had formed. Triethylamine (39 mg, 0.4 mmol) was then added to the polymer solution. In a separate vial 1-amino-2-propanol (14.3 mg, 0.2 mmol) was dissolved in DMSO (1 ml) and this amine solution was then added dropwise to the polymer solution under rapid stirring at RT. After 15 min of stirring 1 M NaOH (4.3 ml) was added to the reaction solution and the resulting solution further stirred for 1 h. Additional water was then added (approx. 50 ml) and the diluted solution was dialysed against water using regenerated cellulose membrane (SpectraPor, MWCO 2000). Lyophilisation of the dialysed solution gave a white solid product 85 (130 mg, 61% yield assuming 100% conversion): \( ^1H\) NMR (D\(_2\)O) \( \delta \) 0.82-1.60 (br m, 35.3H, \( b + g + e \)), 1.60-2.40 (br m, 19.3H, \( a + f \)), 2.50 (br s, unassigned – possible cyclic imide methylene protons, see section 2.2, Chapter 5), 3.15-3.65 (br m, 1.9H, \( c \)), 3.80-4.34 (br m, 1H, \( d \)).

Attempted hydrolysis of conventional PHPMA 1 prepared using AIBN initiator: To a DMSO (5 ml) solution of conventional AIBN prepared 1 (100 mg) in a 10 ml round-bottom flask was added 1 M NaOH (0.5 ml) and the resulting solution stirred for 5 h at RT. A white solid product was isolated by precipitation of the reaction solution into acetone, followed by filtration and drying in vacuo. The product was purified by a further precipitation from a methanol solution (93 mg, 93% yield).

5.2 Chemokine and cytokine release study

The biological experiments described in this section were performed by Dr. Elisabetta Gianasi.
5.2.1 Isolation and culture of cells studied

Peritoneal cells were isolated from the peritoneal drains from HIV-negative patients on CAPD. Informed patient consent was obtained before samples were collected. Patients on CAPD were given daily 1.5 l peritoneal fluid exchanges. Peritoneal cells were obtained from the peritoneal drains of patients on CAPD at several London Hospitals. After a 24 h dwell, the fluid was collected into a standard peritoneal dialysis drain bag (Fresenius systems) and the total drain volume recorded. Bags were delivered to the laboratory within 2 h of collection, sprayed with 70% alcohol and the dialysate aliquoted into 200 ml centrifuge tubes (Greiner). They were centrifuged (400 g, 10 min, RT) and the cell pellet re-suspended in macrophage growth medium (RPMI medium, 10% mixed donor human serum, 200 IU·ml⁻¹ penicillin and 200 µg·ml⁻¹ streptomycin). Cells were enumerated using an automatic cell counter (Coulter ZM). Cells were re-suspended in MGM and the cell density adjusted to 1.0 x 10⁶ cells·ml⁻¹. Aliquots of this suspension (1 ml) were added to 48-well tissue culture plates and incubated overnight at 37 °C with 5% CO₂. The following day the supernatant was aspirated and replaced with the same volume of fresh media or fresh media containing 62, 82, 83, 84 or 85 for analysis.

5.2.2 Toxicity of 62, 82, 83, 84 and 85 on primary cells

Peritoneal macrophages were plated at a concentration of 1 x 10⁶ cells·ml⁻¹ (100 µl) in 96-well plates and left to adhere for 24 h. The cells were then added to fresh medium containing 62, 82, 83, 84, 85, dextran or poly(L-lysine) as negative and positive control respectively (0 to 2000 µg·ml⁻¹), and incubated for 67 h prior to addition of MTT (5 mg·ml⁻¹; 20 µl). The MTT solution was left for a further 5 h and then the medium was removed and DMSO (100 µl) added to dissolve the MTT crystals. The optical density was measured at 550 nm using a plate reader (Vmax kinetic microplate reader, Molecular Devices, Wokingham, UK) (Sgouras and Duncan
1990). The viability of the cell culture was expressed as a percentage of the viability of cells grown in the absence of any compound.

