The synthesis and characterisation of mucoadhesive polymeric systems using synthetic and natural polymers

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Declaration

I hereby certify that this material, which I now submit for assessment, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Date: _____

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Publications

- Duggan, S., O'Donovan, O., Owens, E., Cummins, W. and Hughes, H. (2015) 'Synthesis of mucoadhesive thiolated gelatin using a two-step reaction process', *European Journal of Pharmaceutics and Biopharmaceutics*, 91(0), pp. 75-81.
- Duggan, S., Owens, E., Duggan E., Hughes, H., Cummins, W. and O'Donovan, O., 'Synthesis and characterisation of mucoadhesive thiolated polyallylamine' (Manuscript in preparation)
- Duggan, S., O'Donovan, O., Owens, E., Duggan E., Hughes, H. and Cummins, W. 'Comparing the mucoadhesive properties of thiolated polyacrylic acid to thiolated polyallylamine' (Manuscript in preparation)

Abstract

Mucoadhesion is the binding of a material to a mucosal surface. The mucosal surface has a rate of absorption of up to four times that of the skin and, therefore, has great potential as a route of drug administration. Mucoadhesive polymeric drug delivery devices have been designed to allow for the slow and controlled release of a drug to a specific site, with fewer side effects and greater bioavailability in comparison to other methods of administration.

In this project, mucoadhesive polymers were developed by modification through thiolation. Thiolation can increase mucoadhesive properties by up to 140-fold through the formation of disulphide bonds between the polymer and the mucosal layer. Three different polymers were thiolated to create these mucoadhesive systems: the synthetic polymers polyacrylic acid (PAA) and polyallylamine (PAAm), and the natural polymer gelatin. PAA was thiolated by reaction with L-cysteine and the crosslinker EDC. A novel method of regulating the thiol content of PAA by controlling the pH of the thiolation reaction was achieved which resulted in a range of thiolated polymers with varying degrees of thiolation. Gelatin was thiolated using a novel two-step reaction process whereby gelatin was initially aminated by reaction with EDC and ethylene diamine. This aminated gelatin was then thiolated with Traut's reagent, creating a highly thiolated gelatin product. Finally, PAAm was directly thiolated with Traut's reagent.

Neither thiolated gelatin nor thiolated PAAm have been fully characterised for their mucoadhesive properties previous to this study. All thiolated polymers displayed improved cohesive and mucoadhesive properties in comparison to their unmodified counterparts. Thiol content altered the swelling ability, drug release profiles and thermal properties of the samples. The molecular weight of both PAA and gelatin were proven to have a marked impact on mucoadhesive properties. The potential toxicity of PAAm was lessened by thiolation. Thiolated polymers, both synthetic and natural, with varying degrees of thiolation, and differing swelling ability and drug release profiles were created, allowing for the design of a tailor-made mucoadhesive drug delivery system.

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Chapter 1 Introduction

1.1 Introduction

Mucoadhesion is the process of binding a natural or a synthetic polymer to a mucosal layer. The area of mucosal surfaces of the body far exceeds that of the area of the skin, with the area of the small intestine alone being 100 x greater than the surface of the skin (Khanenko *et al.*, 2009). With this in mind and together with the need for a protective delivery system for drugs such as peptides and proteins (Khutoryanskiy, 2011), the development of mucoadhesive drug delivery systems has been of great interest over the past number of years. Protein and peptide based drugs are potentially susceptible to enzymatic interactions within the body; by incorporating them into a mucoadhesive polymer, the potential loss of pharmacological activity may be avoided. Mucoadhesion to specific target tissue may also be a solution to drugs whose metabolites are more toxic than the parent drug, reducing the potential cell damage of healthy cells caused by such metabolites.

1.2 Mucosal Layer

Mucus lines the gastrointestinal (GI) tract, eye, mouth, nasal cavity, reproductive tract and the respiratory tract. It consists of approximately 95% water, with cholesterol, lipids, proteins and other elements making up the remainder (Bansil and Turner, 2006). The mucosal layer has many different functions including lubrication and hydration of the organs it surrounds. It also acts as a protective barrier, trapping bacteria and other pathogens in its gel-like structure. The viscoelastic properties of the mucosal layer are controlled generally by the glycoproteins, mucins, but water and ion content also contribute (Ensign *et al.*, 2012). The surface will allow small molecules and viruses (capsid virus-like particles) to filter through freely (Cone, 2009) but it may become a potential barrier for larger molecules and, therefore, drug delivery. Mucus is constantly secreted, but the amount and the thickness of the layer varies depending on the organ, with highest levels of secretion in the GI tract (Sigurdsson *et al.*, 2013). Throughout the GI tract, there is also variation in mucus thickness, the thickest layer being found in the stomach due to the need for a protective barrier against the acidic environment of the stomach. Constant turnover of the mucosal layer in the GI tract occurs to protect the organs from pathogens and

damaging compounds taken in through the diet. Thickness of the mucosal layer in the small intestine has also been seen to vary due to diet, with fibre intake influencing both turnover rates and mucus density (Ensign *et al.*, 2012). Mucus secretion alters in some disease states, an example of which is cystic fibrosis, a disease where there is an increase in mucus production. The rate of secretion is important to the development of site specific drug delivery systems, particularly when targeting the GI or reproductive tracts, both of which have faster mucosal layer turnover rates which may affect the absorption of the drug.

1.3 Mucins

The mucoadhesive properties of mucus are due to the presence of glycoproteins called mucins. There are two types of mucins: soluble and membrane bound. Mucins, Figure 1.1, are secreted by goblets cells in the epithelium. Goblet cells in the eye are a component of the conjunctiva and the density of these cells is age related, with children having a higher abundance of cells. This correlates to the prevalence of dry eye syndrome in the elderly. Mucins are highly glycosylated, large molecules which are negatively charged at physiological pH, ~7.4. They have domains which are cysteine rich and can form intermolecular disulphide bonds. Adhesion to a mucosal surface requires the formation of a chemical bond through either hydrogen bonding, Van der Waal's forces, covalent bonding or non-covalent bonding.

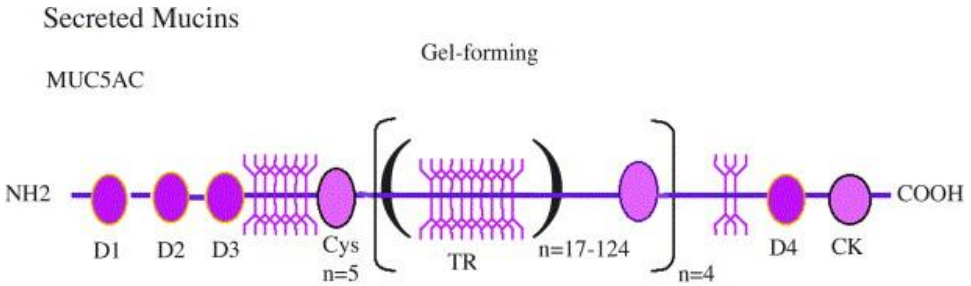

Figure 1.1 Structure of mucin MUC5A, a mucin found in ocular mucus, secreted by conjunctiva goblet cells. D1, D2, D3, and D4 are cysteine rich domains capable of forming disulphide bonds, which allow mucins to form a complex and strong network (Ilene K, 2004)

1.4 Stages of adhesion

In general, there are six theories of adhesion (Roy *et al.*, 2009; Andrews *et al.*, 2009; Khutoryanskiy, 2011):

- Electronic theory: The transfer of electrons between the mucosal layer and the mucoadhesive tablet creating a double layer of electron charge resulting in adhesion
- Wetting theory: Relates to liquid or low viscosity systems penetrating into the irregularities along the surface of the polymeric tablet, hardening within these spaces and creating a bond
- Adsorption theory: Chemisorption interactions such as covalent and ionic interactions, as well as electrostatic interactions such as hydrogen bonding and Van der Waal's forces
- Diffusion theory: The interpenetration of the polymer chain into the mucosal layer. This will depend on the chain length, flexibility and degree of polymer crosslinking
- Fracture theory: The strength of the mucoadhesive bond and the force required to break the bond
- Mechanical theory: The increased surface of the polymer due to the porous nature of surface roughness, allowing for a higher degree of adhesion due to increased contact area

All of the above adhesion theories are valid and it is most likely a combination of all six that result in adhesion. There are two stages of adhesion, shown in Figure 1.2; the wetting/contact stage and the consolidation stage (Smart, 2005). The wetting stage is the intimate contact between the polymer and the mucosal surface, which is required for mucoadhesion (Ludwig, 2005). Interpenetration of the adhesive polymer occurs within the mucus followed by non-covalent bonding, which is due to the presence of hydrophilic groups on the polymer. The extent of the swelling of the polymer and the penetration of the polymer chains into the mucosal surface is often governed by the initial contact time. If there is an increase in the initial contact time, a stronger adhesive bond is created (Asane *et al.*, 2008). In the case of easily accessible areas, this intimate contact can occur by holding the polymer onto the surface until

adhesion is apparent. For less accessible areas, e.g. GI tract, the movement of the muscle itself will allow for adhesion (Smart, 2005).

