Synthesis of an erodible biomimetic hydrogel for drug delivery using native chemical ligation

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Polyethylene glycol

Native chemical ligation

Controlled drug delivery
Abstract

Hydrogels are hydrophilic, three dimensional polymers that imbibe large quantities of water while remaining insoluble in aqueous solutions due to chemical or physical cross-linking. The polymers swell in water or biological fluids, immobilizing the bioactive agent, leading to drug release in a well-defined specific manner. Thus the hydrogels’ elastic properties, swellability and biocompatibility make them excellent formulations for drug delivery. Currently, many drug potencies and therapeutic effects are limited or otherwise reduced because of the partial degradation that occurs before the administered drug reaches the desired site of action. On the other hand, sustained release medications release drugs continually, rather than providing relief of symptoms and protection solely when necessary. In fact, it would be much better if drugs could be administered in a manner that precisely matches physiological needs at desired times and at the desired site (site specific targeting). There is therefore an unmet need to develop controlled drug delivery systems especially for delivery of peptide and protein bound drugs. The purpose of this project is to produce hydrogels for structural drug delivery and time-dependent sustained release of drugs (bioactive agents). We use an innovative polymerisation strategy based on native chemical ligation (NCL) to covalently cross-link polymers to form hydrogels. When mixed in aqueous solution, four armed (polyethylene glycol) amine (PEG-4A) end functionalised with thioester and four branched N-terminal cysteine peptide dendrimers spontaneously conjugated to produce biomimetic hydrogels. These hydrogels showed superior resistance to shear stress compared to an equivalent PEG macromonomer system and were shown to be proteolytically degradable with concomitant release of a model payload molecule. This is the first report of a peptide dendrimers/PEG macromonomer approach to hydrogel production and opens up the prospect of facile hydrogel synthesis together with tailored payload release.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Boc</td>
<td>tert-butyloxycarbonyl</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diazabicyclo [5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N’-diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N’-dimethylformamide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EMP</td>
<td>ethyl 3-mercaptopropionate</td>
</tr>
<tr>
<td>EMPSA</td>
<td>ethyl 3- mercaptopropionate succinic anhydride</td>
</tr>
<tr>
<td>EtOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>food and drug administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluoro isothiocyanate</td>
</tr>
<tr>
<td>Fmoc</td>
<td>N-(9-fluorenyl)methoxycarbonyl</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HOBt</td>
<td>1-hydroxybenzotriazol</td>
</tr>
<tr>
<td>KLK</td>
<td>kallikreins</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption ionisation</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal cells</td>
</tr>
<tr>
<td>NCL</td>
<td>native chemical ligation</td>
</tr>
<tr>
<td>NHS</td>
<td>H-hydroxysuccinimide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>SPPS</td>
<td>solid-phase peptide synthesis</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TIS</td>
<td>triisopropylsilane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
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Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no materials previously published or written by another person except where due reference is made.

Signature: [Signature]
Date: 6/11/2012
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Chapter 1. INTRODUCTION

1.1 Introduction
Interdisciplinary efforts focused on drug design by chemists, pharmacologists and molecular biologists has led to sustained improvement in the pharmacological and therapeutic properties of drugs. Development of new formulations has been driven by imperatives including improved therapeutic efficiency, enhanced safety, expiry of patent and market extensions (Orive et al., 2003). Advances in protein engineering and recombinant protein technology have also led to the identification of protein and peptide therapeutics for disease treatment. However, few peptide drugs make the transition to clinical use due to their rapid elimination times and the poor in vivo stability of these biomolecules.

A potential strategy to overcome these problems is provided by drug encapsulation using biocompatible hydrogels. These three dimensional, hydrophilic polymers imbibe large quantities of water while remaining insoluble in aqueous conditions due to chemical or physical cross-linking (Peppas et al., 2000a). During swelling in water or biological fluids, hydrogels take up and effectively immobilize solubilised bioactive agents (proteins or peptides), whose release is then controlled by passive diffusion out of the hydrogel. In more sophisticated embodiments of this strategy, erodible elements are incorporated into the hydrogel or the hydrogel is designed to respond to environmental changes (the so called “smart gels”) leading to highly defined drug release profiles. These controlled drug delivery systems exhibit perfectly zero order release where in levels of release are independent of the concentration of the drug. This has the desirable effect of maintaining circulating drug levels at a therapeutically useful level. This improved control over drug management eliminates the irregular dosing interval of drugs and allows the administration of tailored levels of therapeutic compounds, leading to fewer side effects from inappropriate therapeutic concentrations in systemic circulation (Skelly and Barr, 1985). Moreover, hydrogels sequester drugs from biotransformation in the liver and kidneys leading to longer effective half-lives.
Hydrogels can be prepared from both naturally occurring and synthetic polymers. Despite natural polymers being characterised by a lack of mechanical strength and their propensity to contain pathogens, they have several inherent properties such as biocompatibility, biodegradability, and biologically recognizable moieties that support cellular activities. These characteristics are also shared by some synthetic polymers which have well-defined structures that can be modified to yield tailorable degradability and functionality which in turn makes them advantageous for synthesis of hydrogels. Because of these properties hydrogel materials resemble natural living tissue more than any other class of synthetic support (Lin and Metters, 2006). Their structural similarity to the extracellular matrix makes hydrogels biocompatible and has led to their uses in contact lenses, artificial heart substrates, artificial skin and drug delivery systems (Neuse, 2008, Lin and Anseth, 2009, Saito et al., 2005). An additional advantage of hydrogels is that they may stabilize drugs, peptides, and especially proteins in potentially harsh environments in the vicinity of the release site (Zustiak and Leach, 2010).

Despite some progress towards production of drug releasing hydrogels, currently available strategies rely on complex synthetic pathways and expensive building-block approaches. Previously used synthetic techniques, such as high energy radiation and click chemistry are limited by their use of cytotoxic intermediates and economic restrictions (Hennink and van Nostrum, 2002, van Dijk et al., 2010). The studies in this thesis address the potential of Native Chemical Ligation (NCL) to covalently cross-link polymers to form hydrogels as an alternative to using toxic and expensive polymerisation reactions.

The NCL method has proven useful in chemical synthesis of large peptides, proteins (Dawson and Kent, 2000) and more recently dendrimers (van Baal et al., 2005). NCL conjugation is typically achieved by mixing two peptides with the N-terminal peptide bearing a thioester group at its carboxy terminus whilst the C-terminal peptide has a cysteine residue at its start. The thioester and cysteine side-chains react to spontaneously form a covalent bond indistinguishable from a peptide bond forming a single polypeptide product at the ligation site. Hu and co-workers further adapted this technology to produce a hybrid polyethylene glycol (PEG) / peptide hydrogel combining the excellent biocompatibility of PEG with synthetic flexibility.
achievable through peptide synthesis (Hu et al., 2009). PEG based hydrogels have an additional advantage in that they are immunologically silent and so are unlikely to provoke an immune response when implanted subcutaneously in the body.

However, the NCL PEG/peptide hydrogel technology is limited by slow reaction rates and difficulties associated with derivatising longer peptides onto the PEG core of the hydrogel. These limitations have led us to explore novel synthetic strategies to allow facile synthesis of these hydrogels. The polymerisation technology developed here in will enable facile loading of peptide drugs into the hydrogel and will potentially allow long term systemic release, overcoming the rapid elimination of this class of therapeutic.

1.2 Hypothesis
We hypothesize that direct NCL driven polymerisation of a peptide dendrimer/PEG hydrogel is possible and desirable since it will allow biomimetic polymerisation of hydrogels containing complex pharmaceutical payloads as opposed to the current scheme described by Hu et al where very simple (dipeptide) cross links are made between PEG macromonomer and payload molecules (eg peptide hormones and cell adhesion signals) are added post polymerisation (Hu et al., 2009). Additionally, we predict that incorporation of peptides bearing sequences that are selectively cleaved by proteases expressed in a tissue-specific or pathology-specific manner will enable highly selective release of payload molecules. Finally, we hypothesise that peptide dendrimer/PEG hydrogels will be easier to synthesise than the corresponding PEG based macromonomer hydrogels, have faster gelation times and superior resistance to shear stress.

1.3 Project outline and outcomes

1. Optimisation of NCL chemistry in the context of cross-linking PEG macromonomers through peptide/thioester linkages.

We used an innovative polymerisation strategy based on NCL to covalently cross-link peptide dendrimers and functionalised PEG macromonomers to form hydrogels. PEG macromolecules bearing thioester functional groups were synthesised using star PEG-amine as a starting point and these were polymerised by addition of a four-
branched peptide macromonomer bearing an RGD motif, a protease-cleavable sequence and N-terminal cysteines to produce a biomimetic hydrogel. This reaction scheme was considerably less complex than the previously published peptide PEG macromonomer/thioester PEG macromonomer strategy from Hu et al.

2. Physical characterisation of peptide dendrimers, PEG macromonomers and the hydrogels produced by their conjugation/gelation.

We used NMR and mass spectrometry to validate synthesis of PEG-thioester macromonomers and peptide dendrimers respectively. We then assessed gelation times for both peptide dendrimer/PEG and PEG macromonomer hydrogels finding a trend towards faster gelation times for the peptide dendrimer gel system. Further physical characterisation revealed that the dendrimer peptide-based hydrogels possess superior mechanical strength compared to the macromonomer based system and trials with purified protease showed that the dendrimers system was proteolytically degradable. Inclusion of a model payload molecule in these assays also revealed that proteolytic degradation could be used as the basis for controlled release with peptide dendrimer-based hydrogels.

3. Assessment of the biological activity of biomimetic hydrogels.

Since these hydrogels are designed for eventual delivery of pharmaceutical peptides and proteins in vivo it was necessary to judge their biocompatibility. This was assessed in a tissue culture-based system. Preliminary data indicate that that the mammalian cells can survive for short periods encapsulated in hydrogels. However, the simple dipeptide cross-linked macromonomer system devised by Hu et al was shown to be toxic to MSC cells.
1.4 Drug delivery systems

In conventional drug delivery systems, the drug potencies and therapeutic effect of the drug are reduced as the concentration of the drug in the systemic circulation fluctuates across the therapeutic index in a ‘peak and valley’ pattern. This irregular dosing interval can either render the drug toxic or make it ineffective. Whilst this fluctuation is ameliorated when drugs are administered by Intravenous (IV) infusions, constant monitoring of drug plasma concentrations by health-care professionals is still required as high plasma concentrations of therapeutics may lead to toxicity and low concentration may be ineffective (Dash and CudworthII, 1998).

In order to achieve the optimum efficacy, drugs with low therapeutic index require delivery in high concentrations at a specific tissue site. Failure to achieve this dosage regimen could prove toxic to the cells in non-target tissues and have adverse immunological effects. Additionally, some drugs display region specific absorption at the biological barriers in the body (blood brain barrier, GIT membrane, “first pass” effect in the hepatic portal vein). The differential absorption rates of drugs greatly reduce the efficacy of the drug before it reaches the target site in the body (Rouge et al., 1996).

In contrast to IV administration, a well-designed sustained release drug system can significantly minimise dosing intervals and maintain a steady state drug concentration in the systemic circulation. In this scheme, drugs are administered orally or by suppository in a form where they are taken up by the body over an extended period. Techniques used to slow down drug release include formation of complexes limiting their solubility and increasing their buoyancy so that the “float” in the stomach (Singh and Kim, 2000). This reduces the fluctuation in steady-state drug levels and improves the safety margin of a potent drug. These systemic release systems have repeatedly been shown to use lower dosages of a given drug to achieve therapeutic outcomes, resulting in reducing the side effects of the drugs, healthcare costs and improving the quality of life (Brazel and Peppas, 1996).

However, in case of drugs with low therapeutic index, sustained release preparations are unsuitable for drug delivery because they suffer from burst release kinetics. This release profile results from an initial concentration-dependent on flux from the stabilising complex with concomitant release of large quantities of drugs into systemic circulation. This can be a risk as it can cause dose dumping which can lead
to toxicity and also prove to be fatal. Once within the gastrointestinal tract (GIT), drug/sustained release complexes are subject to dissolution and absorption by varying pH levels (acidic and basic) occurring along the length of the intestine. Because of the location-specific properties of the small intestine, drugs can be absorbed in a location dependent manner giving rise to the concept of “absorption windows”. Drugs exhibiting an absorption window are unsuitable for sustained drug release schemes as they would be inadequately absorbed by the major portion of the GIT. Once absorbed by the GIT, further important factors influencing effective therapeutic half-lives are absorption, metabolism and excretion of drug in the body which may vary from patient to patient.

In contrast to sustained drug release, controlled release preparations are designed to bring the drug plasma concentration to therapeutic levels by means of a loading dose and then allow maintenance of this level for a required period of time with a maintenance dose (Kumar et al., 2010). The use of controlled drug delivery provides many advantages over the sustained drug delivery systems as it allows a zero-order release, wherein the release of the drug is independent of the concentration of the substance contained in it and can be controlled for periods ranging from days to years. Also, unlike the parenteral administration of large dose drug formulations, the controlled release drug delivery systems (CRDDS) facilitate the continuous administration of small amounts of the same drug. In addition, the drugs that have short half-lives may be protected from degradation. Thus, according to (Sood and Panchagnula, 2003) the dose for CRDDS of large dose formulations with short half-lives can be significantly reduced. Along with these benefits, drug administration may be improved and facilitated in developing countries where good medical supervision is intermittently available (Langer and Peppas, 1981).

1.5 Hydrogels
The unique physical properties of hydrogels render them suitable for CRDDS applications. These polymers swell due to their hydrophilic character, but do not dissolve in biological fluids because of their cross-linked structure. Hydrogels consist of a three dimensional structure made of physical (e.g., entanglements, crystallizations) or chemical cross-links (e.g., covalent bonds) which do not allow the dissolution of hydrogels in an aqueous environment (Peppas and Mikos, 1986). They
are hydrophilic which enables them to absorb up to a thousand times their dry weight in water due to the presence of hydrophilic groups such as –OH, -CONH, -CONH₂, -COOH and –SO₃H on their polymeric backbone. Despite their strong interaction with solvent these networks are insoluble as they are chemically cross-linked by covalent bonds (tie points, junctions) and physically cross-linked by entanglements, crystallites, hydrogen bonds, weak associations such as Van der waals forces and hydrophobic interactions (Brannon-Peppas and Peppas, 1990).

By altering the density of cross-links in the gel matrix the porosity of network structure can be changed. Thus, the release of drug through the gel matrix takes place at a rate dependent on the diffusion coefficient of the bioactive agent (Peppas et al., 2000b). In this way the network structures, mesh size and diffusion property of drugs promote drug release.

In recent times, a number of hydrogels have been synthesised with mucoadhesives or molecular adhesion promoters such as PEG (Peppas et al., 1999). These hydrogels exhibit bioadhesiveness which immobilizes the drug at the site of application making it desirable for peptide and protein delivery. Hydrogels owing to their biocompatible properties (Park et al., 1993) also act as drug protectors shielding peptides and proteins from biochemical degradation in vivo. Further the use of an injectable device such as hydrogels with biodegradable and absorbable properties are preferred due to their ability to degrade within the body. This promotes the safe degradation of by-products and saves the patient from immense pain by avoiding surgery.

Hydrogels with tailorable degradation behaviour make efficient drug delivery systems since they enable better control over degradation and regulated drug release rate can also be obtained (Peppas and Merrill, 1976). Thus a controlled drug delivery system based on hydrogels provides for improved or better steady-state drug levels in the systemic circulation, maximises the utilisation of the drug after absorption, increases the safety margin of potent drugs, reduce healthcare costs as it is more efficacious requiring less frequent dosing intervals and thus improves quality of life for the patients (Kumar et al., 2010).
1.6 Structure and physical properties of hydrogels

1.6.1 Network structure

In order to use hydrogels for drug delivery, it is very important to understand the absorption and diffusion capabilities of the hydrogel structure. Hydrogel structure is mainly characterized by three important parameters: the polymer volume fraction in the swollen state, $V_{2s}$, average molecular weight between two consecutive cross-link points, $M_c$, and the network pore size, $\xi$, between the two points (Fig 1.1).

![Schematic representation of a cross-linked hydrogel](image)

**Figure 1.1:** Schematic representation of a cross-linked hydrogel

The polymer volume fraction, $(V_{2s})$, is the measure of the amount of fluid a hydrogel can incorporate into its structure while its swollen (Peppas, 1987). The molecular weight between two consecutive cross-linked polymer chains, $(M_c)$, provides a measure of degree of cross-linking in the gel. The network mesh size, $\xi$, gives the correlation distance between cross-link points and determines the porosity of the hydrogel matrix through which solute diffusion occurs. It is important to note that these parameters provide only average values since the polymerisation process itself is random.