5.2.3 Endotoxin-free methodology and assay

Polymers \(62, 82, 83, 84\) and \(85\) were treated with active carbon prior to their biological evaluation in primary cells in order to eliminate the presence of endotoxin which could affect the interpretation of the results. An aqueous solution of the compound (20 % w/v) was stirred for 30 min with Norit GSX activated carbon (10 : 1 w/w). Subsequently the solution was filtered (0.22 μm Millex-GS Filter Unit, Millipore, Molsheim Cedex, France), collected in endotoxin-free vials and freeze-dried. The amount of endotoxin still present in \(62, 82, 83, 84\) and \(85\) was determined using the limulus amebocyte lysate (LAL) assay (Coatest plasma-endotoxin kit, Chromogenix, Falmouth, MA, USA).

5.2.4 The effect of on MIP-1β release by different cell types

Peritoneal cells were prepared as described above and endotoxin-free polymers were added to wells in triplicate to a final concentration of 2000 μg·ml\(^{-1}\) for peritoneal macrophages. The cultures were maintained at 37 °C with 5% CO\(_2\). Cell free supernatant was collected at 36 h and stored at -70 °C. The quantity of MIP-1β in the supernatants was analysed by EIA (see below).

5.2.5 Quantitation of MIP-1β in tissue culture supernatants

Levels of MIP-1β were measured in cell supernatants using a commercially available EIA (Quantikine, R&D Systems). The polymers \(62, 82, 83, 84\) and \(85\) were first confirmed not to interfere with the assay by mixing rhMIP-1β to a final concentration of 125 pg·ml\(^{-1}\) with \(62\) at 500 μg·ml\(^{-1}\) in growth medium. Solutions were incubated for 4 h at 37 °C and run as samples in the EIA. The chemokine was captured with a murine monoclonal anti-human MIP-1β antibody immobilised onto the wells of a
96-well microtitre plate. A series of standards was produced by reconstituting a lyophilised pellet of rhMIP-1β in 5 ml and serially diluting it to produce a series containing 1000, 500, 250, 125, 62.5, 31.25 and 15.6 pg·ml⁻¹ rhMIP-1β. Each standard was tested in duplicate. After adding samples to the wells, the plate was covered with adhesive foil and incubated for 1.25 h at RT and then washed 3 times with wash buffer. Horseradish peroxidase conjugated polyclonal anti-MIP-1β antibody was then added to each well for 1.25 h at RT and the plate washed. Colour was developed using a tetramethylbenzidine/hydrogen peroxide solution. After a 20 min incubation, the reaction was stopped with 50 µl of 2 N H₂SO₄. Absorbance was read on a microplate reader at 570 nm (VMAX, Molecular Devices) controlled by computer using dedicated plate reading software (SoftMax, Molecular Devices). Standards were plotted as MIP-1β concentration vs. optical density and a standard curve generated by plotting a linear regression line. The values of MIP-1β in the samples were read off the standard curve.

5.2.6 Detection of TNF-α in cell culture supernatants

After defining the MIP-1β release from peritoneal macrophages, the release of TNF-α from peritoneal macrophages was also determined after incubation for 36 h with 62 and copolymers 82, 83 and 84 at concentrations up to 2000 µg·ml⁻¹.
References


Duncan R.; 1998. Personal communication which took place at the Centre for Polymer Therapeutics, School of Pharmacy, University of London.


Gac-Breton S.; 2000. Unpublished data. Bulk polymerisation at 105 °C (1 h) or in DMF (20 wt% MAOSu 35; at temperatures ranging from 80-120°C; 1-45 h) using the Vladimir nitroxide initiator gave broad MWD polymer 36 ($M_w/M_n > 1.6$).


Müller A.H.E.; 1999. Personal communication which took place at University of Mainz, Germany.


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Sorimachi K., Akimoto K., Hattori Y., Ieiri T., Niwa A.; 1999. Secretion of TNF-
Alpha, IL-8 and Nitric Oxide by Macrophages Activated with Polyanions, and
Involvement of Interferon-Gamma in the Regulation of Cytokine Secretion. *Cytokine*,
11, 571-578.

Soyez H., Schacht E., Vanderkerken S.; 1996. The Crucial Role of Spacer Groups in

Stewart D.R, Callahan E.H, Jacob J.E., Rice J.R., Shannon K.F., St. John J.V.,
Nowotnik D.P.; 2000. Preclinical Development of the Polymer Platinate AP 5280. In
*Programme and Proceedings of the 4th International Symposium on Polymer
Therapeutics, 'From Laboratory to Clinical Practice',* 57.