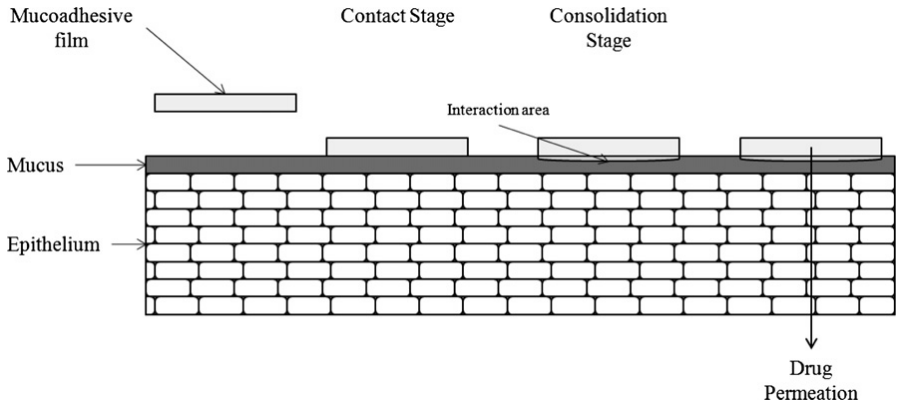

Figure 1.2 The contact and the consolidation stages of mucoadhesion (Morales and McConville, 2011)

The consolidation stage is important where a strong adhesion is required, particularly when the target area is under constant stress, for example targeting the blinking eye. Mucoadhesive strength is strongest when the polymer itself is completely dehydrated and is placed onto the mucosal surface. The polymer will begin to absorb the moisture from the mucosal layer which, once dehydrated, switches from having lubricating properties to having adhesive properties. The moisture will allow the polymeric matrix to become pliable and to adapt to the shape of the surface it is on, thus increasing the contact surface area. It will also allow for the formation of bonds between the mucus and the polymer (Smart, 2005).

1.5 Mucoadhesive Polymers

The transmucosal route of administration has up to 4 x the rate of absorption than skin, depending on the site of administration, making it a much more resourceful route for drug delivery (Madhav *et al.*, 2009). There are many advantages to the development of bio- and mucoadhesive drug delivery systems, including:

- Localised/site specific drug delivery

- Reduced likelihood of systemic side effects
- Better residence time/more intimate contact to the target surface
- Avoidance of peak concentrations due to controlled/slow release of drug (zero order kinetics)

Improved bioavailability of the drug may be observed due to the intimate and increased contact time between the drug-loaded polymer and the target area, thus reducing the time of action of the drug at the site. A lower concentration of drug is also used as the drug is being absorbed directly onto the intended tissue, not systemically, and reduces the potential for toxic side effects. Drugs such as clotrimazole (Bernkop-Schnürch *et al.*, 2003), sodium diclofenac and diclofenac-tris (hydroxymethyl)-aminomethane (Hornof *et al.*, 2003), leuprolide (Dünnhaupt *et al.*, 2012b), antide (Dünnhaupt *et al.*, 2012a) and the compound fluorescein isothiocyanate dextran (Müller *et al.*, 2013; Kafedjiiski *et al.*, 2005) have all been incorporated into thiolated polymers systems and evaluated for their drug release profile.

Both synthetic and naturally derived polymers have demonstrated potential as mucoadhesives. Many synthetic polymers are hydrophobic and most natural polymers are hydrophilic (Yasukawa *et al.*, 2004). Synthetic polymers are known to exhibit a longer duration in the body and, therefore, sustain the release of the drug over a prolonged time period in comparison to the short duration of natural polymers. However, the conditions in which synthetic polymers are formulated, particularly in the formulation of nanoparticulate systems, can be harsher in comparison to natural polymers, using organic solvents in the process (Panyam and Labhasetwar, 2003). Synthetic polymers are also less likely to be biodegradable and may cause an immune response. The majority of polymers used in mucoadhesion are hydrophilic. They are soluble in water and often have a high density of hydrogen groups which are able to interact through hydrogen bonding with mucins, leading to stronger adhesion (Andrews *et al.*, 2009). Specific polymers, both synthetic and natural, will be discussed in more detail in section 1.8.

1.6 Factors affecting mucoadhesion

There are a number of factors that must be considered when designing a mucoadhesive polymer. Environmental factors, including pH, can affect the binding properties of the polymer to the mucosal surface. Properties of the polymer itself, such as molecular weight and structural modification of the polymer, can have an effect on the adhesive properties, the degree of and rate at which the polymer swells and drug release profiles. The biodegradable properties of polymers, specifically synthetic polymers, are important for the removal of the tablet from the body and the degradation rate of the polymer may have an effect on the drug release profile.

1.6.1 Charge on the polymer

Commonly used polymers have either an anionic or cationic charge, but some non-ionic polymers are also utilised. Thiol groups are introduced to both polymer types through different reaction processes.

- Anionic polymers: Amide bonds are easily formed from the carboxylic acid groups on anionic polymers and thiolation can be achieved by the introduction of cysteine, mediated by carbodiimide crosslinkers (Bernkop-Schnürch, 2005). A complication with thiolation is the formation of disulphide bridges during the synthesis. This is due to the unintended oxidation of the thiol groups and may be avoided by performing the reaction under inert conditions or with the addition of EDTA in the buffer solution to chelate any metals that may catalyse the oxidation process.
- Cationic polymers: This is a desirable polymer type to use as it encourages ionic interactions between the positive charges of the polymer and the negative charges, such as the sialic acid residues, of the mucosal layer, (Makhlof *et al.*, 2008), and, in the case of nanoparticles, increases the rate of internalisation into the cell (Kumari *et al.*, 2010). A commonly used cationic polymer is chitosan. Thiolation can occur through the formation of an amide bond, similar to the anionic polymers through the coupling of cysteine in the presence of a carbodiimide, or through the amidine bond with the reaction of Traut's reagent (2-iminothiolane) (Bernkop-Schnürch, 2005).

1.6.2 pH

Studies on the effects of pH changes have been conducted on various aspects of mucoadhesion: On its effect during the production stage of the polymer, and also its effect on the mucoadhesive and thiolation properties of the polymer. Grabovac *et al*. (2005) conducted a study of 19 different mucoadhesive polymers, among which included polyacrylic acid (PAA), chitosan, polycarbophil and the thiolated PAA, thiolated chitosan and thiolated polycarbophil; thiol content of the samples were 498.8 µmol/g, 243.4 µmol/g and 95.7 µmol/g, respectively. The polymers were dissolved in D.I. water and the pH of the solutions was adjusted to either pH 3 or pH 7. The mucoadhesive properties were evaluated in a phosphate buffer at pH 6.8. It was observed that overall the thiolated polymers had a greater level of adhesion in comparison to the unmodified samples due to the formation of intermolecular disulphide bonds between the thiolated polymers and mucin. The thiolated samples which had been adjusted to pH 3 displayed greater mucoadhesive properties than those adjusted to pH 7. It was surmised that the lower pH inhibited the formation of intramolecular disulphide bonds. Once the polymer solutions had been adjusted to pH 7, the tendency towards disulphide bond formation increased, crosslinking the polymers. Therefore, there were fewer free thiol groups to interact with mucins during mucoadhesive testing within the samples adjusted to pH 7. The pH 3 samples were less reactive and, once tested in the phosphate buffer (pH 6.8) for mucoadhesive properties, had a greater ratio of free thiol groups available to bond with mucin, thus increasing mucoadhesion.

Guggi *et al.* (2004) examined the influence pH had on pre-thiolated PAA samples; the thiol content of the samples prior to the pH change was not stated. The PAA-cysteine conjugates were dissolved in water, aliquots of which were then adjusted to different pHs between 3 and 8 and the samples were lyophilised. By reducing the samples with $NaBH_4$, the disulphide bond formation was also measured. After the pH adjustment of the samples, the levels of thiolation were measured and cohesive and mucoadhesive properties were also examined. It was shown that the degree of thiolation decreased when the samples were adjusted to a higher pH. Therefore, the sample adjusted to pH 3 displayed the highest levels of thiolation while the lowest levels were observed when adjusted to pH 8. It was also noted that the disulphide bond content of the samples decreased with increasing pH; therefore, the samples

adjusted to pH 8 displayed lower levels of disulphide bond content than that of the samples adjusted to pH 3. It was thought the formation of sodium carboxylate substructures within the samples which were adjusted to higher pH values increased the molecular weight of those samples, resulting in the lower thiol content observed. The swelling abilities of the higher pH samples were greater than that of the lower pH samples, and this also correlated to an increase in disintegration times with the higher pH samples displaying higher levels of cohesion. Mucoadhesion was greater in lower pH samples. It was concluded that the increase in molecular weight and faster swelling ability of the less acidic PAA-cysteine samples affected the mucoadhesive and cohesive nature of those samples. It was also thought that the lower pH levels of sample below pH 5 prevented the premature oxidation of the thiol groups within those samples, which allowed for higher levels of mucoadhesion and cohesion. It was therefore concluded that higher mucoadhesion could be obtained when the pH of the samples was adjusted to pH 3, but greater levels of cohesion and water uptake would require a higher pH.

1.6.3 Molecular weight

Molecular weight (MW) can influence the mucoadhesion and cohesive properties of a polymer due to the increase in chain length. MWs of between 100 kDa and 4000 kDa were indicted as being optimal for mucoadhesion by Smart (2005). The MW of modified polymers is equally important; thiolated polymers, both anionic and cationic were analysed and it was observed that thiolated polymers with medium MW, between 400 kDa and 600 kDa, had the strongest mucoadhesion (Bernkop-Schnürch, 2005). The MW of the polymer may also effect the diffusion of the polymer into the mucus. It must also be noted, however, that it is difficult to define an optimum MW as each polymer system reacts differently and has a unique structure (Andrews *et al.*, 2009); what is optimal for one polymer, may not be for another.