There are various factors that affect the swelling ratio of hydrogels, among them the cross-linking ratio is the most important as it lowers the mobility of polymer chains by forming cross-links at their cross junctions. Highly cross-linked hydrogels therefore, swell less as compared to lower cross-linked hydrogels. Two methods that are frequently used to determine the swelling behaviour of the hydrogel are the equilibrium swelling theory and the rubber elasticity theory (Peppas *et al*., 2000a).
1.6.2 Equilibrium Swelling Theory

In 1953, Flory and Rehner (Flory, 1953) developed a theory to describe the equilibrium degree of cross-linked polymers. It postulated that the degree to which a polymer network swelled was governed by the elastic retractive forces of the polymer chains and the thermodynamic compatibility of the polymer and the solvent molecules. The physical picture of the equilibrium is described below (Fig 1.2):

![Image of cross-linked polymers formed by relaxed and swollen structures in water](image)

**Figure 1.2: Cross-linked polymers formed by relaxed and swollen structures in water**

When the relaxed network (state of polymer immediately after cross-linking) is moved to a large bath of water it swells to the new equilibrium in accordance with the Flory-Rehner theory (Flory, 1953).

In order to determine the change in free energy as the network is cross-linked and first exposed to a solvent, the total free energy change \( \Delta G_{total} \) upon swelling is written as (1):

\[
\Delta G_{total} = \Delta G_{elastic} + \Delta G_{mix}
\]  

(1)

Here, \( \Delta G_{elastic} \) is the contribution due to the elastic retractive forces in the gel and \( \Delta G_{mix} \) is the result of spontaneous mixing of the polymer with the swelling agent.

Upon differentiation of equation (1) with respect to the number of solvent molecules in the system (at constant T and P), an expression can be derived for the chemical potential change of the penetrating solvent \( (\Delta \mu) \). This is demonstrated in equation (2)

\[
\mu_1 - \mu_{1,0} = (\Delta \mu)_{elastic} + (\Delta \mu)_{mix}
\]  

(2)
Here, $\mu_1$ is the chemical potential of the swelling agent within the gel, $\mu_{i,0}$ is the chemical potential of the pure solvent. At equilibrium, the chemical potential of the swelling agent inside and outside of the gel are equal, therefore the elastic and mixing contributions to the chemical potential balance one another.

$(\Delta \mu_{\text{mix}})$ which is the chemical potential change upon mixing is expressed using heat and entropy of mixing and the change of chemical potential due to elastic retractive forces $(\Delta \mu_{\text{elastic}})$ is determined by rubber elasticity theory developed by Flory (Flory, 1944).

Upon equating these two contributions, the molecular weight between two cross-links of neutral hydrogels prepared without solvent can be expressed as follows (3):

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{(v/V_1)}{\ln (1 - v_{2,s} + v_{2,s} + \chi_1 v_{2,s}^2)}$$

Where $v$ is the specific volume of the polymer, $\chi_1$ is the polymer solvent interaction, $V_1$ is the molar volume of the swelling agent, $v_{2,s}$ is polymer fraction in the swollen state, $M_c$ is the molecular weight between two consecutive cross-linked polymer chains and $M_n$ is total weight of all the polymer molecules, divided by the total number of polymer molecules. If the polymers were cross-linked in the presence of a solvent, the elastic contributions must account for the volume fraction density of the chains during cross-linking.

Peppas and Merill modified the Flory–Rehner theory to account for changes in the elastic contributions to swelling (Peppas and Merrill, 1976). The equation (4) to predict the molecular weight between two cross-links in neutral hydrogels prepared in the presence of solvent is expressed below (4):

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{(v/V_1)}{\ln (1 - v_{2,s} + v_{2,s} + \chi_1 v_{2,s}^2)}$$

$v_{2,r}$ is the polymer volume fraction in the relaxed state which is the immediate state of polymer after cross-linking but before swelling.
To summarise this concept, Flory-Rehner considers the following three ideas: (1) The entropy change caused by the mixing polymer and solvent is positive and favours swelling. (2) The entropy change caused by the reduction in the number of possible chain conformations as the polymer network swells is negative and discourages swelling. (3) The heat of the mixing of polymer and solvent is usually but not always positive and it opposes mixing (Sperling, 1995).

1.6.3 Mechanical properties

Control of mechanical properties is a key factor in engineering hydrogels for drug delivery. These properties include the original rigidity of polymer chains, the polymerisation condition, type of cross-linking molecules, the cross-linking density and swelling as a result of hydrophilic/hydrophobic balance (Lee et al., 2000). Based on values for cross-linking density, another important factor, the network permeability can be determined by calculating the end-to-end distance of the swollen polymers chains between cross-linking points. The network permeation is used to study the diffusion coefficient of solutes through thin membranes of the polymer.

One of the simplest ways to increase the mechanical strength of the copolymer is to increase the relative amount of a physically stronger component. Additional changes to the comonomer composition can include varying the amount and type of cross-linking agent (e.g., Ethylene glycol diacrylate, N, N'-Methylenebisacrylamide and Divinyl benzene).

The mechanical strength of a hydrogel is determined almost entirely by the cross-links in the systems. Particularly in the swollen state where the physical entanglements are nearly nonexistent, the strength of the material increases dramatically with increasing cross-linking density. The reaction conditions will dramatically affect the final polymer product that is formed. Considerations with regard to the polymerisation reactions are the reaction time, temperature, and the amount and type of solvent.

The mechanical behaviour of hydrogels is best understood using the theories of rubber elasticity and viscoelasticity. An analogy with natural rubbers is useful here. These polymers are lightly cross-linked networks with a rather large free volume that
allows them to respond to external stresses with rapid rearrangement of the polymer segments. When a hydrogel is in the region of rubber like behaviour, the mechanical behaviour of the gel is mainly dependent on the architecture of the polymer network. Thus, the time dependence of the applied stress or strain is as important as the magnitude in predicting the material’s mechanical response (Anseth et al., 1996).

1.6.4 Diffusion in hydrogels
The pore size and network morphology of the polymer are the most important parameters in controlling the release rates of drugs in hydrogels. The pore size can be determined theoretically using indirect techniques like mercury porosimetry, rubber elasticity measurements or equilibrium swelling experiments. Direct techniques such as quasielastic laser light scattering or electron microscopy also exist. In order to determine the drug diffusion within a polymeric network it is important to calculate the ratio of the hydrodynamic radius of the drug to the network pore size. Accordingly, hydrogels for controlled release applications are classified according to their pore size. They are Macroporous hydrogels, Microporous hydrogels and Nonporous hydrogels (Langer and Peppas, 1983).

Macroporous hydrogels have large pores, usually between 0.1 and 1 µm. The pores of these gels are much larger than the diffusing species such that the solute diffusion coefficient can be described as the diffusion coefficient of the drug in the water filled pores.

Microporous hydrogels have pore sizes between 100 and 1000 Å. In these gels, the pores are water filled and drug transport occurs due to a combination of molecular diffusion and convection in the water filled pores. Transport in microporous membranes is different from macroporous membranes because the pore size begins to approach the size of the diffusing solutes.

Nonporous gels have molecular sized pores equal to the macromolecular correlation length, ξ (between 10 and 100 Å). Gels of this type are typically formed by the chemical or physical cross-linking of the polymer chains. These gels are made of polymer chains that are densely packed and severely limit solute transport (Langer and Peppas, 1983). The diffusion through nonporous membranes occurs through
free-volume. The free-volume is the area within the gel not occupied by the polymer chains.

(i) Macroscopic Analysis

The transport or release of a drug through a polymeric controlled release device can be described by classical Fickian diffusion theory, which assumes that the governing factor of drug transport in gels is ordinary diffusion (Peppas et al., 2000b). Fick’s 1st Law can be expressed as:

\[ J_i = -D_{ip} \frac{dC_i}{dx} \]

Here, \( J_i \) is the molar flux of the drug (mol/cm² s), \( C_i \) is the concentration of drug and \( D_{ip} \) is the diffusion coefficient of the drug in the polymer.

For many drug delivery devices, the release rate will be time dependent. For non-steady-state diffusion problems, Fick's second law is used to analyse the release behaviour.

Fick's second law is written as:

\[ \frac{\partial C_i}{\partial t} = \frac{\partial}{\partial x} \left( D_{ip} \frac{\partial C_i}{\partial x} \right) \]

This form of equation is for one-dimensional transport with non moving boundaries. This equation can be evaluated for the case of constant diffusion coefficients and concentration diffusion coefficients.

(ii) Determination of diffusion coefficients from Control release experiments

In controlled release experiments, the membranes containing the dispersed drug are placed in drug-free solution and the concentration of the drug in the solution is monitored over time. In order to analyse the drug release behaviour, the following empirical relationship proposed by Ritger and Peppas can be used (see below) (Ritger and Peppas, 1987).

\[ \frac{M_t}{M_{\infty}} = K t^s \]
$M_t$ and $M_\infty$ amount of drug released at time $t$, $K$ is the proportionality and $n$ is the exponent to distinguish and analyse fickian, non-fickian or Case II transport.

By determining the diffusional exponent, $n$, one can gain information about the physical mechanism controlling the drug release from a particular device. Based on the diffusional exponent, the drug transport in a slab geometry is classified as Fickian diffusion ($n=0.5$), Case II transport ($n=1$), non-Fickian or anomalous transport ($0.5<n<1$) (Costa and Sousa Lobo, 2001). In case II transport the dominant mechanism for drug transport is due to polymer relaxation as the gel swells. Anomalous transport occurs due to coupling of Fickian diffusion and polymer relaxation.

1.7 Hydrogel-based drug delivery system

The structural properties of hydrogels such as swelling behaviour and network permeability make it suitable for drug delivery. An additional advantage hydrogels provide is the protection of peptides, proteins from degradation at the vicinity of the release site (Lee and Hashida, 1995). Hydrogels through their polymeric networks allow for controlled drug release at a predetermined rate (constant if desired) over an extended period (at least 12 hrs) (Langer, 1993). Hydrogel based drug delivery systems are classified as diffusion-controlled systems, swelling-controlled, chemically controlled systems, and environmentally responsive systems (Langer and Peppas, 1983). Some of the basic systems are discussed below.

1.7.1 Diffusion-controlled release system

In diffusion-controlled systems, a substance is released from a device by diffusion from its water filled pores to the surrounding medium or through the macromolecular mesh of the matrix system (Huang and Brazel, 2001). These polymeric devices are driven by diffusion and consist of reservoir and membrane devices. Reservoirs consist of a polymeric membrane surrounding a core containing the drug (Fig 1.3). The rate-limiting step for drug release is diffusion through the outer membrane of the device. To maintain a constant release rate of drug release, the concentration difference across the membranes must remain constant. This can be achieved by designing a device with excess amount of drug in the core.
An application of this system for the time controlled release of drugs to gastrointestinal tract has been reported by Ishino and co-workers. The oral tablet was designed such that the outer shell was water permeable consisting of a formulation of hydrogenated castor oil and PEG 6000. During the process of swelling, the drug core was treated with a disintegrant such that there was enough swelling force to break the outer shell. Thus by controlling the lag time of drug release through the outer shell, a rapid release of drug with an appropriate disintegrant was obtained. Based on these studies, pulsatile release of isoniazid tablets with variant lag times were designed (Ishino et al., 1992).

In matrix devices, the drug is incorporated into the gel by equilibrium partitioning where the gel is swollen so that the drug is dispersed throughout the three-dimensional structure of the hydrogel. Release occurs due to diffusion of the drug throughout the macromolecular mesh or water-filled pores. A practical application of this system as injectable hydrogel formulation is reported, where in the initial burst of naltrexone drug incorporated in the poly(lactide-co-glycolide), PLGA was attributed to slow implant gel formation (Shively et al., 1995).

Figure 1.3: Schematic depiction of drug-release from a hydrogel-based reservoir delivery system. Adapted from (Mathiowitz, 1999)
1.7.2 Swelling-Controlled Release Systems

Swelling-controlled release occurs when diffusion of drug is faster than hydrogel swelling. The practical benefit of this theory is demonstrated by Hydroxypropyl methylcellulose (HPMC) tablets (Fig 1.4) which exhibit high swellability when in contact with water or biological fluids. The water diffuses into the polymers of the tablet resulting in the relaxation and expansion of the polymeric networks. Thus the drug diffuses out of the tablet (Siepmann et al., 1999).

The schematic representation of the swelling-controlled release system is described below in Fig 1.5. Most of the hydrogels are glassy in the dehydrated state, the drug (D) is dispersed within the glassy polymer (P). Upon contact with biological fluid (W), the hydrophilic polymer undergoes a glassy- rubbery transition with related swelling of the latter. The drug is able to diffuse out of the swollen area based on the velocity (V) and position of the front dividing the glassy and rubbery partitions of the polymer (Fig 1.5). Therefore the rate controlling factor allowing for drug delivery is the resistance of the polymer to increase in volume and change in shape (Gupta et al., 2002).
1.7.3 Chemically Controlled Release Systems

Chemically-controlled release is used to describe molecule release determined by reactions occurring within a delivery matrix. In hydrogel drug delivery systems, the degradation of cross links occurs due to the hydrolysis of water labile linkages, the enzymatic degradations of polymer backbone linkages or the dissolution of physical cross-links such as entanglements. Degradation could also occur due to the type of chemical reaction occurring during drug release such as the solubilisation of hydrophobic polymers, the protonation of pendent groups along the polymer chain or the liberation of tethered drugs through the degradation of pendent chains (Langer and Peppas, 1981).

Based on these characteristics chemically-controlled release systems are classified into two systems. They are erodible drug delivery systems and pendent chain systems.

Figure 1.5: Schematic diagram of swelling controlled release system. The water (W) penetrates the glassy polymer (P) to form a gel (G). The drug (D) is released through the swollen layer (Mathiowitz, 1999)
i) Erodible drug delivery systems

![Figure 1.6: Schematic diagram of a reservoir erodible drug delivery system. Adapted from (Mathiowitz, 1999)](image)

The two mechanisms of polymer erosion are identified as surface erosion and bulk degradation. Surface erosion occurs when the rate of erosion exceeds the rate of water permeation into the bulk of the polymer (Fig 1.6). In PLA-PEG-PLA hydrogels, the degradation rate can be pre-engineered by changing the polymer content and length of the degradable PLA segment. The slow rate of water permeation into surface eroding devices has a further beneficial effect of protecting water labile drugs up to the time of drug release (Uhrich et al., 1999).

Bulk erosion occurs when water molecules are able to permeate into the bulk of the polymer matrix at a quicker rate than erosion. Thiol acrylate networks consisting of tetrathiol monomer and polyacrylate chains are cross-linked with PLA-b-PEG-b-PLA units to form a degradable hydrogel (Reddy et al., 2005). This hydrogel consists of a backbone made of non degradable tetrathiol polyacrylate chain and the degradable PLA-b-PEG-b-PLA copolymer makes up the bulk. When placed in an aqueous environment this hydrogel exhibits high swelling properties and undergoes bulk degradation due to the hydrolytic cleavage occurring at the PLA unit. This cleavage is assumed to occur homogenously through the system and is independent of the position of PLA and the time it undergoes degradation.

ii) Pendent chain system

The Pendent chain systems consist of linear homo- or copolymers with the drug attached to the backbone chains. The encapsulated or tethered drug is released from the polymer by hydrolysis or enzymatic degradation of the linkages. Zero-order release can be obtained with these systems, provided that the cleavage of the drug is the rate controlling step.
N-(2-hydroxypropyl) methacrylamide (HPMA) copolymers are conjugated pendent systems used for controlled delivery of chemotherapeutic agents. The synthetic HPMA copolymer is synthesised such that a degradable peptide linker exists between the polymer chain and the drug (Fig 1.7). Duncan et al report the effectiveness of HPMA copolymers bound to daunomycin (anticancer drug) via several degradable peptide side chains. The greatest enzymatic release of daunomycin drug (D) occurred when the peptide side-chain Gly-Phe-Leu-Gly (X) was used as a polymer-drug spacer (Qiu and Park, 2001). In this way HPMA copolymer drug conjugates are used for site specific targeting and improve the effectiveness of chemotherapy.