Strohriegl P.; 1993. Esterification and Amidation of Polymeric Acyl Chlorides. A
New Route to Polymethacrylates and Polymethacrylamides with a Variety of

Connecting Poly[N-(2-hydroxypropyl)methacrylamide] Chains by Lysosomal
Cysteine Proteinases. *J Bioact Compat Pol*, 1, 133-146.

Enzymatically Degradable Bonds XII. Release of Daunomycin and Adriamycin from
Poly[N-(2-hydroxypropyl)methacrylamide] Copolymers. *J Control Release*, 18, 123-
132.

Transfer to Monomer. A New Method of Formation of Block Polymers. *J Am Chem
Soc*, 78, 2656-2657.


Appendix I

**Endocytosis and lysosomotropic drug delivery**

There are two types of endocytosis, namely phagocytosis and pinocytosis. Phagocytosis is the uptake of vesicular material by macrophages and monocytes. Pinocytosis is the type of endocytosis responsible for the uptake of soluble macromolecules and involves the uptake of extracellular fluid (including dissolved solutes) by membrane invagination. Depending on the structure of the macromolecule, there are three types of pinocytosis that may occur. Fluid-phase pinocytosis occurs when the macromolecule does not interact with the cell membrane; adsorptive pinocytosis takes place when the macromolecule binds non-specifically to the cell membrane via non-specific hydrophobic or electrostatic interactions and receptor mediated pinocytosis, which involves the binding of macromolecules to ligand-specific membrane receptors.

For a polymer-drug conjugate internalised by fluid phase pinocytosis a vesicle from the cell membrane is formed encapsulating the extracellular fluid including the conjugate. The contents of the vesicles are transferred to the endosomal compartment where the pH falls to ~6.0 compared with ~7.4 in the blood. From the endosome the contents are transferred to the secondary lysosomal compartment where a further decrease of pH to ~5.5 occurs. The increase in acidity observed provides an opportunity to design acid labile linker groups between the drug and polymer. In addition to this acidic environment, the lysosomes contain a battery of hydrolytic enzymes, such as proteases, nucleases and esterases. It has been observed that large quantities of thiol proteases are present in some tumour cells compared with none or very little in normal tissue (Fredrich *et al.*, 1999). It is proposed that a conjugate with a suitable peptidic linker (e.g., optimised for hydrolysis by thiol proteases) will undergo enzymatic hydrolysis to release the drug lysosomotropically (Duncan, 1992) where it can then
penetrate through the lysosomal membrane into the cytoplasm (Figure A). The Gly-
Phe-Leu-Gly linker of the HPMA copolymer conjugate PK-1 is designed to release the drug by this process (Duncan, 1992). Some studies have shown that polymer-drug conjugates with a non-degradable linker, such as Gly-Gly, do not show pharmacological activity (Subr et al., 1992). Drugs that display their pharmacological activity after being internalised and taken up into the lysosomes in this way are termed 'lysosomotropic drugs'. This concept was proposed by deDuve et al. (1974).
Figure A. Proposed mechanism of cellular uptake of (a) low MW drug,
(b) polymer-drug conjugate
Appendix II

Average molar mass and molecular weight distribution

Synthetic polymers are large molecules constructed from smaller molecules called monomers, which are covalently bound together. It is not possible to assign an exact molar mass to a synthetic polymer because there will be a mixture of polymer chains of different lengths in a sample due to the random nature of the polymerisation process. The MWD can be averaged to provide a MW and this can be essential because physical properties, and many biological properties for soluble drug carriers, can be related to the MW. The type of MW average used is determined by the physical averaging process inherent in the method used to measure the property. The number average molecular weight, $M_n$, is determined by experimental methods that count the number of polymer molecules in a sample of the polymer and is defined as the total weight of all the molecules in a sample divided by the total number of moles present.

$$
M_n = \frac{\sum N_x M_x}{\sum N_x}
$$

where $N_x$ is the number of moles whose weight is $M_x$.