1.6.4 Chain length

Chain length of the polymer and flexibility of the chain are considered to play an important role in mucoadhesion. Adhesive properties increase with a longer and

more flexible chain (Roy *et al.*, 2009). This is due to the interpenetration of the chains into the mucosal layer, allowing for greater ease of interaction between functional groups of the polymer and mucins. The diffusion theory of mucoadhesion suggests the diffusion of polymer chains into the mucosal layer and also the diffusion of the glycoprotein chains of mucin into the polymer matrix both occur. However, diffusion occurs more frequently with shorter polymers chains and chain lengths which are excessively long may impede mucoadhesion as diffusion into the mucosal layer is lost (Huang *et al.*, 2000). Crosslinking of the polymer reduces the flexibility of the chain and high degrees of crosslinking can reduce the levels of mucoadhesion due to fewer interactions with and less diffusion into the mucosal layer (Andrews *et al.*, 2009).

1.6.5 Swelling

Once the polymer has adhered to the mucosal layer, it will begin to extract the moisture from the surface and start to swell. This is advantageous as the surface area between the polymer and the mucosal layer will increase (Andrews *et al.*, 2009). By modifying the polymer with crosslinking moieties, which allow the polymer to swell while retaining its structure, or by introducing other components, such as polyethylene glycol (PEG), onto the polymer, the degree of swelling can be controlled (Huang *et al.*, 2000). This is an important consideration when creating a hydrogel type polymeric structure, as the release of drug is due to the uptake of water and swelling of the matrix. Polymers that are activated by moisture, such as hydrogels, risk becoming overly hydrated and may lose their adhesion to the surface. Non-specific bioadhesive polymers are moisture activated and can bind to both the mucosal layer and to cell surfaces (Roy *et al.*, 2009; Smart, 2005). For micro and nanoparticles, diffusion of drugs across the polymeric barrier and biodegradation of the polymer itself is important in the process of drug release. Release rates by diffusion depend on the design of the polymeric system (Soppimath *et al.*, 2001).

1.6.6 Biodegradability

An important characteristic of mucoadhesive polymers is their ability to biodegrade, either by enzymatic or chemical means, without the formation of toxic by-products.

Biodegradation of the polymer can occur in one of two ways: by bulk degradation where the polymer degrades in a uniform fashion, or by surface degradation where erosion rates depend on the surface area of the polymer. This degradation will then allow for renal elimination (Kean and Thanou, 2010). The larger the MW of the polymer, the slower the biodegradation time and, therefore, the longer the drug release time (Yasukawa *et al.*, 2001). Non-biodegradable implants have a more controlled drug release profile but the implant has to be surgically removed. Environmental changes, such as pH changes or ionic strength differences, can cause the drug to be released from the matrix, but it is more common in drug delivery systems that degradation of the carrier controls the release, hence biodegradable (and often natural) polymers offer significant advantages (Tabata and Ikada, 1998).

1.7 Polymeric Drug Delivery Systems

1.7.1 Hydrogels

Hydrogels are made up of a polymer backbone, water and a crosslinking agent which creates a three dimensional complex network. They are highly hydrophilic and capable of absorbing vast amounts of water without dissolving, due to the physical and chemical bonds within the matrix (Bhattarai *et al.*, 2010). Hydrated hydrogels have elastic properties allowing for conformation into a variety of shapes. This elastic quality is also seen to be less irritating to the surrounding tissues after the matrix has been implanted, and fully swollen hydrogels have similar properties to that of natural tissue with a rubbery, soft consistency (Bhattarai *et al.*, 2010). The majority of hydrogels are based on modification of natural polymers, which minimises irritation, but synthetic hydrophilic polymers (e.g. polyvinyl alcohol) have also been used; however, they are more likely to cause an inflammatory response (Bhattarai *et al.*, 2010).

Due to their water content, drug release from hydrogels is by a different mechanism compared to other polymeric systems. The degradation and swelling patterns of hydrogels are important in terms of drug release kinetics (Einerson *et al.*, 2003). Sustained release of a protein based drug is sometimes problematic, as release is due

to diffusion through the pores of the hydrogel. One solution to this uses the theory of the polyion complex: Electrical differences cause the binding of two oppositely charged species i.e. positively charged polymer bound to negatively charged protein (drug-carrier matrix) (Tabata and Ikada, 1998). The protein drug is stabilised and drug release is sustained. Release can be through one of two ways: environmental changes, i.e. increase in ionic strength can cause the release of the drug, or the polymer matrix can degrade and the drug is released.

Basic fibroblast growth factor (bFGF) was radioactively labelled and incorporated into acidic gelatin hydrogels (Tabata and Ikada, 1998). These hydrogels were implanted onto the backs of mice, and the radioactively levels were measured, giving an indication of degradation times. Radioactivity levels were observed to be longer in hydrogels that had lower water concentrations. This implies that the degradation rate of hydrogels depends on the water content of the matrix and the higher the water content, the faster the degradation.

The pore size of hydrogels is also a factor for drug diffusion out of a matrix. Often the pore size is larger than the molecular size of the drug and therefore, slow and controlled release is not possible once the hydrogel has been hydrated.

The loading of the drug into the hydrogel can occur in two ways: diffusion into the hydrogel through the pores or by encapsulating the drug within the polymeric matrix. Both methods have release profiles which are uncontrollable to a certain degree, with an initial burst of drug from the matrix once the hydrogel begins to take on water. Up to 70% of the loaded drug is capable of being released in this initial burst, but crosslinking of the matrix can result in a more controlled release (Bhattarai *et al.*, 2010).

1.7.2 Liposomes and micro-, nano- particles

Both liposomes and micro- and nanoparticles are used as methods for encapsulating drugs. All methods allow for the protection of the enclosed drug which may be advantageous for site specific drug release in the GI tract. Liposomes comprise of artificial lipid bilayers whereas both micro- and nanoparticles are polymeric devices. Liposome encapsulation can reduce toxicity of non-target organs as the encapsulated

drug is confined to the bloodstream (Zeimer and Goldberg, 2001). Similarly, drugs that can also be denatured or modified by pharmacodynamic means may benefit from nanoparticles/liposome encapsulation.

Nanoparticles offer advantages over liposomes in terms of encapsulation as they offer more stability to the drug (Soppimath *et al.*, 2001). Nano capsules have a reservoir of drug surrounded by the polymer. It should, in theory, deliver in a zero order kinetic manner as release is by diffusion.

There are two methods of loading the drug into the polymer nanoparticle: During production, incorporating the drug into the polymer, or after production, absorbing the drug into the polymer from solution. The former method loads a higher drug yield and this is increased when a higher concentration of monomer is present as it will increase drug association (Soppimath *et al.*, 2001).

Encapsulation of drugs into micro and nanoparticles has been seen to increase bioavailability of drugs, particularly protein and peptide based drugs (Ensign *et al.*, 2012). Due to the size of the particles, they can diffuse into the mucosal layer, therefore, increasing residence time in the GI tract and this can be improved further with coating or formation of the particles with mucoadhesive polymers (Makhlof *et al.*, 2008). Nanoparticles have an advantage over microparticles as they can penetrate much deeper into the target tissue due to the submicron size, with typical sizes of nanoparticles ranging from 10 – 100 nm (Panyam and Labhasetwar, 2003). Synthetic polymers, although providing a longer and more controlled release profile, require the use of harsh conditions, such as heat and sonication and organic solvents during formulation (Panyam and Labhasetwar, 2003). The loss of therapeutic function of vascular endothelial growth factor (VEGF) was observed when encapsulated into PLGA nanoparticles (Panyam and Labhasetwar, 2003). There were two theories behind this loss: the exposure of VEGF to solvents during formulation led to the denaturing of the protein, or the inactivity of the protein stimulation due to the acidic nature of the PLGA matrix once it had started to degrade. Solutions to these problems included the addition of bovine serum albumin (BSA) into the matrix, which protected the protein from the oil-water interface formed during the formulation procedure, and secondly, the addition of a buffering base to the matrix neutralised the acid formed during matrix degradation.

1.8 Polymers used as mucoadhesive drug delivery systems

1.8.1 Chitosan

Derived from the deacetylation of chitin (Makhlof *et al.*, 2008), chitosan, Figure 1.3, is the most commonly used material for mucoadhesive polymers. It is a naturally occurring polysaccharide which is cationic and hydrophilic in nature. Cationic polymers are thought to be far superior with regards to their mucoadhesive properties due to the interactions between the positive charge of chitosan and the negative charge of the mucosal layer, such as sialic acid (Ludwig, 2005).