1.7.4 Environmentally-responsive systems

Over the last 30 years there has been significant interest in stimuli sensitive hydrogels or “Smart” hydrogels. Many physical and chemical stimuli have been applied to induce various responses of the smart hydrogel systems. Some of these stimuli include temperature change, UV-irradiation, pH change, pressure change and ionic strength (Qiu and Park, 2001). Environmentally sensitive hydrogels have enormous potential to be used for drug delivery as these hydrogels provide site-specific delivery at varying temperature and pressure in the body. Poly (N-isopropylacrylamide) (pNIPAAm) hydrogels are a commonly studied class of temperature sensitive hydrogels mainly due to their lower critical solution temperature (LCST) close to body temperature (25-32°C). This LCST property facilitates controlled drug delivery based on the sol-gel phase conversion at body temperature. The scientific findings of Ramanan et al demonstrate pNIPAAm
nanoparticles that are incorporated into a hydrogel matrix by a photocuring process using a photo cross linker, polyethylene glycol diacrylate (PEGDA) and a photoiniator (I-2959) (Ramanan et al., 2006). BSA release from these hydrogels in response to temperature changes suggested that this material could be used for controlled drug delivery.

Figure 1.8. Biomimetic hydrogel (Sannino et al., 2009).

The smart behaviour of some cellulose derivatives (e.g., NaCMC, HPMC) in response to physiologically relevant variables (i.e., pH, ionic strength, temperature) makes the resulting hydrogels particularly appealing for in vivo applications (Sannino et al., 2009) (Fig 1.8).

While the concept and strategy of synthesising environmental sensitive hydrogels is plausible, they lack the mechanical strength required for drug delivery. These hydrogels have a slow response time and a fast response is possible only through production of thinner and smaller hydrogel systems. This weakens the physical strength of the hydrogel making it inappropriate for use in in vivo tests. Additionally, the polymers used for synthesis of the hydrogel are required to be biodegradable and biocompatible, constituting a formidable challenge in terms of polymerisation. Thus a hydrogel with biocompatible properties along with favourable cross-linking strategy is required for the polymerisation of hydrogels. These methods are discussed in the next section 1.8.

1.8 Methods of cross-linking

Cross-linking between hydrophilic polymer chains is absolutely essential to prevent the dissolution of hydrogels in an aqueous environment. Once the cross-links between the different polymers are obtained, the network shows both viscous and elastic properties. In chemically cross-linked gels, covalent bonds are present between different polymer chains. In physically cross-linked gels, dissolution is
 prevented by physical interactions, which exist between different polymer chains. A great variety of chemical and physical methods have been used to prepare hydrogels. A representative selection of these methods along with their applications have been summarised in Table 1.1 and 1.2 below (All references have been included in the sections to follow).

### Physical Hydrogels

<table>
<thead>
<tr>
<th>Type of Crosslinking</th>
<th>Method of Crosslinking</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crosslinking by ionic interactions.</td>
<td>Alginate polymer crosslinked by calcium ions.</td>
<td>Release of proteins and encapsulation of cells.</td>
</tr>
<tr>
<td>Crosslinking by crystallization.</td>
<td>Freeze thawing methods to form cross-linked PVA crystallites.</td>
<td>Release of drugs through microspheres.</td>
</tr>
<tr>
<td>Crosslinking by hydrogen bonds.</td>
<td>PAA, PMA and PEG complexes held together by hydrogen bonding.</td>
<td>Release of drugs through polymer complexes.</td>
</tr>
</tbody>
</table>

**Table 1.1: General methods for cross-linking Physical Hydrogels.**

### 1.8.1 Physically Cross-linked Hydrogels

There has been significant interest in physically cross-linked hydrogels because they do not require toxic cross-linking agents for formation. There are many different methods investigated, with the more commonly used strategies listed below.

(i) **Cross-linking by ionic interactions**

Alginate is the most commonly used polymer that can be cross-linked by ionic interactions. The biocompatibility, low toxicity and simple gelation with divalent cations such as Ca$^{2+}$, Mg$^{2+}$, Ba$^{2+}$ and Sr$^{2+}$ make it suitable for drug delivery. Cross-linking occurs at room temperature and physiological pH making this an ideal hydrogel for use *in vivo*. Selective ionic binding of alginate is mainly based on length of Guluuronate or G blocks (Draget *et al.*, 1997). Higher stability of alginate hydrogels with stronger ionic interactions and mechanical strength is seen with longer G blocks. These hydrogels have been used for release of proteins and encapsulation of cells (Kuo and Ma, 2001). The major disadvantage associated with this system is that alginates lack suitable anchoring points for mammalian cells and
they degrade spontaneously through loss of divalent ions in growth medium (Smetana, 1993, Lee and Mooney, 2001).

(ii) Cross-linking by crystallization

Poly (vinyl alcohol) (PVA) gel formation is attributed to crystallization due to physical cross-links in the hydrogel. When aqueous PVA is kept at room temperature, it gradually forms a gel but with weak mechanical properties. However, when the aqueous solutions of these polymers undergo repeated freeze-thawing process, a strong and highly elastic gel is formed. The number and stability of the crystallites are increased as the freeze thawing cycles are increased. PVA hydrogels exhibit bioadhesive characteristics with high mechanical strength, making them suitable to be used in contact lenses, lining of artificial heart and drug delivery devices (Hassan and Peppas, 2000).

(iii) Cross-linking by hydrogen bonding

Poly acrylic acid (PAA) and Poly methacrylic acid (PMA) form complexes with PEG through hydrogen bonds between the oxygen of the PEG and the carboxylic group of PMA. The hydrogen bonds are formed when the PMA carboxylic acid is protonated with the implication that swelling is controlled by pH. PMA and PEG complexes prepared at low pH can be dissolved in ethanol. Upon injecting the PMA-PEG precursor complex, it is in a liquid form, but it gels on reaching its specific site as the precursor complex equilibrates to physiological pHs (Hennink and van Nostrum, 2002). Hydrophobic interactions play a role in joining these complexes together, but they are not stable, with the resulting hydrogel dissociating over time. Hydrogen-bonded networks can dilute and disperse over a few hours in vivo due to the influx of water, restricting their use to relatively short-acting drug release systems unless some other form of cross-linking is also used (Hoare and Kohane, 2008).
1.8.2 Chemically Cross-linked hydrogels

(i) Cross-linking by radical polymerisation
Chemically cross-linked hydrogels can be formed by free radical polymerisation of monomers with cross-linking agent. Poly 2-hydroxyethyl methacrylate (PHEMA) hydrogels can be used for drug delivery as they exhibit high degree of chemical stability and mechanical strength. PHEMA monomers are not suitable biomaterials to adsorb protein and cannot encapsulate cells. To eliminate this effect, simultaneous copolymerisation of PHEMA with a suitable cross-linker was carried out in order to tailor the shape of the hydrogel during gel formation (Peppas et al., 1985). In 1960, using radical polymerisation, Wichterle and Lim cross-linked low molecular weight...
monomers PHEMA with cross-linking agent EGDMA (Ethylene glycol dimethacrylate) to form a hydrogel. The permeability of hydrogels could be easily controlled by the degree of cross-linking. Similar procedures have been used to synthesise many hydrogel systems (Langer and Peppas, 1981). Their similarity to living soft tissues and their efficacy in preventing inflammation to surrounding tissue makes them suitable for drug delivery.

Dextran, a bacterial polysaccharide has been used as a building block for degradable hydrogels (Fig 1.9). Dextran hydrogels can be obtained by derivatising dextrans with double bonds, followed by radical polymerisation of the aqueous solution of this derivatised dextran. Research pioneered by Van dijk et al, reports dextran hydrogels prepared by free radical polymerisation of aqueous glycidyl methacrylate (GMA) derivatized with dextran (Dex-GMA) in the presence of a suitable initiator system N,N,N′N′-tetramethylene-doamine (TEMED) and ammonium peroxydisulphate (APS) as a model system for protein delivery (van Dijk-Wolthuis et al., 1995). The protein loaded hydrogels can be prepared by mixing the protein with the Dex-GMA solution prior to the radical polymerisation reaction. Further, controlling the GMA substitution in the Dex-GMA hydrogel and its swelling property make suitable systems for protein release, delivery of drugs and imaging agents. The drawback associated with this method is that the unreacted TEMED and APS have to be removed from the gel before in vivo application. However care must be taken to add limited amount of this initiator systems as they can cause unwanted oxidation of methionine if it is present in the dextran matrix of the protein (Stenekes and Hennink, 2000).
(ii) Cross-linking by high energy irradiation

The two types of ionizing radiation mainly used in preparing hydrogels are gamma rays and electron beams. Polymers can be made by radiation cross-linking of monomers, both in solid and aqueous solution. Cross-linking in the solid phase has some drawbacks as it requires high doses of irradiation and residual oxygen may be activated and cause unwanted reactions once the gel is formed (Rosiak et al., 2002). The more frequently used method for monomer irradiation is in aqueous solutions. Polymerisation can occur through two distinct but relate mechanisms: direct activation and the indirect (solvent mediated) activation. Direct activation results from irradiation of polymeric chains producing reactive intermediates whereas indirect activation occurs through intermediates generated in water which then propagate macromolecular cross-linking (Rosiak and Yoshii, 1999). This latter effect can be summarized by the reaction.

\[
\text{H}_2\text{O} \rightarrow \text{H}^\cdot, \text{OH}^\cdot, \text{e}^\cdot
\]

The solvent radicals produced in this manner can then act upon soluble polymers to form macroradicals which in turn can drive polymerisation and gelation. Poly (vinyl alcohol), poly (ethylene glycol) and poly (acrylic acid) are well known examples of polymers which can be cross-linked by high energy irradiation (Darwis, 2009). Since many of the reactive intermediates produced are harmful or toxic, particular care has to be taken to remove unreacted radicals in order to avoid unwanted and parallel reaction during the formation of the hydrogel. Additionally, irradiation requires the use of costly specialised equipment.

(iii) Cross-linking by copolymerisation

Water-soluble polymers owe their solubility properties to the presence of functional groups (mainly OH, COOH, NH\textsubscript{2}) which can be used for the formation of hydrogels. The functional groups with complementary reactivity form covalent bonds such as amine-carboxylic acid or isocyanate-OH/NH\textsubscript{2} reaction, or Schiff base formation. Metters et al., demonstrated that the degradation could be accelerated by copolymerisation of PEG-PLA macromers with acrylic acids. The degradation behaviour was found to be more efficient than the linear PLA networks, as in these systems, the PLA segments contribute only a fraction to the molecular weight of the
copolymer. Furthermore, due to the presence of hydrophilic PEG blocks, these gels are highly swollen, allowing more efficient removal of degradation products from the system (Metters et al., 2000). Additionally, Hubell et al. described the formation of a photopolymerised hydrogel with a precursor of PEG copolymerised with α-hydroxy acid and acrylate termini at each end. The degradation and permeability of the hydrogel could be altered by changing the composition of the precursors. In vitro release of proteins and oligonucleotides was observed using these hydrogels (West and Hubbell, 1995).

(iv) Cross-linking by aldehydes
Poly (vinyl alcohol) (PVA) hydrogel membranes with mesh size asymmetry were prepared by cross-linking PVA with glutaraldehyde. The reaction between the hydroxyl groups (OH) of PVA and the aldehyde group (CHO) of glutaraldehyde formed the acetal bridge with the release of water. The reaction produces water, thus no toxic products are formed from the reaction. However the residual glutaraldehyde must be removed from the reaction inorder to use it in biomedical applications. The synthesis of PVA membranes by this method produces high-flux and high selectivity membrane for downstream bioseperation and cell encapsulation (Dai and Barbari, 1999).

(v) Cross-linking by addition reaction
Water-soluble polymers can be converted into hydrogels using bis (or higher) functional cross-linking agents which react with functional groups of water-soluble polymers via addition reactions. The network properties can be easily tailored by the concentration of the dissolved polymer and the amount of cross-linking agent. Coviello et al. report the hydrogel formation between the polycarboxylated derivative of scleroglucan (sclerox) and alkane dihalides (1,6 hexanediibromide). The new hydrogels were analysed in terms of water uptake and diffusion experiments; a possible application for sustained release of tablets is produced (Coviello et al., 1999).
(vi) Cross-linking by condensation reaction
Condensation reactions can occur between hydroxyl groups or amines with carboxylic acids or derivatives to form polyesters or polyamides. This reaction is also used to synthesise hydrogels. Hu et al describes the use of native chemical ligation (NCL) reaction to covalently link soluble polymers into hydrogels. Macromonomers consisting of a four armed poly(ethylene glycol) (PEG) core end-functionalised with either thioester or N-terminal cysteine peptide were designed and synthesised. Upon mixing aqueous solutions of the thioester and N-terminal cysteine macromonomers, rigid hydrogels formed within minutes (Hu et al., 2009) (Fig 1.10).

1.9 Native Chemical Ligation (NCL)
NCL methods were originally developed to prepare proteins with native backbone structures from synthetic unprotected peptide precursors. The N-terminal cysteine – containing peptide is chemically ligated to a second macromonomer possessing an thioester group with the resultant formation of a native peptide bond at the ligation junction (Muir et al., 1998). Dawson et al. have investigated this process in detail as it relates to peptide-peptide conjugation. Initially, a reversible trans thioesterification reaction takes place between the C-terminal thioester from peptide 1 segment and the sulfhydryl group from the N-terminal cysteine from peptide 2 segment (Fig 1.11). This highly chemoselective reaction is performed in aqueous solution at neutral pH. The ligated peptide thioester intermediate, due to intramolecular nucleophilic attack, then undergoes spontaneous rapid rearrangement to form a native peptide bond at
the ligation site. Thus the desired target full-length polypeptide product is obtained (Dawson and Kent, 2000).

![Figure 1.11: Chemistry of chemical ligation (Dawson et al., 1994).](image)

This study focuses on the use of NCL for the synthesis of hydrogels, as it gives a single polypeptide product with a native peptide bond at the ligation site. This hydrogel cross-linking strategy has attractive features such as chemoselectivity under mild aqueous conditions without the use of an additional catalyst. The chemoselectivity of N-terminal cysteine leads to thiol/thioester exchange reactions resulting in a native polypeptide chain in the final form. The mild native chemical reaction combined with peptide self-assembly results in increased stiffness of the peptide hydrogels (Jung et al., 2008). But in the case of multiple peptide fragments, multiple ligation and deprotection cycles are required to assemble very long peptide sequences. An exquisite feature of NCL reaction is that ligation occurs at a unique N-terminal cysteine residue regardless of other residual cysteines present in the reaction. While the need of N-terminal cysteine in the peptide sequence is required to facilitate the NCL reaction, it is seen as a limitation as cysteine is seldom distributed in the peptide sequence. However, it is possible to insert a cysteine using Boc chemistry and Solid phase peptide synthesis (SPPS) by the use of auxillary groups instead of n-terminal Cysteine (Botti et al., 2001).
1.10 PEG

Polyethylene glycol (PEG) is a much used polymer in biomedical application due to its non toxic and biocompatible character (Zhang et al., 2002). PEG represented by HO-CH₂-(CH₂-O-CH₂)ₙ-CH₂-OH is an amphiphilic polymer. The presence of an ether oxygen linkage as a hydrogen bond acceptor makes the polymer vastly hydrophilic and hence soluble in most solvents. To couple PEG to a molecule it is necessary to have a functional group at the terminus to react with some group on the molecule. For this purpose PEG is end functionalised by the addition of amine, thiol, maleimide, acrylate or vinyl sulfone (VS) reactive groups (Cho et al., 2010). PEG compared to other polymers has a relatively narrow polydisperse index (close to 1.00), ensuring a reproducible high quality output (Saboktakin, 2010). PEG due to its non toxic and biological properties has been FDA approved for medical application (Tan and Marra, 2010).

PEG known to be non-immunogenic (Katre, 1990), on covalent attachment to protein or peptides makes the resulting PEG conjugate more soluble. Greenwald et al report Paclitaxel, an insoluble drug to undergo transient modification to become soluble in water when conjugated with PEG (Greenwald et al., 1996). Therefore the shielding effect on proteins, reduces the immunogenicity and the rate of kidney clearance (Bailon and Won, 2009). This makes PEG beneficial for pharmaceutical applications.

In recent times, multifunctional branched PEGs and dendrimers have allowed for high drug loading with longer dosage intervals (Berna et al., 2006). These branched PEG’s have been reported to be more effective than linear PEG in increasing the water solubility, prolonging the plasma half life and preventing proteolytic degradation of the small bioactive material (Caliceti and Veronese, 2003). Further in protein/ peptide drug delivery, the non-immunogenicity of PEG chains is further improved by increasing the molecular weight and the number of polymer chains of PEG (Cho et al., 2010). Thus the pharmacokinetic and immunogenicity potential of the bioactive material is greatly improved by this approach.
Due to these advantages, in our approach we used 4 arm PEG (Fig 1.12). The benign nature and availability of multifunctional PEG derivatives has led to the preparation and application of cross-linked PEG networks or hydrogels. PEG hydrogels are loosely cross-linked and incorporate up to 95% water in total mass. This high hydrophilicity creates high permeability for the encapsulated small molecules. However, this property can sometimes be challenging, as the rapid release of drugs could lead to an overdose of the drug at the target site (Metters et al., 2000).