Methods for measuring $M_n$ are those that measure the colligative properties of solutions, e.g., osmotic pressure using membrane osmometry, where the colligative properties are the same for small and large molecules, for solutions of the same concentration. The weight average molecular weight, $M_w$, can be obtained from light scattering observations. Since scattering is greater for large molecules than it is for smaller ones $M_w$ is biased towards higher molecular weight fractions.

$$
M_w = \frac{\sum N_x M_x^2}{\sum N_x M_x}
$$
Polydispersity, the ratio of the weight and number average molecular weight, i.e., $M_w/M_n$ can be used as a measure of the molecular weight distribution (MWD). For a perfectly monodisperse polymer this ratio is equal to unity and increases with increasing polydispersity. Polymers considered having a narrow MWD typically possess $M_w/M_n$ of around 1-1.2, but what is considered a narrow MWD will depend on the intended application. Biological polymers, such as proteins, have $M_w/M_n = 1$ because all the molecules of that protein have the same structure and hence the same MW.
Appendix III

Termination and chain transfer reactions

The termination of propagating chains can take place in several ways in radical polymerisation.

(1) interaction of two active chain ends

Two possible outcomes are possible:

(a) combination – where two chain ends combine together to form one long chain end increasing the MWD

\[
\begin{align*}
\text{CH}_2 &= \cdot \quad \text{CH}_2 \quad \rightarrow \\
\text{CH}_2 &= \cdot \\
\end{align*}
\]

(b) disproportionation – involves hydrogen abstraction from one end to give an unsaturated group and two dead polymer chains

\[
\begin{align*}
\text{CH}_2 &= \cdot \quad \text{CH}_2 \quad \rightarrow \\
\text{CH}_2 &= \cdot \\
\end{align*}
\]

One or both processes may be active in any system depending on the monomer and polymerisation conditions.

(2) reaction of an active chain end with an initiator radical

(3) termination by transfer of the active centre to another molecule
Premature termination of a propagating chain can also occur by transfer of activity to another species through a collision. This is called chain transfer and involve transfer to monomer, polymer, solvent, initiator, or chain transfer agent (as utilised for the preparation of the semi-telechelic polymer 31 in section 2.3.2, Chapter 2).

\[
\begin{align*}
\left[\text{-M}\right]_n M \cdot + XY & \rightarrow \left[\text{-M}\right]_{n+1} X + Y \cdot
\end{align*}
\]

The free radical is not destroyed by chain transfer, merely transferred. If the new species is sufficiently active another chain will be initiated from the new active centre.
Appendix IV

Example Gel Permeation Chromatogram of PMOSu 36 prepared by ATRP procedures

$M_n = 33,200 \text{ g/mol}^{-1}$, $M_w = 36,300$, $M_w/M_n = 1.09$ (DMF + 0.1% LiCl eluent, PMMA standards)

Reaction conditions:

$[\text{MOSu}]_i:[47]_i:[\text{Cu(I)Br}]_i:[\text{bipy}]_i = 100:1:1:2$, $130 \, ^\circ\text{C}$, 43 wt% DMSO
Appendix V

ATR FT-IR spectrum of PMOSu 36

ATR FT-IR spectrum of PMOSu 36 dissolved in anhydrous DMSO

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Appendix VI

Gly-Phe-Leu-Gly Synthesis

The linker Gly-Phe-Leu-Gly (I) was synthesised by two different methods. The first method was solid phase synthesis and the second method was solution phase synthesis. The second method also incorporates an activation group for drug conjugation.

\[
\text{\begin{center}
\begin{array}{c}
\text{H}_2\text{N} \\
\text{O} \\
\text{N} \\
\text{O} \\
\text{OH}
\end{array}
\end{center}}
\]

Method 1

Wang resin (resin substituted with 4-phenoxybenzylalcohol, 0.83 mol·g\(^{-1}\) loading) and Fmoc (9-Fluorenylmethoxycarbonyl) protected peptides were purchased from NovaBioChem. Wang resin (1.2 g) was loaded onto a sintered glass funnel (connected to a buchner flask and vacuum line) and washed with DMF (2 x 25 ml). The DMF was then drained using the vacuum and the bottom of the sintered glass funnel was sealed with a stopper to prevent further drainage.