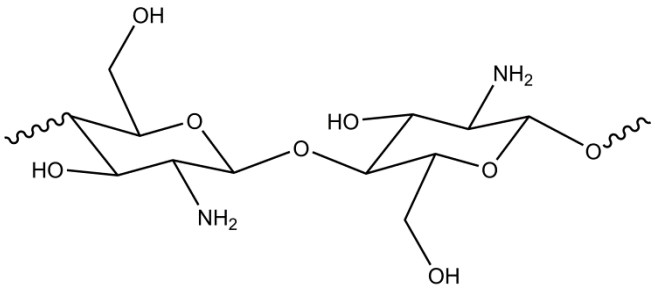

Figure 1.3 Structure of chitosan

Chitosan has many advantages for use in drug delivery, including its biodegradability, biocompatibility and non-toxic properties (Ludwig, 2005). It is also seen to have anti-bacterial properties which is advantageous, particularly in ocular drug delivery where infections are common (Ludwig, 2005). As a polymeric delivery system, it is well tolerated in the body, highly stable, and has the capability to enter epithelial cells making it an excellent carrier for drugs. It is available in a range of MW, with weights between 10 - 450 kDa being normal for drug administration (Makhlof *et al.*, 2008). Chitosan is easily modified under mild conditions and is capable of incorporating macromolecules into the polymeric framework and is, therefore, useful for drug, protein and peptide delivery (Salamanca *et al.*, 2006).

Chitosan's mucoadhesive properties occur due to the interaction between the positively charged amino groups of chitosan with the negatively charged sialic acid residues of mucins (Nagarwal *et al.*, 2009). It is insoluble at neutral and high pHs and soluble in low pH values. Once it is thiolated, it has cohesive properties and also displays gel-like properties in solution (Bernkop-Schnürch *et al.*, 2004b).

Tozaki *et al.* (2002) encapsulated the anti-inflammatory drug 5-aminosalicylic acid (5-ASA) within chitosan. 5-ASA is used for inflammatory disease states including ulcerative colitis. 5-ASA is often delivered in the form of salazosulfapyridine (SASP) which breaks down to 5-ASA and sulfapyridine within the GI tract. However, although 5-ASA is noted as having few side effects and is the therapeutic agent within the SASP dose, the by-product of sulfapyridine is seen to have numerous systemic side effects associated with it. 5-ASA is also absorbed well within the small intestinal and, therefore, for the treatment of ulcerative colitis, a delivery system for site specific release of 5-ASA to the colon is required. This study compared the release profiles of chitosan to another polymer, carboxyl methyl cellulose (CMC). Results showed that no 5-ASA was observed in the large intestine after oral administration of the CMC encapsulated system. This may be due to the instability of CMC in acidic conditions and the polymer may have degraded in the stomach, with absorption of the drug occurring in the small intestine. This was in contrast to the chitosan capsule, which is degraded by colonic bacteria, where a large quantity of the drug was found in the large intestine and only small amounts in the small intestine. 5-ASA was readily released from the chitosan capsule in the large intestine. Plasma concentrations of the drug released from both CMC and chitosan were also measured, and the levels after oral administration of chitosan capsules were lower. This, again, may be due to the absorption of the drug occurring in the small intestine and thence, systemic circulation after CMC capsule administration. The group concluded that chitosan was suitable for the delivery of 5-ASA.

In oral delivery, chitosan is also seen to improve the delivery of hydrophilic drugs, such as peptides and proteins, as it has the ability to open up tight junctions in the mucosal layer by affecting para- and intracellular pathways (actin pathways) (Dodane *et al.*, 1999).

1.8.2 Polyacrylic acid (PAA)

PAA, Figure 1.4, is an anionic polymer. The main method of adhesion of anionic polymers is due to hydrogen bonding between the polymer and mucins (Ludwig, 2005). Interactions of PAA with mucins are strongest at an acidic pH, indicating that the polymer in its protonated state is best for mucoadhesion (Ludwig, 2005). This

was documented further when the addition of urea disrupted the hydrogen bonds between the polymer and mucus resulting in a reduction in mucoadhesion (Mortazavi, 1995). PAA is soluble across a broad range of pHs (Makhlof *et al.*, 2008). It is not absorbed in the GI tract but it has been shown to have anti-enzymatic properties, particularly to the endopeptidase, trypsin (Luessen *et al.*, 1995). This property is ideal for the delivery of protein and peptide drugs through oral administration as the polymer will protect the peptide drug from degradation.

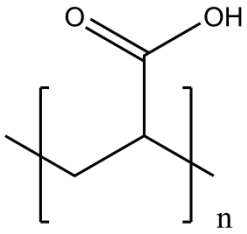

Figure 1.4 Structure of polyacrylic acid

PAA is a commonly used polymer in the production of thiolated polymers, or thiomers as they are also termed. The mucoadhesive properties of a thiolated polymer depend on the degree of swelling and of crosslinking of the polymer. The mobility and the flexibility of the polymer chain are also of great importance for mucoadhesion, as the chain will influence the interactions between the polymer and the mucus. Crosslinking will reduce the flexibility of the chain and may have an effect on the mucoadhesive properties. Leitner *et al*. (2003) investigated the properties of cohesion, mucoadhesion, swelling and disintegration on linear PAA-cysteine conjugates of different MW and, therefore, different chain lengths, ranging from 2 kDa PAA to 450 kDa PAA, and a polycarbophil-cysteine conjugate of MW 750 – 3000 kDa. All polymers had comparable levels of immobilised thiols to allow for a more direct comparison, and results were also compared to unmodified PAA controls. Mucoadhesive studies were analysed by two different methods: Tensile studies and a rotating cylinder method using porcine intestinal mucosa. Both methods concluded that PAA with MW of 250 kDa and 450 kDa exhibited the highest mucoadhesive levels. In addition to these tests, the amount of residual unbound cysteine and its influence on mucoadhesion was measured using 450 kDa

PAA (both thiolated and unmodified PAA). Free cysteine had little to no effect on the mucoadhesion of the unmodified PAA controls, however, the addition of 2.5% free cysteine showed a significant decrease in mucoadhesion time in comparison to 0% free cysteine on the thiolated PAA sample. This means that the complete removal of unbound cysteine by dialysis is vital in the creation of a mucoadhesive drug delivery system. Thiolated PAA 450 kDa sample showed the highest levels of mucoadhesion and showed excellent cohesion. Although the low MW PAA had strong penetration into the mucosa due to the flexibility of the chain, cohesive properties were poor and mucoadhesion was, therefore, affected. Similarly, higher weighted polymers had better cohesion but poor penetration due to the lack of chain flexibility which, again, affected mucoadhesion.

In order to evaluate the effect of varying amounts of L-cysteine bound to PAA, five different PAA-cysteine conjugates were synthesised, each with a different amount of immobilised thiol groups (Palmberger *et al.*, 2007). Levels of thiolation (measured in µmol/gram of polymer) bound to the 450 kDa PAA ranged from 53 µmol/g up to 767 µmol/g. Various studies, including tensile and mucoadhesive studies, were performed. The degree of thiolation was measured using two methods: 1. Ellman's reagent to measure the amount of free thiol groups on the PAA backbone and 2. TNBS (trinitrobenzene sulfonic acid) to measure the amount of remaining unbound cysteine in the reaction solution by measuring the amine groups on cysteine. The total amount of thiol groups on the polymer was also determined by reducing the disulphide bonds with sodium borohydride and then analysing with Ellman's reagent. It was concluded that both mucoadhesion and disintegration of the conjugates strongly depended on the amount of thiol groups, with higher levels of thiolation resulting in longer times of mucoadhesion and disintegration. Mucoadhesion studies were all carried out at pH 6.8 as intermolecular disulphide bonds between the polymer and the mucus can easily be formed at this pH. It was also discovered that without the addition of the crosslinker, 1-Ethyl-3-(3-dimethylaminoprpyl) carbodiimide hydrochloride (EDC), no amide bonds were formed between the polymer and the cysteine.

The swelling capability of PAA is highest when the carboxylate moieties are neutralised. Hornof *et al.* (2003) showed the amount of water increased by up to 1.8-fold at pH 6 in comparison to either pH 5 or pH 5.5. At a pH below 5, the carboxylic

acid groups are protonated and it is this that limits the swelling of the polymer (Varum *et al.*, 2011).

1.8.3 Polylactic acid (PLA)

PLA is a hydrophobic, biodegradable polymer. PLA is often modified, forming a co-polymer with polyglycolic acid (PGA), polylactic-co-glycolide (PLGA), as shown in Figure 1.5. These components are enzymatically broken down to lactic acid and glycolic acids respectively and are metabolised by the Kreb's cycle, minimising any side effects and toxicity caused by the polymer (Yasukawa *et al.*, 2004). Controlled release from PLA is problematic, and, similar to many biodegradable polymer matrices, appears to have a triphasic pattern of release, with an initial burst of drug once the polymer implant is administered into the body followed by a slow and sustained release. During the final stages of degradation, the polymer exhibits a final and uncontrolled burst of drug load, a phenomenon observed more in biodegradable polymers than in non-biodegradable polymers (Kunou *et al.*, 2000; Yasukawa *et al.*, 2004; Yasukawa *et al.*, 2001).