Studies conducted by Anseth and co-workers report the polymerising of PEG core hydrogels by adding methacrylates to both ends of PEG-CAP (caprolactone) tri-block copolymers (Rice et al., 2006). The rate of degradation in this case was controlled by the concentration of the lipase enzyme added to the reaction and the number of CAP blocks in the hydrogel. Hence, this model was used to determine the rate of degradation and mass loss of the material making it suitable to predict development of regenerated tissue in PEG hydrogels. Our study focuses on a similar approach where in the NCL hydrogels are polymerised with KLK 4 protease specific degradation sequence (FCQR). After the polymerisation of these hydrogels, systematic degradation was expected on adding pre-determined concentration of the trypsin enzyme to these biomimetic hydrogels.

1.11 Biomimetic hydrogel

Hydrogels as noted above have been developed for medical use as carriers for novel pharmaceuticals and delivery of drugs, peptides and protein. Recent studies in this arena have led to production of biomimetic hydrogels possessing recognition sites that are structurally and functionally similar to those found in biological tissues. This
advance has resulted from a combination of natural building blocks including peptides, proteins and lipids (Venkatesh et al., 2005).

![Figure 1.13: Synthesis of Biomimetic hydrogels (Duoptix™) to replace the cornea of the eye. (Photo courtesy of Dr Christopher Ta, Stanford University, USA)](image)

Research on biomimetic hydrogels pioneered by David Myung, Curtis Frank and Christopher Ta at Stanford University has focused on the development of biomimetic hydrogels (Duoptix™) with the goal of producing an artificial cornea (Fig 1.13). Their hydrogel consists of a double network of PEG and PAA (poly acrylic acid) forming a central transparent core which is surrounded by a microperforated rim designed to promote peripheral tissue integration within the host eye (Griffith et al., 2008). The double network design (PEG + PAA) provides both biocompatibility along with the advantage of high strength and permeability. This central core also supports cell growth when the surface components of the hydrogel are modified with cell adhesion sequences.

By designing the molecular functionality and structure of a synthetic polymer network to mimic biological systems, three dimensional network structures exhibiting structural similarities can be used for in vivo animal models or drug delivery applications (Lin and Metters, 2006). A recent study conducted by Lutolf et al reports synthetic hydrogels that have been molecularly engineered to mimic the invasive characteristics of native extracellular matrices. A combination of integrin-binding sites and substrates for matrix metalloproteinases (MMP) was required to make the networks degradable and invasive by cells via cell-secreted matrix metalloproteinases (Lutolf et al., 2003). MMP’s were used in this application, as these protease enzymes cleave virtually all constituents of the ECM at specific sites.
Remodelling by proteases is an important feature of hydrogels for use in biomaterials and biomedical applications. Many orthopaedic applications require that foreign implants be colonised and eventually degraded by the host tissues in which they have been implanted (Bramono et al., 2004). A common way of engineering this erodibility into hydrogels is to use cross-linking peptides that bear sequences that are degraded either by matrix metalloproteinases or by other hydrolytic enzymes produced by invading cells (Sintov and Levy, 1997).

Proteases/peptidases are enzymes that catalyse peptide bond hydrolysis and perform vital functions in the human body. Protease action is always irreversible and can involve either indiscriminant and non-specific degradation of protein substrates, as in apoptosis, or highly specific proteolytic processing of target proteins (Borgoño et al., 2004). Serine proteases were found to play a wide range of roles in the human body, right from the regulation of blood pressure and electrolyte balance to tissue remodelling, prohormone processing, neural plasticity and skin desquamation. Recent data suggest that these proteases may causally be involved in carcinogenesis, particularly in tumour metastasis and invasion, and, thus may represent attractive drug targets to consider for therapeutic intervention (Borgoño et al., 2004). In particular a super family of serine proteases, the kallikrein-related peptidases has recently been suggested to play key roles both in carcinogenesis and wound healing (Swedberg et al., 2010).

1.12 Kallikrein peptidase family

The tissue kallikrein including the kallikrein-related peptidases (KLKs) represent a family of 15 chymo trypsin like serine proteases in the chromosomal band 19q13.4 (Clements et al., 2004). All tissue KLK genes consist of five exons of almost identical size and intermittent introns displaying a fully conserved phase (Yousef et al., 2002). Each KLK contains the conserved serine protease catalytic triad (His 57, Asp102, Ser 195) and N-terminal regulatory pre- and pro- sequences, meaning that KLKs are expressed as inactive precursors (zymogens). These inactive zymogens are activated by the removal of the n-terminal pro-peptides on reaching the extracellular space (Swedberg et al., 2010).
Tissue kallikrein are widely expressed in all tissues and fluids of the human body. They perform a diverse range of physiological functions from the regulation of blood pressure and electrolyte balance (Scioli and Carretero, 1986) to pro-hormone processing and skin desquamation (Komatsu et al., 2005). Along with the important role played by kallikrein proteases in normal physiology, dysregulated tissue kallikrein expression has strongly been linked to the development of diseases. The expression of KLKs in lungs, pancreas, colon (Yousef et al., 2004) along with their role in hormone dependent cancers like prostate (Magklara et al., 2000), ovarian (White et al., 2009) and breast (Zhang et al., 2006) have confirmed the role of KLK’s in carcinogenesis. As a result, many kallikreins in addition to hk3/PSA have been identified as promising diagnostic/ prognostic biomarkers for cancer (Yousef et al., 2001).

From previous literature, it has been established that most of the kallikrein-related peptidases are endowed with trypsin–like specificity (Yousef and Diamandis, 2002). Matsumura et al determined KLK4’s substrate specificity, activity and inhibition by using two tetrapeptide positional scanning - synthetic combinatorial libraries (PS-SCL) (Matsumura et al., 2005). They further tested the cleavage rate of B-FVR peptide substrate (KLK4 substrate with greatest activity) by treating it with various protease inhibitors. Trypsin was found to inhibit KLK 4 more effectively when compared to the other protease inhibitors. This result led to the conclusion that the three dimensional structure and active site configuration of KLK4 is similar to trypsin. The trypsin-like specificity for KLK4 was further confirmed as KLK4 demonstrated a strong cleavage specificity when Arginine was placed at the P1 position of the substrate, while the P2-P4 positions were randomized (Matsumura et al., 2005). Given the importance of the kallikreins both as potential targets in anticancer chemotherapy and their potential role in wound healing, we chose to use their proteolytic activity as a driver for designing degradability and controlled drug release into our hydrogel scheme as described in the following chapters.
Chapter 2. MATERIALS AND METHODS

4-armed PEG amine (MW 10 kDa) and 2-chlorotrityl resin were purchased from Iris Biotech GMBH (Germany). Rink amide resin, N,N-diisopropylethylamine (DIPEA) and fluorenlymethyloxycarbonyl chloride (Fmoc) protected amino acids were purchased from Auspep. Ethyl 3- Mercaptopropionate (EMP) was purchased from Spectrum chemicals. Dichloro methane (DCM), N,N-dimethylformamide (DMF), N,N,N',N' Tetramethylene diamine (TEMED), Trifluoroacetic acid (TFA), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBT) and 4-dimethylaminopyridine (DMAP) were purchased from Biorad (Australia). Acetonitrile (ACN) was purchased from Ajax Finechem (New Zealand). Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), diethyl ether, methanol, piperidine, triisopropylsilane (TIS), 1,2 -ethanedithiol (EDT) and Thioanisole were purchased from Sigma-Aldrich chemical company.

2.1 Solid peptide synthesis (SPPS)

SPPS was carried out using (Fmoc) as semipermanent protecting group. 2-chlorotrityl resin (1.3 mmol/g loading) was derivatised with the C-terminal amino acid (Fmoc protected) dissolved in DMF to give a final resin loading of 0.45 mmol/g. Derivatisation was effected through the 2-chlorotrityl group with neutralization of the C-terminal amino acid being acheived by addition of 2 fold molar excess of DIPEA. Further, chain elongation was achieved with 4 eq. 9-fluorenlymethyl carbamate (Fmoc) protected amino acids dissolved in 0.25 M each of HBTU and DIPEA in DMF for 5 minutes using a CEM microwave assisted peptide synthesiser operating at 20 W, 70°C with compressed air cooling. Fmoc deprotection was achieved using 20% piperidine and 5% HOBT, in DMF for 3 mins with microwave assistance as for chain elongation. Note: to limit racemization at histidine and cysteine residues, coupling of these amino acids was carried out at 50°C. Fully protected peptides were cleaved from the solid support by successive changes of 1% TFA in DCM followed by ether precipitation. Fully deprotected peptides were cleaved from the solid support by treatment with 95% TFA containing scavengers (H2O 1.25%, TIS 1.25% and Thioanisole 2.5 %) for one hour at room
temperature. Cleaved peptide was isolated by trituration with 10 volumes of ice cold ether.

2.2 Synthesis of Thioester PEG macromonomer

The procedure for the synthesis of the thioester PEG macromonomer was adapted from a previous protocol (Hu et al., 2009). The schematic of the synthesis is provided in Figure 2.1. EMP (4.5 g, 44mmol) was added under nitrogen (N₂) gas to a stirring solution of succinic anhydride (3.0g, 20mmol) and DMAP (122mg, 1mmol) in 25 ml of acetonitrile-pyridine ratio (9:1). The reaction mixture was stirred at room temperature overnight. The solution was concentrated under reduced pressure and dried in vacuo. The residue was then dissolved in 50ml Ethyl acetate (EtOAc). The EtOAc solution was washed three times with 30ml 0.1 N HCl aqueous solution and H₂O. The solution was dried over anhydrous magnesium sulphate (MgSO₄). After filtration the solution was concentrated to dryness under reduced pressure in vacuo.

A solution of EMPSA (0.0234 g, 1mmol) in DCM (2ml) was added into a vial containing PEG-4A (1g, 0.4mmol of amine) and BOP (0.221g, 1mmol) followed by addition of DIPEA (0.348 ml, 2mmol). The mixture was vortexed for 5 min and rocked for 2 hours. The purification of product was performed by dilution with

![Figure 2.1: Synthesis of Thioester PEG macromonomer. EMP reacts with succinic anhydride to form EMPSA. The EMPSA reacts with PEG 4A to form a Thioester.](image-url)}
Methanol to a final volume of 50 ml. The solution was mixed thoroughly and frozen at -20°C. The precipitate was collected by centrifugation (Avanti J-30 I) (-9°C, 6000 rpm, 20 min) and decanting the solvent. The purification cycle was performed at room temperature by dissolution in Methanol to a final volume of 50 ml, freezing at -20°C, centrifugation at -9°C, and decanting the Methanol four times. This was followed by precipitation in diethyl ether, and drying in vacuo.

2.3 Synthesis of N-terminal Cysteine PEG Macromonomer

The Boc-Cys-(Trt)-OH (0.50g, 0.5mmol) was dissolved in DCM (2ml). In a 14ml glass microwave synthesiser vial, PEG 4A (0.50g, 0.5mmol) and BOP (0.11g, 0.5mmol) were dissolved in DIPEA (44µl, 0.5mmol). The coupling reaction was carried out using a microwave peptide synthesiser according to protocol (CEM Discover SPPS). The cyclisation was carried out at 40°C for 30 minutes. Post synthesis the precipitate was dissolved in 25 ml Methanol and frozen at -20°C. The precipitate was collected using a bench top centrifuge (Avanti J-30 I) (-10°C, 3000 rpm, 10 min). The pellet was scratched and washed in diethyl ether. The diethyl ether was tipped off and the precipitate was dried under Nitrogen (N2) gas. The protected cysteine-PEG4A conjugates were then vortexed and treated with 30ml of TFA containing TIS (1ml) and EDT (1ml) at room temperature for 2 hours.

![Figure 2.2: Synthesis of cysteine PEG macromonomer. The Boc-cysteine (Trt)-OH was treated with PEG 4A solution to form a Cysteine PEG macromonomer.](image)
The bulk of the TFA was then removed leaving 3ml of the solution. It was then placed on heating block at 45°C and later dried under N₂. The precipitate was dissolved in 50ml MeOH, frozen at -20°C and spun down in benchtop centrifuge (-10°C, 3000 rpm, 10 min) (repeating twice). The ninhydrin test gave blue colour indicating the Boc protection group was removed. The next step was precipitation in diethyl ether (40ml) (repeated twice). The precipitate was redissolved in 25 ml 0.1M NH₄HCO₃. Argon was bubbled through the solution for 20 mins. Finally lyophilisation was carried out overnight to give a total yield of 76% salt free conjugates N-terminal cysteine macromonomers.

2.4 Synthesis of PEG/ comb-peptide dendrons (Mass spec)

10 mg NHS PEG and 10 mg Fmoc-amino PEG₃₅₀–COOH was weighed out in two tubes. The NHS PEG (N-Hydroxysuccinimide) and Fmoc-amino PEG- COOH was treated with 80 µl dicycloexylcarbodiimide (DCC) and 2.3 µl chloroform and stirred overnight at room temperature. The deprotection was carried out by treatment with 50% Piperidine in DCM (200 µl). This was followed by resuspension in 75% methanol. The final precipitation step was done in 50% diethyl ether. A 1/10 dilution of products was done and analysed using Surface Enhanced Laser Desorption Ionisation (SELDI). In order to prepare the Diamino-bis-PEG with a suitable spacer, Fmoc Lysine was coupled onto the 2-chloro trityl chloride resin (300mg). The Fmoc lysine (23mg) was added to 10 ml tube containing 1 ml DMF and 40 µl DIPEA. It was nutated on a rocking platform for 1 hour. 5 ml of methanol was added to the reaction vessel and swirled for 5 mins. A fully protected 3 amino acid spacer lysine, β-alanine and glycine (0.18mM) was synthesised according to CEM discover SPPS protocol and coupled onto Fmoc lysine. Fmoc-NH-PEG-COOH (150 mg) was coupled onto 100 mg (Fmoc Lys) using a Peptide synthesiser (CEM discover SPPS protocol). Deprotection and cleavage was carried out with 1 ml 3% TFA. Precipitation was observed overnight in 40 ml diethyl ether.
2.5 Synthesising the lysine core peptide dendrimer

A two part strategy was used to synthesise the dendrimeric macromonomers for later hydrogel preparation. Initially a tetrameric lysine core was produced to which peptides were later grafted following a second independent synthesis. This approach enabled mass spectrometric evaluation of the peptides which would not have been possible if peptides were directly synthesised onto the polylysine core. Dendrimeric assembly was carried out using conventional Fmoc SPPS as described above with the exception that lysine was Fmoc protected at both the amino terminus and the ε-amino group. Hence on deprotection both amino groups would be available for coupling producing the dendimeric growth pattern shown above (Fig 2.3).

2.6 NCL polymerisation

The first batch of NCL hydrogels was polymerised by mixing, 10 % w/v 4 arm thioester PEG macromonomer with 4 arm 10% w/v cysteine PEG macromonomer in an aqueous solution. To polymerise the second batch of NCL hydrogels, 10 % w/v 4 arm thioester PEG macromonomer was mixed with 4 arm 10% w/v peptide dendrimer combination in an aqueous solution. Reactions were carried out in a total
volume of 100 µL in PBS buffer at room temperature, with gelation being tested for by inversion of reaction tubes. Gelation was assessed as having occurred when the contents of reaction tubes were no longer mobile. Polymerisation results from covalent linkage of macromonomers through native chemical ligation, a reaction scheme for which is shown below.

Native chemical ligation proceeds through a thioester intermediate which rearranges through an intramolecular S,N-acyl shift producing an amide (peptide bond). These reactions occur at the termini of reacting macromonomers and drive polymerisation of hydrogels.

2.7 HPLC
Cleaved peptides were purified from remaining synthetic by-products by reverse phase HPLC (rp-HPLC) across a gradient of 20-100% isopropanol 0.1% TFA using a Jupiter 4 µ Proteo 90AC-18 column (Phenomenex running at 3 mL per . Peptide elution was monitored by optical absorption at 280 nm and peptide containing fractions were collected and lyophilized prior to storage at -20°C.

2.8 NMR
The PEG 4A- EMPSA (0.1 g) was dissolved in deuterated chloroform (CDCl₃) in a glass vial. After the sample was dissolved, it was passed through the NMR tube as a solution through a cotton filter. The ¹H NMR spectrum of the PEG-4A thioester macromonomer was taken on a 400 MHz instrument in deuterated chloroform.

2.9 Trypsinization of mouse bone-marrow derived mesenchymal stromal cells (MSC) and encapsulation/culture in hydrogels
96 well plate (Nunclon ™, Denmark), Fetal bovine serum (FBS), Dulbecco Modified Eagle Medium (DMEM), Phosphate buffered saline (PBS), Trypsin (1ml), β
mercaptoethanol (2 µl), propidium iodide (PI), cysteine macromonomer (40 mg) and thioester macromonomer (40 mg). The macromonomers were prepared as described in section 2.1 and 2.2.