Coupling of first amino acid - Glycine: Fmoc-Gly-OH (1.49 g, 5.0 mmol) was added to the resin along with 1,3-diisopropylcarbodiimide (DIC, 0.32 g, 2.5 mmol). After careful stirring, 4-dimethylaminopyridine (DMAP, 0.12 g, 1.0 mmol, dissolved in 2 ml DMF) was then added drop-wise and stirred. After the addition the mixture was
left for 1 h followed by the drainage of the excess coupling mixture. The resin was then washed with DMF (10-20 ml x 10), treated with PIP (20% v/v piperidene/DMF solution, 20 ml) for 5 min and then washed with further DMF (10-20 ml x 10).

_Coupling of next amino acid:_ Fmoc-Leu-OH (0.88 g, 2.5 mmol), TBTU (2-[1H-benzotriazole-1-yl]-1, 1, 3, 3, tetramethyluronium tetrafluoroborate, activating agent, 0.79 g, 2.5 mmol) and _N,N-diisopropylethylamine (DIPEA, proton scavenger, 0.32 g, 2.5 mmol)_ were added to DMF (5 ml) creating a slurry, which was then added to the resin on the sintered glass funnel. After 30 min with occasional stirring, the resin mixture was then drained and the resin washed with DMF, treated with PIP and further washed with DMF as described for Fmoc-Leu-OH previously. The coupling procedure was repeated for Fmoc-Phe-OH (0.97 g, 2.5 mmol) and Fmoc-Gly-OH (0.74 g, 2.5 mmol). The resin was then washed with DCM (HPLC grade, 10-20 ml x 5) and ether (spectrophotometric grade, 10-20 ml x 3) and allowed to dry. Cleavage of the oligo-peptide from the resin was achieved by transferring the resin to a small flask to which a TFA solution (100 ml of 95% v/v TFA/water) was then added. After 90 min, the mixture was then filtered retaining the filtrate. The resin was further washed with TFA (20 ml x 3) and the filtrates pooled. Removal of the TFA under vacuum left an oily residue, which was then triturated with diethyl ether (100 ml) to precipitate a white solid product (I). The solid was isolated by filtration, further washed with ethanol and then dried in vacuo (0.05 g): _1H NMR (CD3OD) δ_ 0.92 (dd, 6H, (CH3)2), 1.54-1.61 (m, 2H, (CH3)2CHCH2), 1.61-1.75 (m, 1H, (CH3)2CH), 2.94 (dd, 1H, CH-aromatic), 3.19 (dd, 1H, CH-aromatic), 3.59 (d, 1H, H2NCH2), 3.71 (q, 2H, CHCOOH), 3.89 (d, 1H, H2NCH2), 4.41-4.46 (m, 1H, NHCHCH2CH), 4.64 (q, 1H, CHCH-aromatic), 7.18-7.23 (m, 1H, ring), 7.27 (s, 4H, ring).
**Method 2**

In this second method, Gly-Phe-Leu-Gly was synthesised with a succinimidyl ester activating group. The molecule, Z-Gly-Phe-Leu-Gly-O-Su (II) was synthesised as an example of the type of molecule required for the preparation of a peptidyl linker-drug for conjugation to the active ester homopolymer developed to produce polymer-drug conjugates. After reaction of the succinimidyl ester with a drug, the Z- protection group (benzyloxycarbonyl) would be removed (e.g., by catalytic hydrogenation (Bodansky, 1993)) to reveal the primary amine of the first glycine residue to enable conjugation to the polymer