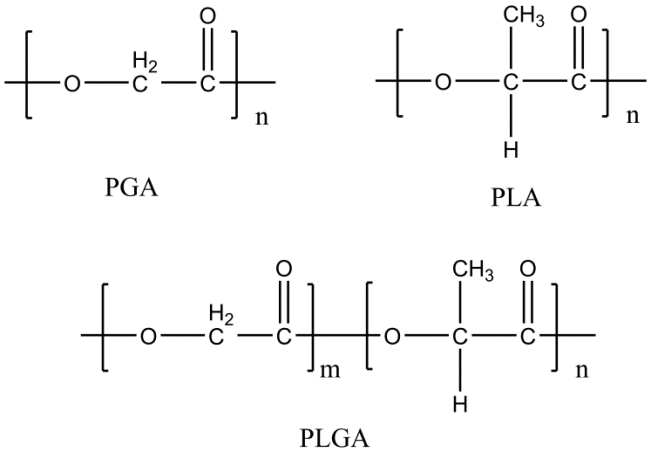

Figure 1.5 Structures of PGA, PLA and PLGA

Kunou *et al.* (2000) showed the use of an implant which combined PLA of two different MWs, medium (PLA 70000) and low (PLA 5000). The combination allowed for a more controlled release of the drug ganciclovir which is used in the treatment of an inflammatory retinal condition, cytomegalovirus retinitis. This

blended polymer matrix removed the issue of the second burst of drug, creating a pseudo zero order kinetic like property. The study used a variety of ratio blends and discovered that the best ratio of medium: low MW PLA was 80:20 which successfully eliminated the second burst of drug. Thus, the rate of degradation of a PLGA matrix depends on the ratio of PLA to PGA, with higher quantities of PGA resulting in a faster rate of degradation.

Due to the strong hydrophobic nature of PLA, it is not suitable for the delivery of certain drugs like peptides, proteins and some anticancer drugs (Hu *et al.*, 2002). To improve its hydrophilicity, it is often modified with polyethylene glycol (PEG), which is highly hydrophilic. PEG also increases the hydrogen bonding between mucins and the polymer matrix. One limitation in the creation of these hydrophilic-hydrophobic entities is the use of organic solvents in their preparation (Hu *et al.*, 2002). Modification with PEG also affects the binding properties of the polymer to serum proteins, particularly opsonins, upon systemic administration. By limiting the binding of the polymer to unwanted serum proteins, the availability of the polymer and, therefore, the drug to the site of action will be increased (Kommareddy and Amiji, 2007). In mucoadhesive drug delivery, PLGA nanoparticles were synthesised and coated with either chitosan or PAA to form mucoadhesive drug delivery systems (Kawashima *et al.*, 2000). The nanoparticles were loaded with protein-based drugs and the mucoadhesive properties of the particles were tested on the intestinal tissue of a rat. The particles coated in chitosan showed improved mucoadhesion in comparison to those coated with PAA and it was surmised that the increase in mucoadhesion was due to the electrostatic interactions between chitosan and the mucosal surface. Similarly, Grabovac and Bernkop-Schnürch (2007) coated PLGA nanoparticles with thiolated chitosan to increase its mucoadhesive properties further.

1.8.4 Pectin

Similar to chitosan, pectin, as shown in Figure 1.6, is a polysaccharide which is isolated from citrus peel or from apples. As a natural polymer, it has non-toxic properties and it is often used within the food and pharmaceutical industries. Pectin is a complex heteropolymer which contains residues of galacturonate and rhamnose. It is anionic in nature with high levels of carboxyl groups along its backbone. Due to

the levels of hydroxyl groups, pectin is also highly hydrophilic making it ideal for swelling in drug delivery (Pratt and Cornely, 2011).

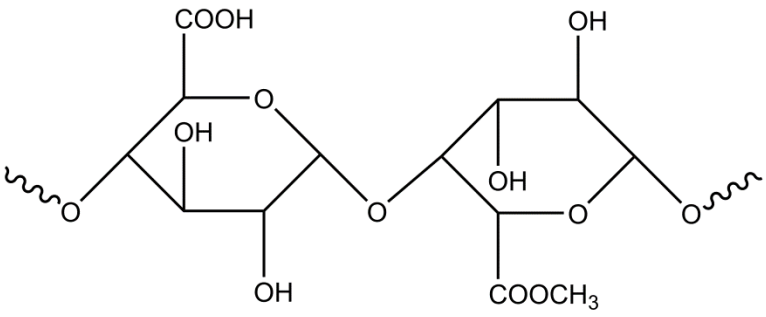

Figure 1.6 Structure of pectin

Modification of the pectin backbone often occurs through either methoxylated or amidated carboxyl groups, and it is the degree of modification that can allow for altered levels of mucoadhesive properties (Joergensen *et al.*, 2011). Pectin also has the benefit of being water soluble allowing for modifications to occur under mild conditions. The MW of the pectin samples have been proven to be important in mucoadhesive properties; using pectins of varying MW ranging from 80 – 200 kDa and also with varying levels of modification, Thirawong *et al.* (2007) investigated the mucoadhesive properties of pectin using a texture analyser. Samples were classified as either high methoxy pectin or low methoxy pectin and one of the low methoxy pectin samples also had a degree of amidation. In general, the high methoxy pectin samples showed improved mucoadhesion in comparison to the low methoxy samples. The addition of amide groups to the low methoxy sample also improved mucoadhesive properties. The results were in contrast to previous studies where low methoxy samples had higher mucoadhesive properties and it was suggested that the MW of the samples used by Thirawong *et al.* had a marked influence on the mucoadhesive properties of the samples; previous studies examined mucoadhesive properties of varying methoxylation but comparable MW (Liu *et al.*, 2005). The highest level of methoxylation and the highest MW demonstrated the greatest mucoadhesive properties, thus showing the importance of polymer properties in mucoadhesive drug delivery. Interestingly, the area of GI tissue that the samples were tested on was also of importance. Tissue was taken from the whole

length of the GI tract, from the mouth to the large intestine, and pectin samples were tested on each section for mucoadhesion. Samples of chitosan and carbomer946P, known mucoadhesive polymers, were also tested as positive controls in the mucoadhesive testing. Each tissue sample was submersed in media relevant for the area, i.e. lower pH buffer for stomach tissue in comparison to intestinal tissue etc. Mucoadhesion was noted to be highest in the large intestine and there was little variability between the buccal, stomach and small intestine tested. It was thought that the variability in mucosal levels between the different areas of the GI tract played a marked role in the mucoadhesive levels of the samples; the large intestine has a high ratio of goblet cells resulting in high levels of mucus.

Similar to PAA and chitosan, pectin is often thiolated to improve its mucoadhesive properties. Sharma and Ahuja (2011) thiolated pectin using thioglycolic acid and examined the thiolated pectin product for its mucoadhesive properties. Beads of both thiolated and unmodified pectin were synthesised and were loaded with the drug, metformin. It was observed that thiolation improved the mucoadhesive nature of pectin by up to 2-fold. Pectin, due to the presence of hydroxyl groups, is naturally mucoadhesive with electrostatic interactions occurring with the mucosal layer and, in a wash-off bioadhesive test on goat intestinal tissue, the unmodified pectin beads remained adherent for up to 2.5 h. This is in comparison to the thiolated beads which remained adherent for up to 5 h using the same test procedure. Similarly, in the swelling tests, the thiolated beads had a lower rate of swelling in comparison to the unmodified samples and it was also observed that the unmodified samples eroded at a faster rate than the thiolated samples. Therefore, the lower levels of adhesion observed by the unmodified pectin beads were deemed to be due to increased swelling ability and faster erosion in comparison to the thiolated samples. Interestingly, in drug release studies of metformin, the release profiles of both the unmodified and thiolated beads were comparable. Therefore, the improved swelling ability of the thiolated sample did not result in a more controlled release of drug over time.

1.8.5 Gelatin

This is a natural polymer derived from Type 1 collagen (which contains no cysteine) of animals and fish. Gelatin is hydrophilic, is degraded enzymatically and, as it is a natural polymer, it does not induce an immune response. These properties make gelatin an excellent component for potential drug delivery applications.

Depending on the pre-treatment process from the collagen, two different forms of gelatin can be produced: acidic (Type A) or basic (Type B). Each process creates a gelatin form with a different isoelectric point (IEP), the point at which a molecule carries no net charge. The acidic process produces an IEP similar to that of collagen (7 - 9) whereas the basic process is much lower (4.8 – 5.2). The alkaline process converts the amide groups of asparagine and glutamine in collagen to carboxyl groups in gelatin, resulting in a higher number of carboxylic acid groups in gelatin B in comparison to gelatin A. The resulting gelatin B has a lower IEP. The varying IEP for gelatin allows for a greater flexibility when it comes to drug delivery systems; it allows the gelatin carrier to be of opposite charge to the drug, depending on the drug in question (Young *et al.*, 2005). This allows for the potential formation of a polyion complex, a strong bond formed due to electrostatic interactions between oppositely charged entities (Young *et al.*, 2005; Tabata and Ikada, 1998).