MSC cells derived from a transgenic mouse stably expressing GFP (gift from Dr Mike Doran, QUT) were cultured in tissue culture flasks. The MSC’s were washed with PBS (5ml) and trypsinised by incubation with 1ml of 0.05% trypsin at 37°C for 5 mins followed by trypsin neutralisation through addition of DMEM containing 10% FBS. A cell count was performed by haemocytometer. (The MSC cell count was determined to be 1.38 X 10⁶ cells per well) and the remaining MSCs were collected by centrifugation (3000 rpm, 400G) for 5 mins. The supernatant was aspirated and the cell pellet was resuspended in 500 µl PBS (1x PBS). About 3X10⁴ cells were added to the cysteine macromonomer solution (200 µl PBS + 40 mg cysteine) containing 30 mM β-mercaptopethanol and then mixed with thioester macromonomer solution (50 mg thioester + 200 µl water). Droplets (10 µl) of the resulting cell suspension were pipetted into 96 well plates and immersed in 250 µL of DMEM. After incubation for 15 mins, images of the cultures were captured under an inverted microscope (Nikon Eclipse TE 2000-U). Encapsulated cells were maintained at 37 oC, 5% CO₂ and constant humidity for 24 hours. After which the fluorescent red DNA stain propidium iodide (PI) was added to each well to visualize dead cells.

3.0 Hydrogel degradation assay

4 arm Poly (ethylene glycol) thioester (40 mg) and 4 arm cysteine peptide (40mg) were synthesised according to the protocols detailed in chapter 2 section 2.2 and 2.3. Phosphate buffered saline (PBS, pH 7.4), Trypsin (1ml), Coomassie Brilliant Blue dye (CBB), Ponceau dye, Elisa Cell plate reader (Biorad Benchmark Plus).

The peptide dendrimer used for cross linking PEG macromonomers contained an arginine residue at position 6. This basic residue interacts strongly with the anionic protein stain Coomassie Brilliant Blue when part of a longer peptide chain. However, proteolytic cleavage at the C-terminus of this residue would produce a C-terminal carboxylate that would neutralise the cationic arginine side chain, greatly reducing its interaction with coomassie blue. Given the occurrence of arginine in the peptide dendrimer cross-linking macromonomer, we reasoned that peptide cleavage at
arginine would cause degradation of the peptide/ hydrogel hybrid and additionally release coomassie blue giving a realtime readout of gel degradation and would also act as a model of molecular release.

The hydrogel (a 5 mm cube) was stained in (500µl) coomassie blue (dissolved in 50:5:45 methanol, acetic acid and water). It was placed on a laboratory rotator (Ratek) for 2 hours in order for the hydrogel to absorb the stain. Following staining the gel was washed in 50:5:45 methanol, acetic acid and water to remove unbound dye and equilibrated with Tris buffered saline (TBS). The gel was then cut into 6 equal pieces and added to 6 wells of a 96 well plate. 100 µl TBS was added to the 6 wells and mixed well using a multichannel pipette. 20 µl of trypsin (0.5µg/250µl) was added to wells 4, 5 and 6. The cell plate reader (Biorad Benchmark Plus) was used to measure the rate of degradation of hydrogel by comparing the gels treated with trypsin to those which were not.

Chapter 3. MACROMOLECULAR SYNTHESIS AND GELATION

Summary: The ease of polymerisation associated with the NCL approach coupled with it’s essentially biomimetic nature and lack of toxic initiators has provoked considerable interest in the biomaterials area. However, the published methods are both laborious and constrained in terms of the way cargo molecules can be incorporated into the polymerised hydrogel. Accordingly, a series of alternative polymerisation strategies were explored with a view to making NCL hydrogels more accessible for the wider research community and to increase their utility in terms of cargo loading and delivery. Three distinct strategies were explored during this study: 1. Polymerisation driven by a bifunctional cysteinyl pendant peptide system and thioester macromonomer ; 2. Combination of a PEG/comb-peptide dendrimer with a thioester PEG macromonomer; and 3. A peptide dendrimer/thioester PEG macromonomer combination. Of these variants, only the last proved to be technically feasible and capable of offering a viable alternative to the published NCL methods currently available.
3.1 Introduction

As outlined in Section 1.10, PEG was selected as the basis for hydrogel production because of its low immunogenic potential and general biomimetic properties. However, PEG by itself is non-reactive and has no net charge, which makes it essential for this hydrophilic polyether to be functionalised prior to peptide conjugation or other form of derivitisation. For this reason, PEG molecules functionalised with amine groups were used as the platform for development of thioester macromonomers for eventual hydrogel formation with dendrimeric peptides.

Four (4arm) PEG amine was selected for thioester macromonomer production by virtue of its low immunogenicity, larger molecular weight and propensity to form three dimensional structures. Protein /peptide drug delivery studies carried out by Cho et al., demonstrated that the non-immunogenicity of PEG chains is further improved by increasing the molecular weight and the number of polymer chains of PEG (Cho et al., 2010). Additionally, studies conducted by Yamaoka et al provide evidence that the urinary and tissue clearance of PEG from the body, decreases with increasing PEG molecular weight, while the liver clearance of PEG increases with the increasing PEG molecular weight (Yamaoka et al., 1994). Therefore high molecular weight PEG has been shown in human and animal models to prevent both liver and kidney damage (Webster et al., 2009).

More recently, multifunctional branched PEGs have allowed for high drug loading with longer dosage intervals (Berna et al., 2006). These branched PEG’s have been reported to be more effective than linear PEG in increasing the water solubility, prolonging the plasma half life and preventing proteolytic degradation of small bioactive molecules (Caliceti and Veronese, 2003). Thus the pharmacokinetic and immunogenicity potential of the bioactive material is greatly improved by this branched (also known as star) PEG approach.

The choice of PEG amine as starting point was driven by the routine use of Fmoc SPPS in this laboratory. Clearly, conjugation to amine groups via the C-terminal carboxylate of a peptide is chemically feasible. Indeed, previous studies (Stetsenko and Michael, 2000, Ollivier et al., 2005), have successfully demonstrated the utility of this approach. However, thioesters are intrinsically less reactive than the
corresponding peptide carboxylates necessitating a higher level of chemical activation than that achievable with uranium ester formation (HBTU/HOBT coupling). In other arenas a common route to increasing the activity of species for conjugation is to first make a succinic anhydride adduct. This is routinely performed for reactions coupling proteins to nucleophiles as shown below in Figure 3.1.

Dicarboxylic acid anhydrides, such as succinic anhydride, are able to react with nucleophiles, causing the ring structure of the anhydride to open. This results in the formation of an acylated product, modified to contain a newly formed carboxylate group (Hermanson, 1996). Acylation of ethyl 3- Mercaptopropionate (EMP) with succinic anhydride results in the formation of EMPSA (EMP succinate), the carboxylate of which is considerably more active than the starting compound’s free thiol. This activated carboxylate is then capable of derivatising the PEG 4 amine as shown in figure 3.2 below.

**Figure 3.1:** Protein containing amine reaction with succinic anhydride to form carboxylate groups.

**Figure 3.2:** Thioester PEG macromonomer synthesis.
The synthesis of the thioester PEG macromonomer was carried out in solution phase since the PEG molecule is too large to carry out solid phase organic synthesis. Problems with SPPS synthesis of large PEG-based dendrimers have been reported by Alesso et al. This study used polystyrene resin (PS) as a support for PEG modification reactions, but found low levels of resin substitution during synthesis. Furthermore, PEG molecules swell extensively during synthesis in protic solvents such as DMF. In turn this causes aggregation of PEG macromonomer which limits reagent accessibility and reduces the efficiency of derivatisation steps. There are also potential problems with the long PEG chain complexing Lewis acids (eg preactivated Fmoc amino acids) as well as issues with PEG leakage following cleavage and the high cost of the grafted PS-PEG supports which makes them the less preferred method for PEG thioester macromonomer synthesis (Alesso et al., 2003).

To synthesise cross linking peptides, microwave SPPS was used. This new technique for the derivatisation of peptide was employed since it provides increased deprotection and coupling reaction rates (upto 1000 fold). In contrast, the traditional SPPS is a very slow process and is plagued with drawbacks such as intermolecular aggregation, β-sheet formation, steric hindrance from protecting groups and premature termination of the peptide sequence (Collins et al., 2003).
Additionally, microwave synthesis provides efficient internal heating, resulting in even heating throughout the sample. This enhances solvent accessibility and reagent access, since the tendency for the initiation of boiling is reduced and superheating above the boiling point of the solvent is possible even at atmospheric pressure. This results in enhanced reaction rates and faster conversions of the chemical reactions when compared with conventional techniques of peptide synthesis (Willis, 2004).

For the quantitation of the amount of peptide present in the solution, spectroscopic analysis was used. Absorption is usually measured by varying the frequency (or wavelength) of the applied radiation. Absorbance is directly proportional to the path length, \( l \), and the concentration, \( c \), of the absorbing species. Beer's Law states that \( A = \xi l c \), where \( \xi \) is a constant of proportionality, called the absorptivity.

The peptide bond absorbs strongly in the far UV with a maximum at about 190 nm (Aitken and Learmonth, 1996). The most useful UV range for proteins is at wavelengths greater than 230 nm where the aromatic side chains of Phenylalanine (Phe), Tyrosine (Try) and Tryptophan (Trp) contribute to the absorption. Trp has the most intense absorbency, which is the basis for the measurement of protein concentration by UV spectrometry. The number of moles of Trp per mole of protein is determined from the absorption of the protein solution measured at 288 and 280 nm (Tsai, 2007).

Microwave energy directly activates any molecule with a dipole moment and enables rapid heating at the molecular level (Loupy, 2004, Hayes, 2002). This allows for higher resin substitution and less excess reagents to be used that increases scale up potential when compared to traditional methods. Furthermore, aggregation, \( \beta \)-sheet formation and steric hindrance can be overcome by microwave energy allowing for the possibility of purer and longer peptide sequences (Collins et al., 2003).

3.1.1 NMR characterisation
Nuclear magnetic resonance (NMR) has been regularly utilised by scientists to determine the physical, chemical, and biological properties of matter. Some of its
applications include the structural determination of organic molecules, confirmatory tests to determine if a reaction has occurred at the correct site of the molecule, assistance for research chemists in checking the contaminants in food, cosmetics and medications and assessment of / diagnosis of different stages of certain diseases such as cancer and osteoarthritis (Lacitignola *et al.*, 2008).

The NMR phenomenon occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field (Hornak, 2003). The technique relies on the ability of atomic nuclei to behave like a small magnet and align themselves with an external magnetic field. The most common nuclei observed using this technique are protium (\(^{1}\)H) and Carbon-13 (\(^{13}\)C), but the isotope of \(^{19}\)F, \(^{31}\)P, \(^{29}\)Si and \(^{77}\)Se can also be observed by NMR since they possess magnetic moments.

The atomic nuclei spin about an axis and generate their own magnetic field, or magnetic moment. When a strong magnet is placed next to a sample, the randomly oriented hydrogen atoms orientate/align in one of the two possible spin states. They are referred to as \(\pm 1/2\) spin state (lower energy), the hydrogen's magnetic moment is aligned with the direction of \(B_0\), while in the \(-1/2\) spin state (higher energy) aligned opposed to the direction of \(B_0\), where \(B_0\) represents the applied field of the strong magnet (Fig 3.4). The difference in energy between the two spin states increases with increasing strength of \(B_0\) (Hornak, 2003).

![Figure 3.4: The two spin states of hydrogen](image)

When the number of times per second the proton revolves in a complete circle equates to the exposed electromagnetic radiation of frequency \(v\), the proton is said to attain resonance frequency. This frequency corresponds to the energy difference between the proton’s two spin states. With the strong magnetic fields generated by the superconducting magnets used in modern NMR instruments, the
resonance frequency for protons falls within the radio-wave range, anywhere from 100 MHz to 800 MHz depending on the strength of the magnet. Nuclei which are close to one another exert an influence on each other's effective magnetic field. This effect shows up in the NMR spectrum when the nuclei are nonequivalent. If the distance between non-equivalent nuclei is less than or equal to three bond lengths, this effect is observable. This effect is called spin-spin coupling or $J$ coupling (Hornak, 2003). In most of the NMR spectra recorded, the compounds are dissolved in a solvent. In order to avoid spectral signals generated from these solvents, a deuterated solvent (chloroform, methanol) are used. However 100% deuteration is not possible, so signals for residual protons are still observed.

$^1$H NMR was preferred to $^{13}$C-NMR for determining the magnetic moment of PEG-peptide macromonomer since the signal produced by an $^1$H nucleus is much stronger than that of an $\alpha$ $^{13}$C nucleus. For example, peaks corresponding to carbonyl carbons are much smaller than those for methyl or methylene (CH$_2$) peaks. Also, the low natural abundance of $^{13}$C, means it would be more difficult to observe the carbon signals and hence more sample would be required. Further the data from hundreds of scans would have to be averaged in order to bring the signal-to-noise ratio down to acceptable levels. Thus by integrating the signals from these proton transitions, we learn about the number of hydrogens present in the chemical environment of each atom, along with the information of neighboring atoms.

### 3.2 Results

Whilst synthesis of the PEG thioester macromonomer proved relatively straightforward and was confirmed by NMR, production of cysteinyi macromonomers proved to be more challenging. Despite several attempts, both the bifunctional pendant peptide and comb dendrimer synthetic approaches proved to be intractable. However, the dendrimeric peptide macromonomer was successfully synthesised and used to produce self-gelating hydrogels that were resistant to dissolution in reducing agent confirming their production by NCL.
3.2.1 NMR characterisation of PEG thioester macromonomer

An NMR spectrum appears as a series of vertical peaks/signals distributed along the x-axis of the spectrum (Fig 3.5). Each of these signals corresponds to an atom within the molecule being observed. The splitting pattern seen in these signals gives information as to how many hydrogens are present in the neighbouring carbon.

Analysis of PEG 4A thioester by $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 4.15 (2H, q, J=7 Hz, -CH$_2$-CH$_3$), 3.79-3.41 (m, O-CH$_2$CH$_2$-O), 3.12 (2H, t, J=7 Hz, -S-CH$_2$), 2.92 (2H, t, J=7 Hz, -S-CO-CH$_2$), 2.61 (2H, t, J=7 Hz, -S-CH$_2$-CH$_2$), 2.52 (2H, t, J=7 Hz, -CH$_2$-COOH), 1.252 (3H, t, J=7 Hz, -CH$_2$-CH$_3$).

The spectrum contains seven signals. The downfield signal, with a chemical shift of 4.15 ppm, integrates to two hydrogens, and is a quadruplet with a coupling constant of J=7 Hz. The middle signal between 3.79-3.41 ppm is a multiplet, and integrates one hydrogen. The signal with a chemical shift of 3.12 ppm integrates two hydrogens, and is a triplet with a coupling constant of J=7 Hz. The signal with a chemical shift of 2.92 ppm, integrates two hydrogens, and is a triplet with a coupling constant of J=7 Hz.
The signal with a chemical shift of 2.61 ppm integrates two hydrogens, and is a triplet with a coupling constant of $J=7$ Hz. The signal with a chemical shift of 2.52 ppm integrates two hydrogens and is a triplet with a coupling constant of $J=7$ Hz. The upfield signal, with a chemical shift of 1.252 ppm integrates three hydrogens, and is a triplet with a coupling constant of $J=7$ Hz.

There were extra singlet peaks generated at 2.21 ppm and 6.48 ppm. These signals may have been generated due to the impurities (solvents, water, oil) present during the preparation of the sample (Gottlieb et al., 1997). The peak at 7.26 ppm is ignored as it represents the proton from the chloroform solvent. The figures 3.6 and 3.7 provide a clearer view of the different peaks present in the PEG thioester macromolecule.
3.2.2 N-terminal cysteine peptide synthesis
The structure of the n-terminal cysteine peptide sequence (CSGFVQRGD) was confirmed by mass spectrometry (Fig 3.8). The major peak corresponding to 1056 Da indicates the exact calculated mass for the molecular peptide.

3.2.3 PEG 3500 NHS synthesis for PEG comb synthesis
The mono-methoxy PEG (0.5 g) was mixed with NHS (7.5 mg) in a DCC and chloroform solution to form the Fmoc-PEG-succinimide product (10 mg). Products were diluted 1:1 in 50% acetonitrile/water and analysed using mass spectrometry (SELDI) as shown in Fig 3.9. The PEG 3500 produced a “forest” of peaks centred on the expected mass of 3500 daltons. Post NHS derivatisation, this mass shifted with a new central mass of ~3600 indicating derivatisation with NHS.