1. **Z-Gly-Phe-O-Su**: To a cooled anhydrous DMF (2 ml) solution of Z-Gly-Phe-OH (1 g, 2.8 mmol) and N-hydroxysuccinimide (0.32 g, 2.8 mmol) in a 25 ml round bottomed flask, was added DCC (0.58 g, 2.8 mmol, 2 ml DMF) and the resulting solution was placed in a refrigerator (0°C to 5°C) overnight. The white precipitate that had formed was removed by filtration (0.55 g) and the solvent removed from the clear filtrate under vacuum to leave a white sticky product. The product was purified by triteration with acetone leaving an insoluble white solid (40 mg) that was removed by filtration. The solid was further purified by dissolving in EtAc (10 ml) and washing this organic phase with 0.1 M aqueous NaHCO₃ (8 ml) and
water (8 ml). After drying of the organic phase with MgSO₄, filtration and removal of the solvent under vacuum a white solid remained (1.0 g, 78% yield): ¹H NMR (CDCl₃) δ 2.78 (s, 4H, (CH₃)₂), 3.18 (dd, 1H, CHCH₂), 3.28 (dd, 1H, CHCH₂), 3.77 (dd, 1H, NHCH₂), 3.84 (dd, 1H, NHCH₂), 5.09 (s, 2H, CH₂O), 5.21 (q, 1H, CHCH₂), 5.51 (br s, 1H, NH), 6.67 (br s, 1H, NH), 7.25-7.32 (overlaid m signals, 5H, Phe ring protons) 7.34 (s, 5H, Z ring protons).

2. Z-Gly-Phe-Leu-Gly-OH: To Z-Gly-Phe-O-Su (1.0 g, 2.2 mmol) dissolved in anhydrous THF (7 ml) in a 25 ml round bottomed flask was added under stirring aqueous NaHCO₃ solution (0.22 g, 2.2 mmol in 5 ml water) of Leu-Gly-OH (1 g, mmol). After stirring for 45 min at room temperature the reaction solution was acidified using aqueous NaHSO₄, which resulted in the precipitation a white product. This was extracted into EtAc (20 ml) and this organic phase was washed with water (10 ml x 2) and then dried over MgSO₄, filtered and the solvent removed under vacuum to leave a white solid product (0.9 g, 78% yield): ¹H NMR (CD₃OD) δ 0.91 (d, 6H, (CH₃)₂), 1.60-1.67 (m, 2H, CH₂CH(CH₃)₂), 1.85-1.90 (m, 1H, CH(CH₃)₂), 2.95-2.99 (m, 1H, CHCH₂-ring), 3.14-3.19 (m, 1H, CHCH₂-ring), 3.68-3.78 (m, 2H, NHCH₂COOH), 3.86 (s, 2H, CH₂CONH), 4.41-4.44 (m, 1H, CHCH₂CH(CH₃)₂), 4.61-4.65 (m, 1H, CHCH₂-ring), 5.08 (s, 2H, CH₂O), 7.19-7.30 (overlaid m signals, 5H, Phe ring protons), 7.35 (s, 5H, Z ring protons).

3. Z-Gly-Phe-Leu-Gly-O-Su: To a cooled anhydrous DMF (2 ml) solution of Z-Gly-Phe-Leu-Gly-OH (0.9 g, 1.7 mmol) and N-hydroxysuccinimide (0.20 g, 1.7 mmol) in a 25 ml round bottomed flask was added DCC (0.35 g, 1.7 mmol, in 2 ml DMF) and the resulting solution was placed in a refrigerator (0°C to 5°C) overnight. The white precipitate that had formed was removed by filtration and the solvent removed from the clear filtrate to leave a white solid product. The product was
purified by dissolving in EtAc (10 ml) and then washing this organic phase with 0.1 M aqueous NaHCO₃ (10 ml) and water (10 ml). After drying of the organic phase with MgSO₄, filtration and removal of the solvent under vacuum a white solid product remained (0.9 g). In a further purification step, the solid was dissolved in acetone and filtered to remove an insoluble white solid. Removal of the acetone under vacuum afforded a white solid product II (0.85 g, 79% yield):

**¹H NMR (CDCl₃)** δ 0.87 (d, 6H, (CH₃)₂), 1.49-1.56 (m, 2H, CH₂CH(CH₃)₂), 1.66-1.76 (m, 1H, CH(CH₃)₂), 2.73 (s, 4H, (CH₂)₂), 2.98-3.08 (m, 2H, CHCH₂-ring), 3.86 (d, 2H, CH₂CONH), 4.23 (dd, 1H, CH₂C(=O)O), 4.35 (dd, 1H, CH₂C(=O)O), 4.52-4.62 (m, 1H, CHCH₂CH(CH₃)₂), 4.70-4.78 (m, 1H, CHCH₂-ring), 5.04 (s, 2H, CH₂O), 5.77 (br s, 1H, NH), 6.94 (br s, 1H, NH), 7.12-7.22 (overlaid m signals, 5H, Phe ring protons), 7.35 (s, 5H, Z ring protons), 7.50 (br s, 1H, NH);