The amino acid composition of native gelatin is 16% carboxylate and 13% primary amine. Gelatin is comprised mostly of proline, glycine and hydroxyproline. It has a general structure similar to collagen containing repeating sequences of glycine–X-Y triplets, where X is often proline and Y is hydroxyproline, as shown in Figure 1.7. Due to the presence of these glycine–X-Y triplets, gelatin can exist in a triple helical structure as its most stable conformation (Pratt and Cornely, 2011)

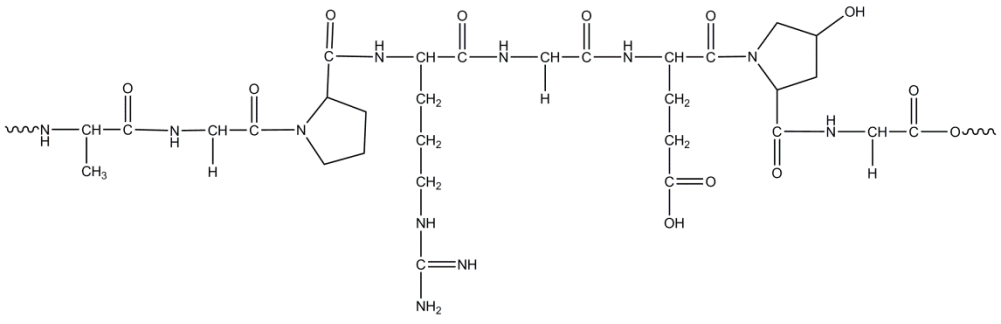

Figure 1.7 Structure of type B gelatin (Elzoghby, 2013)

The gel strength of gelatin is measured in terms of bloom strength; therefore, the higher the bloom value, the stiffer the gel. Bloom strength also correlates to MW so again, the higher the bloom strength, the higher the MW. Gelatin dissolves in aqueous solutions at approximately body temperatures (37 °C). Here it forms single, flexible coils and will become transparent upon cooling (Vlierberghe *et al.*, 2011). Because of this ability to dissolve, gelatin must be chemically crosslinked in order to become insoluble at body temperature (Dinarvand *et al.*, 2005). Gelatin is easily modified under mild conditions, which is ideal for drug delivery carriers. Mild conditions can also be used to load the drug into the polymer matrix (Young *et al.*, 2005). However, when a protein based drug is present during the production of gelatin hydrogels, the proteins are seen to lose pharmacological activity due to the chemical crosslinking required in the creation of the hydrogel (Tabata and Ikada, 1998). This problem can be avoided by allowing the protein to absorb into the hydrogel post-production.

Dinarvand *et al.* (2005) created a crosslinked gelatin based microsphere and loaded it with lactic acid. The crosslinking agent used was gluteraldehyde and loading of lactic acid into the spheres occurred after crosslinking. A biphasic release pattern was observed with up to 80% of lactic acid being released initially. This was seen to be advantageous, as the initial burst was at therapeutic levels while sustained release was still realised. The effect of the crosslinking concentration levels over time was evaluated. It was concluded that a shorter crosslinking time, one hour, and lower concentrations of gluteraldehyde, 12% v/v, gave optimal results with regards to swelling of the gelatin matrix.

A similar study involved a gelatin based mucoadhesive delivery system for the delivery of amoxicillin to the gastric mucosa (Wang *et al.*, 2000). The bacteria, *Helicobacter pylori*, are the main cause of ulcers and gastritis. A constant level of antibiotic in the gastric mucosa is required to treat the condition. This study compared the mucoadhesive and drug release properties of gelatin microspheres versus modified gelatin microspheres which were firstly aminated with ethylene diamine and secondly crosslinked using varying levels of gluteraldehyde (0.06%, 0.12%, 0.18%). Different crosslinking times were also measured. The unmodified gelatin microspheres had an initial burst, releasing up to 80% of its drug load within 30 min. The varying levels of crosslinked microspheres showed decreased bursts

within the same time period. The highest level of crosslinkage, 0.18% gluteraldehyde, had a slower and more controlled release of amoxicillin but the increased levels of crosslinkage affected the mucoadhesion, with adhesion levels being similar to that of the unmodified gelatin. Highest levels of mucoadhesion were observed with 0.06% gluteraldehyde. This indicates that a compromise between levels of crosslinkage was required to establish an efficient mucoadhesive system which will allow for slow, controlled release.

Using ethylene diamine aminated gelatin, Seki *et al.* (2005) examined the absorption enhancing effects in the intranasal delivery of protein and peptide drugs. Without causing damage to the epithelial membrane, absorption enhancers increased the drug permeability and were particularly useful in the permeation of peptide and protein drugs which are prone to degradation upon administration and reduced bioavailability. Gelatin samples with varying amine content were analysed and insulin was used as the model drug for nasal absorption in rats. By measuring the plasma glucose levels of the rats, a level of absorption was determined. Unmodified gelatin samples demonstrated glucose levels similar to that of control samples, i.e. an administration of PBS solution without insulin. Aminated gelatin samples demonstrated a decrease in plasma glucose, with higher levels of amination showing higher decreases in plasma glucose concentration, suggesting the absorption enhancing effects of aminated gelatin.

Vandervoort and Ludwig (2004) prepared gelatin microspheres for ophathalmic drug delivery. The microspheres encapsulated one of two drugs, pilocarpine HCl, a hydrophilic drug, and hydrocortisone, a hydrophobic drug. Both type A and type B gelatins were used to encapsulate both drugs and were compared using different pH levels: pH 4 or pH 6. At pH 4, both gelatin types were positively charged, whereas at pH 6, type B was negatively charged. In order to increase the solubility of hydrocortisone, cyclodextrin complexes were prepared using either hydroxyl propyl-β-cyclodextrin, a neutral molecule, or 2-hydroxy-3-trimethyl-ammoniopropyl cyclodextrin, a cationic molecule. As shown in Figure 1.8, the release of pilocarpine from the gelatin microspheres was slower than that of the control, with 30% drug released after 3 h from the microsphere versus 50% from the control over the same period. The gelatin type used had little to no difference on the drug release profiles of the pilocarpine encapsulated microspheres, with similar amounts of drug being

released from both the type A and B gelatins at specific time points. The pH levels had no marked effect on drug release rates. Release of hydrocortisone was slower from the microspheres than from the control and there was a slight difference in release rates between the gelatin types used. This may be due to the cyclodextrin complexes interacting with the gelatin types.

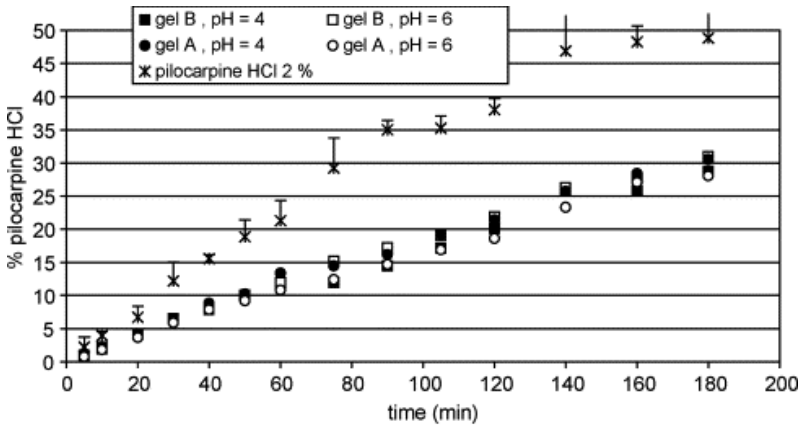

Figure 1.8 Pilocarpine HCl release from type A and type B gelatin nanoparticles and control solution (Vandervoort and Ludwig, 2004)

1.8.6 Polyallylamine

Polyallylamine is a synthetic, cationic polymer, shown in Figure 1.9 as (A) its free base form, and (B) its salt form. It is a linear polymer with primary amines along its backbone. Polyallylamine hydrochloride (PAH) is commonly used in the treatment of hyperphosphatemia in patients with end-stage renal failure. Crosslinked with epichlorohydrin and known in this form as sevelamer hydrochloride, it acts as a phosphate binder, removing excessive phosphate ions as it is excreted (Hudson *et al.*, 2012). It is non-biodegradable but is not absorbed into the blood stream. It is also excreted unchanged and therefore, is not metabolised to toxic by-products or metabolites.

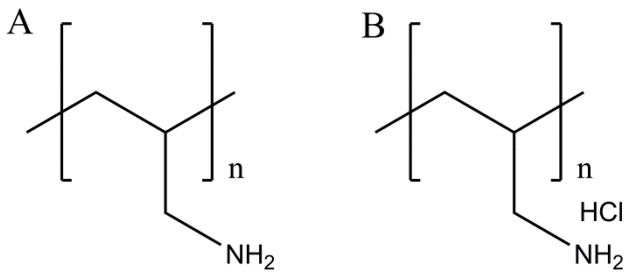

Figure 1.9 Structures of polyallylamine (A) and polyallylamine hydrochloride (B) (Vigl *et al.*, 2009)

In the form of a polymeric micelle, PAH was used as a delivery system to the GI tract for siRNA (small interfering RNA) (Guo *et al.*, 2013). Polymeric micelles are emerging as a platform in the oral delivery of gene based drugs as the drug is protected in the hydrophobic centre of the micelle. PAH was modified with cholesteryl and palmitoyl moieties and also a hydrophilic ammonium moiety, creating a so called quaternised PAH product. This product contained a hydrophobic interior and hydrophilic exterior, which was thought to be more stable in an acidic environment such as the stomach; amine groups on an unmodified PAH sample would be affected greatly by this acidic environment. siRNA bound to the PAH by electrostatic interactions and its stability in simulated GI fluids was measured. Stability of siRNA in the quaternised PAH was improved in both simulated gastric fluid and simulated intestinal fluid in comparison to an unmodified PAH system, implying the protective nature of this modified PAH polymeric micelle. Although not analysed in this paper, the quaternised PAH was also seen to interact with the mucosal layer in the GI tract, again through electrostatic interactions between the polymer and mucins. This resulted in increased residence and contact time.