3.2.4 Lysine core synthesis
The dendrimeric peptide macromonomer was constructed in two stages. Initially a branched lysine core was constructed which was later derivatised with fully protected peptide synthesised as described in chapter 2 section 2.1. The advantage of this approach lies in the ability to produce a single large scale synthesis of the lysine core for later derivatisation with a variety of different peptides or mixtures of peptides. Additionally, separate synthesis of the lysine core and cross linking
peptides allows for mass spectrometric validation of the peptide cross linkers which is not possible once linked to the lysine core. The lysine core itself was synthesised using conventional SPPS techniques except that Fmoc was used for both sidechain and N-terminal protection. Thus, when a lysine residue was added to the growing core and deprotected, two additional amines were exposed as potential points for further elongation as shown in figure 3.10.

Figure 3.9: Mass spectometry of PEG 3500. A. PEG A only B. PEG A post treatment with NHS

Figure 3.10: Synthesis of the Lysine core dendrimer.
The 2-Chlorotrityl Chloride resin (300mg) was loaded with the Fmoc-Fmoc Lysine spacer (20mg) and then conjugated with the Fmoc-PEG-succinimide product (10mg). SELDI was used to detect the shift in the Fmoc-PEG-succinimide peak. The major product formed was mono PEG with a higher molecular weight (3700 daltons). The solution coupling of Lysine spacer to the mono-methoxy PEG resulted in the formation of Diamino-bis-PEG (Fig 3.11).

3.2.5 Polymerisation of the NCL hydrogel
The first batch of NCL hydrogels was polymerised by mixing, 10 % w/v 4 arm thioester PEG macromonomer with 4 arm 10% w/v cysteine PEG macromonomer in an aqueous solution. In order to polymerise the second batch of NCL hydrogels 10 % w/v 4 arm thioester macromonomer was mixed with 4 arm 10% w/v cysteine peptide macromonomer in an aqueous solution (Fig 3.12).

Gelation occurred between 2 and 3 minutes with an initial cloudiness appearing as gelation progressed. Longer polymerisation times were apparent as reagents aged and there was a pronounced swelling of the hydrogel when stored in PBS buffer. Hydrogels were consistently stable to prolonged incubation with the mild reducing agent 2-mercaptoethanol (50 mM in PBS) indicating that gelation was not due to cysteine oxidation.

Figure 3.11: Addition of Lysine spacer to PEG 3500.

Figure 3.12: Polymerisation of NCL hydrogel.
3.3 Discussion

For purification of the PEG thioester macromonomer, solution phase extraction was performed to separate the sample from the two immiscible solvents (water and chloroform). In this procedure, the eluate is purified by passing it between the complementary solvents. For complete separation, the sample is re-extracted by passing through the solvents again. The downside of this procedure is that it is very time consuming and leads to incomplete phase separations along with less than quantitative recoveries (Parris, 1976). In some instances less than 10% of the starting material was recovered largely due to incomplete partitioning between solvents. This could have led to the low yield of the PEG macromonomer when separated by solution phase extraction.

The final solvent extracted thioester macromonomer was insoluble in water and required the addition of 50 mM 2-mercaptoethanol before forming a clear colourless solution. Further, on mixing the PEG thioester with the PEG cysteine macromonomer in an aqueous solution, the hydrogel formed instantly. The reaction was carried out at low temperature and followed up with multiple DMF washes mostly for Fmoc-removal and to avoid any unwanted multiple reactions. Therefore the synthesis of peptide sequences was improved by intermediate acid washing steps with polar solvents.

The hydrogels polymerised by the NCL strategy remained insoluble even on the addition of a reducing agent (100 mM 2-mercaptoethanol in water). Thus showing the gelation was not due to disulfide bonds forming between the macromonomers, but was a result of NCL reaction.

One approach discussed in the literature (Berna et al., 2006), was to conjugate monodisperse PEG-Dendrons to the protein and thus synthesise branched PEG based architectures with higher molecular weights. A similar approach to conjugate the n-terminal peptide to the PEG dendrons (Combination of a PEG/comb-peptide dendrimer with a thioester PEG macromonomer approach) was utilized in our study but the PEG dendrimer was not able to attach to the large peptide sequence (CSGFVQRGD). The reason attributed to this can be steric hindrance between PEG
/peptide chains. The changes in conformation and electrostatic binding properties between these macromolecules increases the molecular weight and could have influenced the binding of these macromolecules (León-Tamariz et al., 2007).

To solve this problem, a novel approach of directly linking the 4 arm PEG thioester macromonomer to the 4 arm N-terminal peptide macromonomer (CSGFVQRGD), containing a lysine core (to avoid steric hindrance) was used to polymerise the hydrogel (A peptide dendrimer/thioester PEG macromonomer combination). The N-terminal peptide sequence contained the KLK 4 degradable sequence (FVQR) and the cell adhesion sequence (RGD). These sequences had twin benefits of introducing degradability as well as cell attachment into the hydrogel. To this end, the hybrid peptide/PEG hydrogel was polymerised by mixing them in an aqueous solution at room temperature.

The hydrogel was polymerised at a physiological pH and at room temperature in less than 2 minutes on mixing the peptide dendrimer/thioester PEG macromonomer in an aqueous solution. But this gel formation was dependent on concentration of the macromonomers. On adding 2-mercaptoethanol (reducing agent) to the hydrogel, it remained insoluble showing the hydrogel remained resistant to reduction post polymerisation. Thus confirming the hydrogel formation proceeded through NCL.

In order to confirm the structure of the hydrogel, the splitting pattern found in the $^1$H NMR spectrum for the PEG-4A thioester sample matched the reference spectra already recorded in the literature (Hu et al., 2009). This is consistent with observations made in the laboratory which pioneered NCL ligation as a means of driving hydrogel formation. Whilst there are some extraneous peaks present in the spectrum the majority of the signals are clearly integrated and match with both theoretical predictions and the empirical data presented in the Messersmith group’s publications (Hu et al., 2009). Those peaks that do not match correspond with previously identified contaminants. This data confirms and validates the cysteine macromonomer synthesis.

During the preparation of the sample for $^1$H NMR, the PEG/Peptide macromonomers were insoluble in the chloroform solvent. This could potentially be due to the formation of aggregate chains by the thioester moiety. Whilst it was difficult to
solubilise precipitated material by addition of a reducing agent, it resulted in unacceptable increases in noise. Thus the formation of the insoluble compounds and the size constraints stoichiometry between thioester PEG macromonomer and the dendrimic peptide adduct made it difficult to characterise the sample by $^1$H NMR. Apart from these difficulties, the length and complexity of the thioester PEG/dendrimer peptide conjugates would create a spectrum with considerable analytical challenges.

The spectrum produced from the peptide dendrimer/thioester PEG macromonomer combination was too complex to view by one dimensional NMR. So for this reason, in the future, two-dimensional (2D) solid-state NMR could be used to determine the three dimensional connectivities within the molecule. The types of 2D NMR include correlation spectroscopy (COSY), J-spectroscopy, exchange spectroscopy (EXSY) and Nuclear Overhauser effect spectroscopy (NOESY). The end result is a plot showing an intensity value for each pair of frequency variables. The intensities of the peaks in the spectrum can be represented using a third dimension. More commonly, intensity is indicated using contour lines or different colours.
Chapter 4. PHYSICAL CHARACTERISATION OF NCL HYDROGELS AND PROTEOLYTIC DEGRADATION

Summary: The previous chapter described the synthesis of a novel hydrogel based on polymerisation of a peptide dendrimer with a PEG-thioester macromonomer. This strategy circumvented a series of synthetically complex and time consuming steps inherent to the previously published NCL strategy. Additionally, the peptide dendrimer approach gave seemingly faster polymerisation times than those observed qualitatively for cysteinyl pendant peptide system/thioester-PEG macromonomers. Given these synthetic enhancements, a series of in vitro studies were carried out to assess the new hydrogel’s suitability as both a potential substrate for tissue engineering and a platform for drug delivery. These studies included an estimation of the hydrogel’s Young’s modulus of compression, its fine structure, and its ability to release a model compound following proteolytic degradation. Data for the novel hydrogel compared well with literature values for both young’ modulus and pore dimensions. Furthermore, the hydrogel proved to be proteolytically erodible and showed delayed burst kinetics for release of a model compound.

4.1 Introduction

4.1.1 Mechanical characterisation
Hydrogels are usually biocompatible and an understanding of the physical properties of the hydrogel provides important information to allow evaluation of hydrogel suitability as a support for tissue engineering and drug delivery. A variety of tests of physical and physicochemical properties of the hydrogel are required if the biomimetic constructs are to be used for tissue engineering or controlled- release formulations.

However, the principal drawback associated with hydrogels is their poor mechanical characteristics. The physical properties such as mechanical strength, mesh size, diffusivity and degradation rate play an important role in drug delivery.
The highly porous structure of the hydrogel enables the introduction of relatively large drugs into the system. The porosity of the cross-linked polymers can be tuned by controlling the density of cross-links and their affinity towards the swelling environment (Canal and Peppas, 1989).

As explained by the equilibrium swelling theory (section 1.6.2), the hydrogel when placed in solvents, swells, and the thermodynamically driven force is counterbalanced by the retractive force of the cross-linked structure, leading to an equilibrium state. As the gel swells, the cross-link chains widen, increasing the mesh size and allowing the solute transfer through the gel. The transport or release of solutes/drugs through the swollen hydrogel is controlled by classical Fickian diffusion resulting in drug transport by ordinary diffusion (section 1.6.4).

From the swelling experiments, the mesh size of the gel can be determined. Once the mesh size has been calculated, it can be compared to the hydrodynamic diameter of solute molecules to determine whether diffusion of solute is possible. Hydrogels are fabricated with small intrinsic pores, ranging from the order of a few microns to tens of nanometers (LaNasa et al., 2011). The mesh size determines the mechanical strength, molecular weight proteins, peptides and oligonucleotides maybe hindered due to the larger hydrodynamic radii of these biomolecules. Therefore effective designing of the mesh size in the hydrogel results in desired rates of macromolecule diffusion.

The cross-linking structure of the hydrogel has a direct relation to the mesh size. On increasing the molecular weight and cross linking density between the polymers, the resulting hydrogel has greater mechanical strength but smaller mesh size. However, the increase in modulus coupled with the decrease in mesh size has undesirable effects on the diffusivity and swelling of the network.

In this study PEG hydrogels were selected since they possess network structure and mechanical properties that can be controlled by varying the cross-linking density between the polymers. The 4-arm lower molecular weight PEG hydrogels (10kDa) have a decreased chain length between cross-links and therefore possess a higher cross-link density. Thereby increasing the modulus/strength of the hydrogels (Temenoff et al., 2002). Additionally, the mechanical strength of the hydrogels,
namely the Young’s modulus and the tensile strength can be tailored to make them suitable for tissue engineering applications (Schmedlen et al., 2002).

The primary objective of this study was to evaluate the Young’s modulus and hence stiffness produced by the number of pores, pore diameter, and polymer composition on the gross mechanical properties of hydrogels. The hydrogels compared in this study were formed by cross-linking tetra PEG to the cysteine macromer and by cross-linking the tetra PEG macromer to the 4 arm cysteine peptide (Hu et al., 2009).

4.1.2 Proteolytic degradation assay and release kinetics

The study of in vitro dye release from the hydrogel gives us an idea of its ability to function as a drug delivery system. Model drug release from the hydrogels, was assessed following proteolytic treatment showing the effect on the release kinetics through the self assembled peptide hydrogels. To mimic drug delivery of small peptides, Ponceau dye and Commmassie Brilliant Blue (CBB) were used to study controlled release from the hydrogel. Various researchers have studied swelling and diffusion of solutes in different synthetic hydrogels (Hoffman, 2002). This research project focuses on model drug release from synthetic hydrogels by diffusion following enzymatic degradation.

CBB was chosen as model drug due to its potential to bind to proteins with high affinity through the strongly ionized sulfonic groups of the dye that interacts electrostatically with positively charged amino acid residues (arginine, lysine and histidine ) (Nagai et al., 2006).

Figure 4.1: Coomassie Brilliant Blue (CBB) dye and arginine interaction.
Arginine is present on the peptide dendrimer forming part of the RGD integrin binding motif. It will be positively charged at physiological pH and will interact strongly with the sulphonic anion group on the CBB backbone (Fig 4.2). CBB has two negatively charged sulfonic groups and a positively charged quaternary nitrogen-carbon group in its anionic species and one, proton-neutralized, sulfonic group in its neutral species.

Arginine can react with the neutral or the anionic species because of its positively charged guanidino group and also destabilize its other, proton-neutralized, sulfonic group in the pH range (0–1.3) of their formation (Georgiou et al., 2008). However, once the peptide backbone of the dendrimer is cleaved to the C-terminal side of arginine, a new C-terminal carboxylate is revealed which will effectively neutralise the arginine side chain, greatly reducing the interaction with CBB and causing it to loose its affinity for the peptide dendrimer. Thus, when self-assembling hybrid PEG/peptide hydrogels containing the RGD sequence are cleaved at arginine residues there should be a concomitant release of any bound CBB dye.

4.1.2.1 Hydrogel preparation method (Peptide dendrimer/thioester PEG macromonomer combination)

The polymerisation of NCL hydrogels was carried out by direct linkage of peptide dendrimer (4 arm) combined with the 4 arm PEG thioester in an aqueous solution (see Fig 4.2). We reasoned that the direct linkage of the peptide to the PEG macromonomer would eliminate the need of decorating the hydrogel with bioactive compounds (hormones, growth factors) post polymerisation. Due to the biomimetic
nature of the hydrogel formation a higher efficiency of the peptide drug would be possible.

4.2 Mechanical characterisation- Modulus measurements
The mechanical properties were calculated by performing a compression test on the hydrogels. The time-dependent temporary deformation was measured in-situ using a modification of a method described by Ahearne (Ahearne et al., 2008). Briefly, a disposable syringe was modified to become a uniaxial compression analysis tool as follows: The silicon plunger at the end of the syringe insert was removed leaving a light cylinder of plastic that moved with minimal friction within the syringe’s body.

A sample of hydrogel with carefully defined dimensions was placed between the head of the insert and the outlet of the syringe. The whole arrangement was then clamped in a free standing restraint in a draft-free environment and the syringe insert was loaded with increasing masses of water (100 µL increments) measured with a p200 Gilson pipette. The accuracy of the pipette had previously been calculated to be ±0.50 µL at 100 µL. High resolution (8 mPixel images) were acquired of the syringe insert and body with each load increment and the displacement of the syringe insert was measured against a reference point marked on the exterior of the syringe.

At the end of loading experiments, the test sample was unloaded to check for irreversible deformation. The data presented were all acquired within the limits of elastic deformation and the applied force and the compression of the material at the reference point were used to obtain the stress-strain chart. The deformation/displacement was then used to quantify the Young’s modulus of the hydrogels from the slope of the stress to strain graph (Fig 4.4).
The gel precursors (Thioester PEG + Peptide dendrimer) were loaded into a syringe and mounted using a custom syringe extrusion. In each test, gel was extruded by depressing the syringe piston at a constant rate controlled by the gravitational force (g-force). The g-force associated with an object is its acceleration relative to free fall (9.8 m/s²). Throughout the test, the piston of the syringe moved a total of 3.2 mm from the reference point (Fig 4.3). This causes stresses and strains on the hydrogel, which are felt as weight on the surface (weight per unit mass). The plots of force against strain were recorded and Young's modulus was derived as the slope divided by corresponding cross-sectional area of the hydrogel.

4.3 Scanning electron microscopy and dimensional analysis

The values of the Young's modulus/elastic modulus $E$ in the swollen state were obtained from uniaxial compression measurements using a syringe. The apparatus was equipped with a probe of diameter 10 mm, and was loaded sequentially. The hydrogel samples of diameter 2.5 mm, with thickness of 20–30 mm were submitted to unidirectional compression. The applied strain was small enough (<10%) in order to remain in linear region of the stress-strain response.
4.4 Results
Physical characterisation of the hydrogels produced by NCL polymerisation between the peptide dendrimers and PEG-thioester macromonomers revealed a system that shared many of the key features of hydrogels already being used in the biomaterials area. Young’s modulus for the hydrogel was assessed to be 2.69 kPa comparing well with literature values for other hydrogels as did pore size measurements. Initial analysis of erodibility showed that trypsin was capable of degrading the hydrogel and also drove quantitative release of a model compound (CBB) loaded into the hydrogel during polymerisation.