**MS (ESI) 646.3** (M + Na); analysis calculated for C₃₁H₃₇N₅O₉ (found): C, 59.70 (59.57); H, 5.98 (6.18); N, 11.23 (11.24).
List of Publications


Abstracts for conferences with selection committee:


Patents:

### Abbreviations

<table>
<thead>
<tr>
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<tr>
<td>AAS</td>
<td>atomic absorption spectroscopy</td>
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<tr>
<td>AIBN</td>
<td>2,2'-azo-bis-isobutrylnitrile</td>
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<tr>
<td>AOSu</td>
<td>N-acryloxy succinimide</td>
</tr>
<tr>
<td>ATR</td>
<td>attenuated total reflectance</td>
</tr>
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<td>ATRP</td>
<td>atom transfer radical polymerization</td>
</tr>
<tr>
<td>bipy</td>
<td>2,2'-bipyridine</td>
</tr>
<tr>
<td>CAPD</td>
<td>continuous ambulatory peritoneal dialysis</td>
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<tr>
<td>CRP</td>
<td>controlled radical polymerization</td>
</tr>
<tr>
<td>DCC</td>
<td>1,3-dicyclohexyl carbodiimide</td>
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<tr>
<td>DCM</td>
<td>dichloromethane</td>
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<td>DIVEMA</td>
<td>pyran copolymer prepared from divinylether and maleic anhydride</td>
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<td>DMA</td>
<td>N,N-dimethylacrylamide</td>
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<td>N,N-dimethylformamide</td>
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<td>dimethylsulfoxide</td>
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<td>DP</td>
<td>degree of polymerisation</td>
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<td>EAA</td>
<td>2-ethylacrylic acid</td>
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<tr>
<td>EI</td>
<td>electron ionisation</td>
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<td>EIA</td>
<td>ELISA [enzyme-linked immunsorbent assay] inhibition assay</td>
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<td>ethyl acetate</td>
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<td>equiv.</td>
<td>reactive group molar equivalents</td>
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<td>FAB</td>
<td>fast atom bombardment</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>Fmoc</td>
<td>9-fluorenlymethoxycarbonyl group</td>
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<td>FT-IR</td>
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<td>Gly</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HOBT</td>
<td>1-hydroxybenzotriazole</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>N-(2-hydroxypropyl)methacrylamide</td>
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<td>IR</td>
<td>infra-red</td>
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<td>MCP</td>
<td>methyl-2-chloropropionate</td>
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<td>tris(2-dimethylaminoethyl)amine</td>
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<td>MIP-1β</td>
<td>macrophage inflammatory protein-1-beta</td>
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<td>number average molecular weight</td>
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<td>MS</td>
<td>mass spectrometry</td>
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<td>N-hydroxysuccinimide</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<td>PAOSu</td>
<td>poly(N-acryloxy succinimide)</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PEAA</td>
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<td>phenylalanine</td>
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<td>poly(N-[2-hydroxypropyl]methacrylamide)</td>
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<tr>
<td>PK1</td>
<td>HPMA copolymer-gly-phe-leu-gly-doxorubicin conjugate</td>
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Abbreviations

PK2 PK1 with addition of galactosamine as a targeting moiety
PMAA poly(methacrylic acid)
PMMA poly(methyl methacrylate)
PMOSu poly(N-methacryloylsuccinimide)
PVP poly(N-vinylpyrrolidone)
PMDETA N,N,N',N',N''-pentamethyldiethylenetriamine
pTSA para-toluene sulphonic acid
SFRP stable free radical polymerisation
SMANCS styrene-maleic acid/anhydride neocarzinostatin
Su succinimide
RAFT reversible addition-fragmentation chain transfer
RT room temperature
TFA trifluoroacetic acid
THF tetrahydrofuran
TLC thin layer chromatography
TNF-α tumour necrosis factor-alpha
Z- benzyloxycarbonyl group