Cationic polymers are often used as vectors for gene transfer and polyallylamine is one such polymer that is utilised. Polyallylamine, however, can be toxic to cells. Therefore, used in its free base form, polyallylamine (PAAm) was modified to reduce its toxicity and to increase its buffering capacity for enhanced DNA transfer (Oskuee *et al.*, 2015). PAAm of varying MW, 15 kDa and 65 kDa, was used and the polymer was modified by the substitution of the primary amines along the polymer backbone to acrylate derivatives of varying chain length: butyl, hexyl and decyl acrylates. Grafting of the acrylate groups also occurred at 10, 30 and 50% of the

polymer backbone. Oskuee *et al.* showed that upon modification, the cytotoxicity of PAAm was markedly reduced in comparison to the unmodified PAAm sample and the transfection ability of the modified samples was improved in comparison to the unmodified samples. It was also noted that MW and grafting percentage were important to gene transfer; the 15 kDa modified samples displayed improved transfection efficiency in comparison to the 65 kDa sample, and 50% grafting percentage of hexyl acrylate on the 15 kDa was deemed to have the highest gene transfer ability. Modification of PAH to decrease toxicity and increase gene transfer was also performed by Boussif *et al.* (1999). PAH was glycolylated and both cytotoxicity and transfection efficiency were tested and compared to that of unmodified PAH. Glycolylated PAH displayed decreased cytotoxicity in comparison to unmodified PAH. A range of modified PAH samples were synthesised with a degree of glycolylation ranging from 50 – 85%. The samples were tested for transfection efficiency, which was noted to improve after glycolylation in comparison to unmodified PAH. The degree of glycolylation was deemed to be important, as 70% and 75% modification displayed improved efficiency over other modified samples. The importance of modification of polyallylamine was displayed by both Boussif *et al.* (1999) and Oskuee *et al*. (2015) as the toxic effects of the polymer were reduced after modification.

Vigl *et al.* (2009) investigated the effect thiolated PAH had on efflux pump inhibitory properties. Initially, PAH was crosslinked by reacting the polymer with triethylamine followed by the addition of dimethylsuccinate. This crosslinked polymer of 70 kDa MW, along with two uncrosslinked samples of 15 kDa and 70 kDa MW, were thiolated using N-acetlycysteine in the presence of the carbodiimide crosslinker, EDC. Thiol content was measured as 77.6 µmol/g in the 15 kDa sample, 83.1 µmol/g in the 70 kDa uncrosslinked sample and 162.5 µmol/g in the crosslinked 70 kDa sample. Although cell cytotoxicity was low and both cell permeation and efflux pump inhibition were positively affected by the thiolated PAH polymers, it was acknowledged that a higher thiol content would have vastly improved the results, as seen by Clausen and Bernkop-Schnürch (2000).

1.9 Modifying polymer techniques

A common way of modifying polymers is by crosslinking, which can be done by chemical or physical means.

Physical methods include UV irradiation and dehydrothermal treatments. It is hard to control these methods and the end result is often less efficient than that of chemical crosslinking. Chemical crosslinking can be separated into zero and non-zero length crosslinkers. Most chemical crosslinking methods are irreversible, which is unfavourable when using the polymers as delivery systems. Commonly used chemical crosslinkers in polymeric drug delivery systems include gluteraldehyde, carbodiimide and thiolating moities. Thiolation is a reversible method of crosslinking.

1.9.1 Thiolated Polymers (Thiomers)

Andreas Bernkop-Schurch pioneered research in the area of thiolated polymers using a range of different approaches (Bernkop-Schnürch and Steininger, 2000; Hornof *et al.*, 2003; Bernkop-Schnürch *et al.*, 2003; Bernkop-Schnürch *et al.*, 2004a; Bernkop-Schnürch *et al.*, 2004b; Grabovac *et al.*, 2005; Palmberger *et al.*, 2007; Schmitz *et al.*, 2008; Vigl *et al.*, 2009; Iqbal *et al.*, 2011; Wang *et al.*, 2012; Hauptstein *et al.*, 2013; Hauptstein *et al.*, 2014; Hauptstein *et al.*, 2015). By thiolating well established mucoadhesive polymers, mucoadhesive properties have been greatly improved, with up to a 140-fold increase in adhesive properties (Bernkop-Schnürch, 2005). This is due to the formation of inter- and intra- disulphide bridges with the cysteine rich areas of the mucins. These covalent bonds formed with mucins are much stronger than the non-covalent bonds formed by non-thiolated polymers, and mirror the natural bonds formed by mucins themselves. Ideally for drug delivery systems and mucoadhesion, a thiol content value of at least 400 µmol/g of polymer is required, although mucoadhesion is much better with a higher value. The degree of intra- and inter-crosslinking depends on the amount of free thiol moieties present on the polymer backbone; the more thiol groups attached, the more cohesive and adhesive the polymer. However, unbound thiol will disrupt mucoadhesion, so it is imperative that molecular residues which possess free thiol groups be removed from the polymer prior to use. Leitner *et al.* (2003) investigated the mucoadhesive properties

of thiolated PAA in comparison to a control PAA sample. All samples were dissolved in water with and without the addition of 1% cysteine (m/m), therefore, the affect unbound cysteine may have on mucoadhesive properties of thiolated polymers was also examined. The samples were frozen and lyophilised. Mucoadhesive testing was conducted on the tabletted samples with and without the addition of cysteine and, as shown in Figure 1.10, the presence of unbound cysteine had a significant influence on the mucoadhesive properties of the thiolated sample, decreasing adhesion by up to 50% due to the interruption of the polymer-mucin disulphide bond. The addition of cysteine to the control sample had no effect on mucoadhesion, thus highlighting the importance of the removal of unbound thiols from the thiolated polymer matrix.

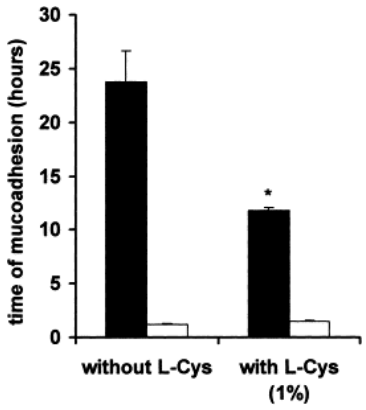

Figure 1.10 Mucoadhesive testing on thiolated and control PAA samples, with and without the addition of 1% cysteine (m/m) (Leitner *et al.* 2003)

Adhesion of the polymer to the surface is futile if there is no cohesion in the polymer itself (without the polymer itself binding together, it is unlikely to interact and bind with mucus). Thiomers show a high level of cohesion. Bernkop-Schnürch and Steininger (2000) compared the cohesive properties of thiolated polycarbophil and carboxymethylcellulose and unmodified samples. It was shown that the disulphide bonds formed within the thiolated polymers created a much more effective and beneficial delivery system than the unmodified versions.

The crosslinking action of thiolation changes the degree of swelling of the polymer, theoretically allowing for a more sustained and controlled release of drug. Thiolation

will prevent over hydration and change the degradation pattern of the polymer, particularly important in hydrogels (Tabata and Ikada, 1998). However, high degrees of crosslinking may affect the mucoadhesive properties of the polymer, as the chain length and flexibility are altered. A commonly used molecule in the creation of a thiomer is L-cysteine (Figure 1.11).

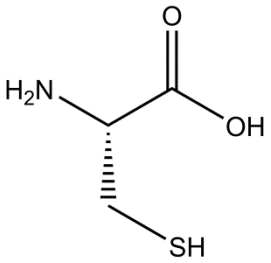

Figure 1.11 Structure of L-cysteine

1.9.1.1 Thiolation with cysteine

Kafedjiiski *et al.* (2007) showed the significant difference between crosslinked polymers and non-crosslinked polymers in relation to rates of degradation and adhesion times. The polymer used was hyaluronic acid (HA) and was crosslinked using L-cysteine ethyl ester hydrochloride. Degradation of HA is due to hydrolysis by hyaluronidase. Both the thiolated and unmodified HA rates of degradation were slow but the thiolated polymer rate was significantly slower, indicating that the crosslinked disulphide bonds formed in the thiolation process inhibited enzymatic hydrolysis.

Grabovac *et al.* (2005) showed thiomers, in general, had higher mucoadhesive properties than their equivalent unmodified polymers and that the mucoadhesion was pH dependent. At a pH over 5.0, the thiol groups in the polymer were more reactive and began to form intramolecular disulphide bonds and in turn did not form them with the cysteine rich domains of the mucins. At a lower pH, pH 3.0, the thiols groups were much less reactive and formed bonds with the mucosal layer. Bernkop-Schnürch (2005) explained that the pH of the medium surrounding the surface of the polymer controlled the reactivity of the polymer as a whole, whereas the pH of the polymeric framework controlled the reactivity of the thiol groups. With regards to

drug delivery systems, the pH of the epithelium would always be around pH 7 and the thiol groups would be reactive enough to penetrate mucus. Thiomers form inter- and intramolecular disulphide bonds at physiological pH due to the oxidation of the thiol groups, classified as in situ gelling properties. Polymers that demonstrate a phase transition, sol-to-gel transition, due to the change in the environment or physiochemical changes (pH, temperature) have been designed. Srividya *et al.* (2001) synthesised PAA based eye drops that were pH triggered and changed phase (liquid to gel) once they had come in contact with the eye. The carrier drug used was ofloxacin, an ocular anti-bacterial agent usually given topically as an eye drop. PAA was used in this experiment due to its capacity to form a stiff gel at elevated pH. The trigger in this case was the change from the pH at formulation, pH 6, to the pH of the eye, pH 7.4. The irritation levels of the PAA formulation was measured using the Draize technique on rabbits. No ocular irritation was observed.