4.4.1 Young’s modulus calculations
The values of the Young's modulus/elastic modulus $E$ in the swollen state were obtained from uniaxial compression measurements using a syringe. The apparatus was equipped with a probe of diameter 20 mm, and ran at 0.1 mm/s. The hydrogel samples of diameter 2.5 mm, with thickness of 20–30 mm were submitted to unidirectional compression. The applied strain was small enough (<10%) in order to remain in linear region of the stress-strain response.

The modulus strain calculations were done as follows:
Stress = Force/area
\[ Stress = 1 \times 10^{-4} \text{N/area in m}^2 \]
Area = \((1.25 \times 10^{-3})^2 \times \pi = 4.96 \times 10^{-6} \text{ m}^2\]
Stress = 20.37 N/m$^2$ or Pascals per 100 uL loading
Strain = original length-new length/original length
Where, F = applied force
\(A = \text{cross-sectional area of hydrogel, } \sigma = \text{stress} \) - the restoring force caused due to the deformation divided by the area to which the force is applied.
\(\varepsilon = \text{strain} \) - the ratio of the change caused by the stress to the original state of the object.
E = Young's modulus - the tendency of an object to deform along an axis when opposing forces are applied along that axis; it is defined as the ratio of tensile stress to tensile strain.

The Young’s modulus (E) = 2693.212, was determined from the Stress vs Strain graph (Fig 4.4). The Young’s modulus for the PEG- Peptide hydrogels was determined to be 2.69 kPa (Kilo Pascals).
4.4.2 Stress-strain characteristics of PEG - Peptide hydrogels (10%)

The compression curve may be described as a non-linear function with the decreasing slope representing the increased mechanical stress countered by the hydrogel while the decrease in strain curve represent the reduction in strain. When the hydrogel counters a stress of 700-800 N/m$^2$, higher strain is observed in the range 0.2%, but decreases significantly as the force decreases. But the hydrogel ultimately attains its elasticity property without being deformed at 0.1% strain.

![Scatter plot representation of deformation on hydrogel](image)

**Figure 4.4: Stress kPa Vs Strain %scatter plot**

The compression curve may be described as a non-linear function with the decreasing slope representing the increased mechanical stress countered by the hydrogel while the decrease in strain curve represent the reduction in strain. When the hydrogel counters a stress of 700-800 N/m$^2$, higher strain is observed in the range 0.2%, but decreases significantly as the force decreases. But the hydrogel ultimately attains its elasticity property without being deformed at 0.1% strain.
4.4.3 Pore size determination

The morphological investigation with SEM imaging (HITACHI TM 3000) was employed to elucidate the pore size of the hydrogels. The surface images of hydrogels polymerised by bifunctional cysteinyl pendant peptide system and thioester macromonomer mixing and thioester PEG-Peptide hydrogel are shown in Fig 4.5. The cysteinyl peptide-PEG hydrogel showed a comparatively smooth surface when compared to the rough surface of PEG-peptide hydrogels.

The SEM images of Cysteinyl peptide-PEG hydrogel and thioester PEG-peptide hydrogels revealed spherical, interconnected pore structures throughout the surface. The size and shape of pores vary greatly depending on the fabrication technique and often result in irregularly shaped pores spanning a range of sizes. The mesh size of cysteinyl peptide-PEG hydrogel was calculated to be 3 µm (left) and the mesh size of PEG-peptide hydrogel was calculated to be 1.5 µm (right) (Table 4.1).

![Figure 4.5: Comparison of the pore size between Cysteineyl peptide-PEG hydrogel (left) and the PEG-Peptide hydrogel (right).](image)

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Table 4.1: Calculation of NCL hydrogel pore size.
4.4.4 Hydrogel degradation by trypsin
As an initial step towards determining the erodibility of the hydrogels produced by peptide dendrimer/thioester PEG macromonomer combination system were challenged by incubation with the protease trypsin which would be expected to cleave cross-links between the peptide dendrimer and PEG macromonomers at arginine residues of the RGD sequence. Mixing the PEG thioester and the peptide dendrimer resulted in very rapid polymerisation of hydrogels. To aid visualisation, hydrogels were stained red with ponceau S dye. In figure 4.6 (below), the clumps of the hydrogel formation can be observed in the eppendorf tube. On adding the protease enzyme, clumps of the hydrogel degraded to form a clear solution.

Figure 4.6: Hydrogel degradation monitored by staining with red dye (Ponceau stain). Images (from left to right) hydrogel formation; clear dye solution on addition of protease enzyme.
4.4.5 Quantitative dye release experiments

To further quantify these results more hydrogel was polymerised and this time stained with a blue compound called coomassie brilliant blue (1%) which is used here as a model for drug release. The gels were cut, and measured length wise and weighed on an analytical balance to assure the gels were of equal size. The gels were placed in 96 well plate reader containing PBS buffer (0.2ml). The gels in wells 4,5,6 where treated with trypsin protease enzyme (0.2% final concentration) and the wells 1, 2, 3 were without the protease enzyme (Figure 4.7). The dye release from the gels was spectrophotometrically monitored in an ELISA plate reader.

Figure 4.7: NCL hydrogel stained with (1%) coomassie blue and treated with PBS only, lanes 1-3 or Trypsin (0.2 % w/v), lanes 4-6
In figure 4.8, the absorbance values on the Y axis corresponds to rate of CBB released over a time period of 1 hour. It can be clearly seen that the rate of CBB release increased massively in wells 4, 5 and 6 on adding the trypsin protease enzyme.

![Figure 4.8: Hydrogel degradation monitored by coomassie blue dye release on addition of protease enzyme (trypsin).](image)

**4.4.6 Dye release kinetics**

Close analysis of data from dye release shows an unexpected feature. In the absence of the protease the rate of dye release follows a slow linear trend (Figure 4.9). On addition of protease (0.2%) at 25°C, there is linear release phase, which is faster and accelerates even faster, showing delayed release kinetics.
The mechanical properties of a hydrogel play an integral role in determining its load-bearing application. Its suitability to be used as a biomedical device is determined by mechanical testing. It has already been established in the literature that drug release rates are a function of the degree of cross-linking of the polymer, larger mesh sizes correlate with greater diffusion of drug molecules through the polymer (Peppas and Khare, 1993).

While compressive and tensile tests have been explored to determine the mechanical properties of hydrogels, few comprehensive studies have investigated the effects of...
polymer composition and pore diameter simultaneously on the mechanical strength of the hydrogel (LaNasa et al., 2011). In the current study, the mesh size of peptide dendrimer/thioester PEG macromonomer combination was compared to cysteiny1 pendant peptide system and thioester macromonomer combination using SEM imaging. This experiment was followed by compression testing of the thioester PEG-peptide dendrimer hydrogels to determine their mechanical strength.

The mesh size of peptide dendrimer/thioester PEG cross-linked hydrogel was smaller in size compared to thioester PEG/cysteiny1 pendant peptide hydrogel (Table 4.1). This matched up with the reasoning that the ether bond between the cross-links would be shorter in distance due to the higher cross-linking between the polymers. This led to a stiffer hydrogel because of the strong cross-links between the polymers.

In order to evaluate the maximum diameter of a molecule that could diffuse through the network, the gel porosity or the mesh size was calculated. The pore size between thioester PEG-Peptide cross-linked polymers were found to be in the range of 1.5-3 µm. Similar studies conducted by Hubbell and co-workers on proteolytically degradable PEG based hydrogels (MMP-PEG) were compared to well-established ECM mimetics fibrin and collagen to assess cell migration through the ECM model system. The cell viability of the three materials was found to be in the range of 90-95%, but interestingly the MMP enzyme degradable hydrogel the cell survival rate critically depended on the gelation duration (Raeb er et al., 2005).

The pore sizes of these three different ECM model systems (PEG hydrogels, fibrin and collagen were found to be in the range of 0.1µm, 1µm and 10µm respectively. This pore size is within the range of 1.5 - 3 µm determined in our experiments and thus can be suitable to be used for cell culture in the future.

As reported by (Almany and Seliktar, 2005) the stress-strain characteristics of the PEG- fibrinogen hydrogels are nonlinear and the elastic modulus is also determined from the stress-strain curve, and is dependent on the percent polymer, the molecular weight of PEG- peptide hydrogel. Our results (Fig 4.4), is in agreement with these finding, after pore collapse in a weaker part, more peaks or a plateau, may be obtained on the stress-strain diagram by further compression due to subsequent deformation of stronger parts of the hydrogel. This may explain why the elastic modulus increases considerably more than the stress (Linde et al., 1988).
Previous studies on the 4 arm PEG hydrogel report the storage modulus to be strongly dependent on the molecular weight and the number of arms of the star-shaped PEG derivatives (van Dijk et al., 2010). Interestingly, on maintaining an equal polymer concentration and increasing the number of arms from 10% 4 arm PEG-20K to 8 arm PEG-20 k, an increased storage modulus from 15.5 ± 0.6 kPa to 39.8 ±1.0 kPa was reported. However on comparing a lower polymer concentration 4 arm PEG-10 K (24.9 ± 2.3 kPa) to a 4 arm PEG-20 K (15.5 ± 0.6 kPa) resulted in a lower cross-linked density hydrogel that possess a lower storage modulus. Therefore the polymer concentration plays a vital role in deciding the mechanical strength of the hydrogel. This decrease in the storage modulus values when compared to the studies conducted by Van Dijk et al go to suggest that the direct linking of the peptide to PEG polymer decreases its mechanical strength. However the hydrogel was still able to maintain its shape after the temporary deformation.

To further support this findings, Phelps and co-workers report the RGD-functionalized 4-arm PEG macromers to be cross-linked to four different end group substitutions, namely, PEG-maleimide (PEG-4MAL) macromer, PEG-acrylate (PEG-4A), PEG-vinylsulfone (PEG-4VS) and UV cross-linked PEG-diacrylate (PEG-DA) hydrogels. On comparison of the Young’s modulus measurement for 10% (wt/v) PEG gels were in the range 0.5 -2 kPa. Importantly, the comparison of the same polymer weight percentage among the Michael-type addition hydrogels, showed a high modulus (1.5kPa) for the PEG-4MAL hydrogel. Therefore robust networks of PEG-4 MAL with low polymer weight percentages (4-5% wt/v) were used for 3D cell culture and in vivo delivery (Phelps et al., 2012).These finding very much coincide with our results, as the combination of peptide dendrimer/thioester PEG macromonomer resulted in a Young’s modulus reading of 2.69 kPa. Cumulatively, these results demonstrate that the mesh size and elastic modulus of the PEG-Peptide hydrogel determine the distance between the cross-links, controls the degree of swelling and diffusion in the polymer and thus makes it suitable for 3D cell culture and drug delivery. Some of the sources of error could be that the hydrogels were made with points that had to cut off. Another important point to consider is that the hydrogel slices were not perfectly symmetrical and thus could influence the results of the mechanical strength of the hydrogel.
The degradation data reveals several interesting patterns regarding the proteolytic degradation of the cleaved fibrinogen (RGD) hydrogels in the presence of trypsin. In Figure 4.6, the gel formation is observed on peptide dendrimer/thioester PEG macromonomer combination in an aqueous solution. The red (ponceau S) dye was added to allow the gel to be visible. But on adding trypsin, rapid degradation of the hydrogel clumps was observed ultimately giving a clear solution. In the absence of protease, the gel was stable in suspension for days, indicating that the included RGD sequence was cleaved by trypsin.

Erodibility and release of model compounds (Brilliantindocyanin 6B and 3-Hydroxy-4-[2-sulfo-4-(4-sulfophenylazo) phenylazo]-2,7-naphthalenedisulfonic acid) were studied spectrophotometrically. The erodibility of the peptide dendrimer/thioester PEG hydrogel is due to the proteolytic degradation of the cleaved fibrinogen (RGD) at the arginine (R) residue. The diffusion release mechanism of the hydrogel was supported by a linear relationship between the absorbance values and the release time of the coomassie brilliant blue dye [Fig 4.9 (B1, B2, B3)]. But upon adding trypsin there was a sharp increase in the absorbance values of the hydrogels which followed an exponential release pattern [Fig 4.9 (B4, B5, B6)]. The increased absorbance reading meant more of the coomassie blue dye was released into the hydrogel supernatant and hence more degradation of the hydrogel was observed. Therefore the model drugs were retained in hydrogels but released upon proteolytic treatment in a time-dependent manner. A few outliers were seen at the 2000 sec and 3000 sec mark in all the wells but this could be due to a system error in the microplate reader.
Chapter 5. BIOLOGICAL ASSESSMENT

Summary: The advantage with NCL polymerised hydrogels is their biomimetic synthesis. Thus they can be used as in vitro models for drug delivery and even have the potential to be cell culture constructs. The PEG/peptide macromonomers inherited characteristics of non immunogenic behaviour and their non toxic properties make them suitable to explore their cell interactive properties. In this work, two hydrogel design strategies were studied, one by bifunctional cysteiny1 pendant peptide system and thioester macromonomer while the other by peptide dendrimers/ thioester PEG combination. MSC’s cultured on Cys-thioester PEG hydrogels showed lower cell viability whereas the combination Peptide/ PEG thioester hydrogel was expected to show higher cell viability, but in preliminary experiments was found to be toxic. This hydrogel’s potential requires further studies to confirm its utility as a model system for drug release.

5.1 Cell encapsulation in NCL hydrogel to test for cytotoxicity

5.1.1 Introduction

Hydrogels owing to their physical characteristics and favourable properties such as high permeability and good biocompatibility make excellent candidates for use as biomaterials. As explained previously (section 1.8), hydrogels are formed through various gelation mechanisms where polymer chains are cross-linked by physical or chemical bonds. However, chemically cross-linked hydrogels are preferred due to their ability to control the macroscopic properties of the hydrogel such as swelling, mechanical properties, diffusion and degradation. But a limitation with chemically cross-linked hydrogels is that additional components are introduced into the system during polymerisation as a result of carryover from macromonomer synthesis (which is impossible to completely eliminate) and thus must be examined for cytocompatibility.

Overall hydrogels show good biocompatibility, providing the embedded cells with high permeability for oxygen, nutrients, and other water-soluble metabolites. This allows the diffusion of nutrients and other biological signalling molecules from the surrounding medium to the encapsulated cells, making them suitable for tissue engineering (Nguyen and West, 2002).
Since the pore sizes of PEG hydrogels are usually between 40-200Å, as established by equilibrium swelling theory, this pore size is significantly smaller than the size of the cell and is able to retain the cells inside the gel (Nicodemus and Bryant, 2008b, Bryant and Anseth, 2002). In addition, incorporating biological factors such as biological macromolecules, growth factors or biologically active peptide sequences initiate cell migration, proliferation, and guided differentiation (Dikovsky et al., 2006). Therefore, strategies focusing on mimicking the native extracellular matrix (ECM) provide biological cues to promote cell-matrix interactions leading to tissue growth.

One of the major research focuses in this area is the provision of encapsulated Islet of Langerhans cells for treatment of types 1 and 2 diabetes. Islet cell transplantation and production of an artificial pancreatic tissue provide a permanent alternative to insulin injections for treatment of this disease but both approaches are complicated by autoimmune activity (type 1 diabetes) and host rejection (both type 1 and type 2 diabetes). These issues have shifted focus to islet cell encapsulation in order to protect implanted cells from immune attack whilst at the same time allowing the insulin they produce to disseminate into the general circulation. PEG hydrogels have been extensively investigated in relation to this. Weber (Weber et al., 2009) used PEG gels formed via the photopolymerisation of varying molecular weight PEG macromers (2000-10000 g/mol) and were encapsulated with islet of Langerhans cells at varying cross-linking densities. The viability studies conducted on these islets showed that these cells not only remained viable, but also released insulin after 14 days of culture in each gel condition. Chondrocytes photoencapsulated in PEG gels with varying cross-linking densities were used as cell-hydrogel constructs for cartilaginous tissue formation on the constructs (Bryant and Anseth, 2002). Similarly, osteoblasts photoencapsulated in PEG hydrogels showed that the majority (90-100%) of the cells remained viable depending on the initial concentration of the macromer (10-20%) PEGDA (Bryant et al., 2003, Nicodemus and Bryant, 2008b).

Hu and coworkers report the polymerisation of dipeptide PEG hydrogel using the NCL strategy (Hu et al., 2009). The PEG gels apart from being completely synthetic, are also biomimetic since the polymerisation is driven by NCL of peptides and hence are suitable for cell culture. However in our approach, the PEG hydrogel was not
post functionalized with maleimide peptide since the aim of the experiment was to
test for the cytocompatibility of MSC’s within the PEG hydrogels itself.

Based on previous studies conducted on encapsulation of MSCs in PEG hydrogels, it
was hypothesised that the hydrogel would present a 3D environment conducive for
cell growth. Therefore, in our study, MSC’s transfected with GFP were directly
cultured on NCL derived biomimetic PEG hydrogels. GFP transfected MSCs were
employed for cellular identification in the hydrogel since GFP is efficiently
expressed in MSC’s and can be detected easily by its highly sensitive and specific
expression (Harting et al., 2009).

5.1.2 Bone marrow-derived mesenchymal stromal cells (MSCs)
Bone marrow-derived MSCs proliferate as fibroblastic spindle shaped cells. They are
pluripotent adult stromal cells capable of differentiating into different cell types such
as osteoblasts, chondrocytes and adipocytes (Pittenger et al., 1999). As with Islet
cells there is considerable interest in their encapsulation for the production of
artificial tissues (Nicodemus and Bryant, 2008a). Along with their ability to
differentiate into different lineages they exert immunomodulatory effects, escape
immune recognition and are able to inhibit immune responses (Rasmusson, 2006).
Accordingly, we used this as a model system to assess the suitability of our
hydrogels for cellular encapsulation. Through collaboration with Dr Michael Doran
(QUT) we were able to access an MSC cell line derived from a mouse bearing a
green fluorescent protein transgene. This conferred the advantage of being able to
visualise MSC cells fluorescently whilst encapsulated in polymerised hydrogels as
opposed to using phase contrast techniques which would have been confounded by
the refractive index changes between tissue culture medium and hydrogel
encapsulated cells.

5.1.3 Results
MSC’s were encapsulated in NCL hydrogels as described in the general methods
(Section 2.9). Figure 5.1, shows one of the previously polymerised hydrogel by the
NCL strategy. It was polymerised by cross-linking PEG thioester with bifunctional
cysteiny1 pendant peptide in an aqueous solution. Cells were visualised using the
intrinsic fluorescence of GFP from the transfected vector and can be seen to form an
interconnected sheet implying that cells were viable for at least a short time
following encapsulation. Longer term viability of the cells was determined using a vital dye approach. Following 24 hours incubation in a tissue culture incubator with (5% CO₂ and 37°C temp), encapsulated cells were stained with propidium iodide.

This fluorescent dye is excluded from living cells but can freely diffuse into dead cells and stain the nuclear DNA a bright red when visualised at 475 nm. However, despite the indication that cells were initially viable on hydrogel encapsulation, vital dye exclusion was not seen after propidium iodide staining with all the cells showing brightly fluorescent nuclei.

5.1.4 Discussion

To investigate the biocompatibility of the hydrogel, MSC fibroblasts (Figure 5.1) were encapsulated into hydrogels polymerised by combining thioester PEG and bifunctional cysteinyi pendant peptide system in an aqueous solution. MSC’s transfected with GFP were cultured in this experiment in order to monitor the growth of MSC cells within the hydrogels. On evaluating the cells for attachment and proliferation on the hydrogel, most of the cells stained red, indicating the Ponceau S dye had entered the cell nucleus and the cells were dead.

Figure 5.1: Culture of Mouse fibroblast mesenchymal cells stably expressing GFP on NCL hydrogel. a.) MSC’s visualised fluorescently with an excitation wavelength of 475 nm and emission of 509 nm immediately after encapsulation. b.) MSC’s after encapsulating in NCL hydrogel, staining with Propidium Iodide and visualisation after 24 hours with excitation wavelength 536 nm and emission wavelength 617 nm. Scale bar = 200 µm
The viability of the MSCs might have decreased since the 3D environment of the hydrogel leads to limited nutrient delivery, waste removal and gas exchange. The MSCs poor viability could be accounted to the low adsorptive properties of PEG surfaces to cell adhesion proteins and exposure of cysteine from the hydrogel to the cell surface. Alternatively, the use of mercaptoethanol (reducing agent) may have been toxic to the cells, but it is very unlikely due to the repeated washing with PBS during hydrogel formation.

Previous studies conducted by Lui et al report MSC’s cultured on hydrogels with 1mM RGD concentration expressed high levels of aggrecan and Type II collagen as compared to hydrogels without RGD sequence (Liu et al., 2010). In conjunction with this Nuttelman et al demonstrated that tethering RGD sequence on PEG hydrogels enhances the integrin-matrix interaction, and thus improves the viability of human mesenchymal stem cells (hMSCs) (Nuttelman et al., 2005). Furthermore, Anseth and co-workers also report that tethering RGD moieties to a PEG hydrogel, makes it cleavable and releasable leading to the enhancement of chondrogenic encapsulated hMSCs (Salinas and Anseth, 2008). Lei et al describe hyaluronic acid (HA) hydrogels where mMSC proliferation occurred in the absence of cell spreading, this goes to show that mMSC could only spread when both RGD and MMP degradation sites were present in the hydrogel (Lei et al., 2011).

Taking these research studies into account our next strategy was to incorporate a peptide macromonomer (CSGFVQRGD) into the PEG hydrogels. This approach (peptide dendrimer macromonomer/thioester PEG macromonomer combination) has twin benefits as the RGD sequence provides an adhesion site for cells to bind to the biomimetic hydrogels at specific cell-surface integrins and the hydrogel is rendered degradable by enzymatic reaction (trypsin). The trypsin cleavage of peptide bonds following basic amino acids such as arginine, lysine and histidine is due to negatively charged aspartate in the substrate-binding pocket (Matsumura et al., 2005).

Given previous findings in this field it is quite likely that the incorporation of a cleavable RGD sequence into the hydrogel would improve both adherence of the MSC’s and improve long term viability of cells encapsulated into the hydrogels.
However due to time constraints we were unable to conduct a side by side comparison of these two hydrogels.
Chapter 6. CONCLUSION AND FUTURE DIRECTIONS

This thesis presents the design, synthesis and initial characterisation of a novel hydrogel system based on polymerisation of a customised dendrimeric peptide with a thioester derivatized PEG macromonomer. To the investigator’s knowledge, this is the first report of a hybrid macromonomer approach to hydrogel formation and is currently the only scheme which allows both determinants of programmed hydrolysis and a payload molecule to be incorporated during gelation. The use of NCL to drive polymerisation makes the resulting macromonomer linkages entirely biomimetic since they are indistinguishable from naturally occurring peptide bonds. The uniqueness of this approach derives from the use of the dendrimeric peptide as a macromonomer, rather than the previous schemes reported by the Messersmith Lab where two PEG monomers were conjugated either with thioester or cysteine, with gelation being driven by NCL of these two groups which was followed by later decoration with additional peptides through the free thiol remnant of NCL. As a proof of concept the investigator was able to demonstrate proteolytically stimulated release of model compounds using proteolytic cleavage sequences with relevance to cancer (cleavage sequence defined for KLK4 which is overexpressed in prostate cancer).

The model compounds Ponceau S (3-Hydroxy-4-[2-sulfo-4-(4-sulfophenylazo) phenylazo]-2,7-naphthalenedisulfonic acid) and Commassie blue (Brillantindocyanin 6B) were employed as proxies for chemotherapeutic small molecules. Spectrophotometric monitoring of commassie blue allowed quantitative determination of release and demonstrated that the model compound was largely retained by hydrogels in the absence of proteolytic activity retained but released in a time dependent manner on protease treatment.

Thus the investigator has been able to significantly enhance the existing PEG hydrogel schemes in the following ways:

1. Gelation and payload incorporation into a single module. The NCL method described by Hu et al. revolves around establishing dipeptide cross links between functionalized PEG macromonomers with payload molecules (eg peptide hormones and cell adhesion signals) being added post polymerisation (Su et al., 2010, Hu et al., 2009). In contrast the strategy
employed here involves the direct cross-linking of 4 PEG thioester macromonomer with four peptide macromonomers. The reactive side chains of the dendrimeric molecule allow direct functionalisation of the dendrimer during its solid phase synthesis rather than post hydrogel formation.

2. Peptide dendrimer/PEG macromonomers show enhanced reaction kinetics compared to conventional PEG-macromonomer only schemes.
The previously described NCL PEG/peptide hydrogel technology is limited by slow reaction rates and difficulties associated with derivatising longer peptides onto the macromonomer’s PEG core. Indeed, this investigator was unable to derivatize 4-Arm PEG with 8-12 amino acid peptides but was successful in producing cysteine derivatized macromonomers. In contrast, production of peptide dendrimers is relatively straight forward and they show very rapid gelation kinetics when mixed at concentrations equivalent to those for the conventional PEG macromonomer-based technology. This may reflect the greater reactivity of the dendrimeric peptides compared to the PEG-cysteine macromonomers. It might also be speculated that the cysteines at the N-termini of peptide dendrimers would be more accessible than the equivalent cysteines on a PEG macromonomer as a result of the greater stiffness of the peptide backbone in the later molecules. Thus the cysteines would be more effectively “displayed” compared to those attached to longer PEG chains with their greater degree of freedom. In the work reported here, direct NCL driven polymerisation of star PEG macromonomers with four branched peptide macromonomers yielded biomimetic hydrogels which could be used to deliver complex pharmaceutical payloads.

3. Erodibility directly linked to payload release.
The peptide macromonomer, (CSGFVQRGD)₄-polylysine renders the peptide dendrimer/thioester PEG hydrogel degradable by enzymatic action (trypsin) and the RGD sequence provides an adhesion site for cells to bind to the biomimetic hydrogels through integrins. In the current study, erodibility of the NCL peptide/hydrogel is due to the proteolytic degradation of the cleaved fibrinogen (RGD) at the arginine (R) residue through a preceding KLK4 specific cleavage sequence (FVQR) which is also recognised by
trypsin. Although a non-covalently bound molecule was used for proof of concept experiments, this strategy could easily be adapted for release of compounds that are chelated by peptide backbones eg cisplatin (Tauro and Gemeinhart, 2005).

4. **Enhanced physical characteristics.** One of the drawbacks of all PEG based macromonomer strategies is an inherent lack of mechanical strength. This in turn reflects the higher flexibility of polyethylene glycol polymers. In contrast the amide linkage of peptide backbones produces a much more rigid structure with considerable constrained degrees of freedom. This is in turn drives the superior Young’s modulus of deformation as measured for the peptide-PEG macromonomer hybrid gels.

The NCL method in general has advantages over the other methods reported in the literature. It provides an alternative to toxic polymerisation techniques such as copper ion-catalysed cycloaddition reaction in which the hydrogels formed are not biocompatible because of the presence of the metal copper catalyst in the reaction (Lutz, 2008). In case of thiol-ene coupling reaction, the drawback lies in the sensitivity of the free thiol functionality towards oxidation to a disulphide (van Dijk et al., 2009). On the other hand stimuli-sensitive based hydrogels have the common weakness of slow response rate and low mechanical strength to be used in biomedical applications (Qiu and Park, 2001), both limitations are addressed by the current innovative hydrogel *in vitro*.

Notwithstanding the novel peptide dendrimer’s performance in vitro, there are a number of questions still to be investigated if this hydrogel is to be further developed as a useful medium for drug delivery or tissue engineering endeavours. Some of the suggestions include using different cell lines, changing the gelation conditions and varying the PEG arm length so as to create different mesh/pore sizes which would affect the various aspects of cell encapsulation. The goal of this section is to establish an experimental strategy to answer the fundamental in vivo questions which require elucidation prior to further development of hybrid peptide dendrimer/PEG macromonomer NCL based hydrogels.
Five essential questions that need to be answered are:

1. What is the basis of the apparent toxicity described in chapter 5?

2. Is the hydrogel capable of providing controlled release of a clinically relevant drug rather than a simple model compound?

3. Will the combined peptide dendrimer/PEG macromonomer provoke an immune reaction when implanted subdermally?

4. How will the PEG macromonomer break down when faced with the complexities of mammalian metabolism and biotransformation?

5. What is the molecular toxicology of the hydrogel and its breakdown products?

The question of toxicity is a vital one for the future of this hydrogel. Residual synthesis reagents are the most likely culprits in this process. As a preliminary step towards their identification, polymerised hydrogels should be extensively washed and tested as substrates for tissue culture rather than encapsulation. Comparison with unwashed hydrogel and a lack of toxicity in the washed material will confirm the presence of soluble toxins. Individual synthesis reagents and by products can then be tested to examine which is responsible for the toxicity.

From our *in vitro* studies conducted on NCL hydrogels, the model drugs (coomassie blue and ponceau S) were retained in hydrogels but released upon proteolytic treatment in a systematic time-dependent manner. The cleavage sequence (FCQR) defined for KLK4 is overexpressed in prostate cancer. The NCL hydrogel has been designed to incorporate specific oligopeptide sequences (RGD) in order to be biorecognizable by intracellular enzymes (trypsin). This strategy can be utilized for site-specific release of anti-cancer drugs (5-fluorouracil, Doxorubicin) by binding the drug to the polymer carrier. Low molecular weight drugs could then be attached to a high molecular weight PEG/peptide hydrogel via a peptide linkage affording the possibility of tailored release through a given protease recognition site. As a first step, cytotoxic drug-releasing hydrogels could be tested in mammalian tissue culture systems in combination with a conventional viability assay, trypan blue or propidium iodide (Altman *et al.*, 1993) as used here for example. In order to test for selective toxicity hydrogels could be tested in a co-culture system consisting of a tumour...
derived cell line compared with a co-cultured “normal cell line”. The two cell lines could be distinguished by permanently transfecting the tumour cell line with GFP as used for the MSC cells described here. Biological activity could be supplemented by carrying out analysis of cell culture supernatants to assess concentrations of drug released from the hydrogels under investigation. This later aim is simplified by the existence of numerous mass spectrometry and ELISA-based assays for commonly used drugs.

Immunogenicity studies need to be carefully and prospectively designed since the immune response of the MSCs on PEG hydrogels could have detrimental effects on the cell viability for in vivo applications. The immune response is activated by the adsorption of proteins onto the biomaterials, which will subsequently stimulate the recruitment of immune cells, such as macrophages. The in vivo biocompatibility and inflammatory responses of NCL hydrogels can be determined by histological and morphological analysis of the tissue adjacent to the implant. But multifunctional peptide dendrimer/PEG macromonomer used in this study provide inherited advantages such as shielding effect on proteins, lower immunogenicity and reduced kidney clearance making them suitable for pharmaceutical applications.

Because of their extensive use as a form of molecular camouflage for biological molecules [see (Harris, 1992) for review] PEG based molecules have enjoyed considerable attention from the pharmaceutical community. As a result of this there is a wealth of information on the toxicology of PEG and branched-chain PEG molecules. The overwhelming impression is one of a molecule that is inherently non-toxic when introduced into biological systems (Fruijtiter-Pölloth, 2005) with the caveat that small PEG molecules (<10 kDa) can cause kidney and liver damage at very large doses (3 g/kg/day)(Working et al., 1997).

Although the peptide portion of the hydrogel will be subject to proteolytic breakdown (indeed this is part of the overall hydrogel design) it is unlikely that the individual dendrimeric peptides will cause toxicity. Notwithstanding these comments, future development of 4 arm PEG-based hydrogels, and the hybrid strategy described here will have to be systematically tested for toxicity. These experiments will be complicated by the fact that PEG is difficult to both detect and quantify. Without prior modification PEG is effectively UV transparent, has no
intrinsic fluorescence and gives ambiguous signals on mass spectrophotometric analysis because of its polydisperse nature. Accordingly, an accurate picture of its metabolic disposition will require analysis of $^{14}$C or tritium incorporated PEG molecules. Potentially other radio tracers could be used such as $^{125}$I or $^{111}$In. However, these atoms do not naturally occur in PEG molecules. This same complication will also apply to tracing the breakdown pathway for PEG.

Whilst some information is available for general PEG metabolism, knowledge of branched-chain PEG molecules is much more sparse. Extrapolating data from straight chain PEG molecules would indicate that the most likely breakdown pathway would transition through oxidation of alcohol groups to form carboxylic acids. These carboxylic acids would then most likely be excreted in urine (Hunt et al., 1982). Happily, this simplifies their detection as they can easily be discerned using mass spectrometry in negative ion mode.

In conclusion, PEG compared to other polymers has a relatively narrow polydisperse index (close to 1.00), ensuring a reproducible high quality output (Saboktakin, 2010). PEG due to its non toxic and biological properties has been FDA approved for medical application (Tan and Marra, 2010). Therefore, future in vivo experiments can be designed such that these NCL hydrogels are implanted subcutaneously into the animal. Drug release from hydrogels can be monitored by the rate at which hydrogel breaks down, depending on concentration of protease enzyme (trypsin) present within the animal’s body.

The biocompatible and amenable molecular design considered in the polymerisation of these NCL hydrogels can make them suitable to be used for biomedical applications. They are easy to synthesise, non toxic, non-immunogenic and biodegradable which could make them suitable for localised therapies through injection at a particular tissue (in vivo). But prior to that application, trying out different cell lines, varying the PEG arm length and gelation conditions could give us more useful results. This is something to work on in the future applications of this strategy.
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