Krauland and Bernlop-Schürch (2004) showed a 3-fold improvement in residence time of thiolated PAA, as shown in Figure 1.12, versus unmodified PAA in the small intestinal mucosal layer. This study used insulin as the model peptide drug for delivery and combined a copolymer of Eudragit RS® (a copolymer consisting of ethyl acrylate and methyl methacrylate) into the thiolated PAA matrix. Previous studies using insulin and thiolated PAA without Eudragit RS® had observed that the insulin had burst-released within a number of minutes, before the polymer had adhered to the surface. A more controlled and prolonged release of insulin was observed when Eudragit RS® was incorporated into the microparticles. The delivery of insulin is a constant problem, with oral delivery of the peptide not being viable due to the denaturisation of the protein. Current delivery is only by subcutaneous injection.

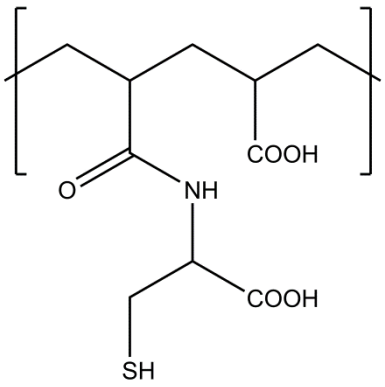

Figure 1.12 Proposed structure of PAA-cysteine conjugate (Bernkop-Schnürch and Steininger, 2000)

Grabovac *et al.* (2008) designed a three layered mucoadhesive patch for delivery of insulin by oral administration and mucoadhesion to the small intestine. The mucoadhesive patch consisted of a polycarbophil-cysteine matrix within which was a mixture of insulin, mannitol and gluthatione, all dissolved in water. Due to the small surface area of the patch and low levels of water uptake and, therefore, limited swelling of the polymer at the site of action, a single dosage of insulin was created by sticking two separate polymeric matrices together which were then surrounded by ethylcellulose and then Eudragit, which acts as an enteric coating and has a pH threshold of 5.5. Plasma insulin levels and blood glucose levels over time were examined in three separate cohorts of rats; one cohort receiving the polymeric patch, another had subcutaneous delivery of insulin and finally, oral administration of an insulin solution used as the control group. The polymeric matrix showed a more sustained insulin level in the blood plasma, which is to be expected as the insulin was released in a more controlled manner in comparison to the subcutaneous injection. After oral administration of insulin solution to the control group, there was no change in the insulin levels in the blood. This shows the efficiency of the mucoadhesive polymeric system.

Clausen and Bernkop-Schnurch (2000) also demonstrated the permeation enhancing effects of thiolated polymers on tight junctions in tissues. In this study, the permeation of thiolated polycarbophil with peptide based drugs on mucosal tissues was investigated. It was thought that these permeation enhancing effects were due to the bonding of the thiolated polycarbophil with extracellular Ca^{2+}, therefore allowing the tight junctions to open. They also observed a decrease in the transepithelial

electrical resistance (TEER), which is the measurement of tightness between mature epithelial cell junctions (Sigurdsson *et al.*, 2013). They concluded this decrease in TEER as the loosening of the membrane's tight junctions, which contributed to the passive diffusion of the peptide drugs across the membrane.

More recent advances in thiomer synthesis include the development of so called preactivated and S-protected thiomers. The concept of preactivated thiomers using PAA (Iqbal *et al.*, 2012) came to light due to the oxidation of thiols at pHs above pH 5, and since then, the mucoadhesive properties of many other preactivated and S-protected polymers have been investigated including chitosan, pectin, crosslinked PAA and alginate (Dünnhaupt *et al.*, 2012b; Hintzen *et al.*, 2013; Bonengel *et al.*, 2014; Hauptstein *et al.*, 2015). The preactivation of thiomers uses theory based on covalent chromatography where proteins were successfully linked to thiol-bearing resins once they were preactivated with pyridyl structures (Iqbal *et al.*, 2012; Brandt *et al.*, 1977). The preactivation of the thiol group means it was essentially protected by a non-toxic aromatic leaving group attached by a disulphide bond, protecting the thiol from oxidation. This bond was broken by a disulphide exchange between the thiol groups of the mucosal layer and the polymer thus releasing a free thiol which binds to the mucosal layer, improving mucoadhesion. The oxidation effect of thiols was seen to lessen the mucoadhesive properties of certain thiomers, therefore, protecting the thiol groups of the polymers allows for greater stability over a broader range of pH. Iqbal *et al.* (2012) observed enhanced stability of preactivated PAA blends in comparison to unmodified and thiolated PAA. Higher levels of interactions with mucin glycoproteins were also observed with the preactivated thiomers, which was due to the presence of hydroxyl groups on the preactivated thiomers. This was shown through rheological testing where higher levels of apparent viscosity in preactivated polymer and mucin mixtures were measured in comparison to both the unmodified, and thiolated (non-preactivated) polymer blends.

A similar study for vaginal delivery using preactivated chitosan thiolated with thioglycolic acid was conducted (Friedl *et al.*, 2013). The use of thiolated polymers for drug delivery to more acidic regions of the body, gastric or vaginal, has also been impeded due to the reactivity of the thiol group in acidic pH, lessening mucoadhesion. Gastric drug delivery with preactivated thiomers was examined in greater detail using pectin (Hauptstein *et al.*, 2013). Pectin was initially thiolated

with cysteine and was then activated with dimers of 2-mercaptonicotinic acid to create the preactivated thiomer product. Swelling, disintegration and mucoadhesive properties were all improved in comparison to the unmodified polymer. Mucoadhesion was tested in both acidic and basic conditions, simulating gastric and intestinal adhesion. Mucoadhesion was vastly improved in the acidic conditions using the preactivated polymer over the thiolated counterpart, due to the increased stability of the preactivated thiomer at lower pH.

1.9.1.2 Thiolation with Traut's reagent (2-iminothiolane)

Traut's reagent, Figure 1.13, reacts with primary amines groups on the polymer backbone and converts them into thiol groups. Bernkop-Schnürch *et al.* (2003) used Traut's reagent to thiolate chitosan. Chitosan had previously been thiolated using thioglycolic acid, creating a thiomer with an uncharged amide bond linkage (Kast and Bernkop-Schnürch, 2001); by thiolating with Traut's reagent, a cationic linkage bond was formed which had the potential to form ionic interactions within the mucosal membrane with residues such as sialic acid or sulfonic acid. Mucoadhesion time on porcine mucosa tissue using the rotating cylinder method was increased by up to 140-fold after thiolation with Traut's reagent.

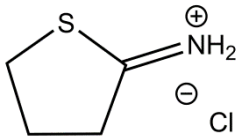

Figure 1.13 Structure of Traut's reagent (2-iminothiolane)

Vlierberghe *et al.* (2011) compared two different thiolating agents in their reaction with gelatin. The two compounds used were N-acetylhomocysteine thiolactone and Traut's reagent. The main body of analysis in this paper was conducted using N-acetylhomocysteine thiolactone, however, thiolation levels using both compounds, N-acetylhomocysteine thiolactone and Traut's reagent, were compared. Both thiolated gelatin products were analysed using UV spectrometry and size exclusion chromatography. Thiolation levels were analysed using both Ellman's reagent and

using the ortho-phthalic dialdehyde (OPA) method; Ellman's reagent analysed the thiol content whereas the OPA method measured the free amine content, which when compared to unmodified gelatin, gave the degree of substitution. The OPA method indicted higher levels of substitution being present than the Ellman's method, implying the presence of disulphide bonds in the product. When one equivalent of Traut's reagent was added to the reaction, 70% degree of modification was achieved. This is in comparison to only 30% with N-acetylhomocysteine thiolactone. In a subsequent part of the experiment, the product thiolated with N-acetylhomocysteine thiolactone was examined. In the form of a hydrogel, and thiolated at different levels, various factors including swelling tests, rheology and texturometry were investigated. Rheological results showed that the storage modulus (G') was higher in the thiolated samples in comparison to unmodified gelatin and that as thiol content increased, so did G'; this was due to the ability of the thiolated samples to crosslink and form a gel. When comparing G' at different temperatures, room temperature (21 °C) and 50 °C which is the temperature above the sol-gel transition of gelatin, it was shown that disulphide bond formation contributed alone to G' at 50 °C whereas at 21 °C, the physical properties of gelatin itself also contributed. Swelling properties decreased as the thiol content increased showing that crosslinking of the polymer influences its swelling ability.

Bacalocostantis *et al.* (2012) thiolated polyallylamine (PAAm) with Traut's reagent, the proposed structure of which is in Figure 1.14. Different ratios of Traut's reagent were added to the reactions to result in an overall 5%, 13%, or 20% conversion of amines to thiol groups. The resulting thiolated polymer was used as a delivery vector for DNA in which the cationic domains of the polymer were electrostatically bound to anionic charged phosphate groups on DNA. Each thiolated polymer and unmodified polymer was tested for DNA binding and release rates, and protection of DNA. The 13% conversion rate showed the greatest potential, with higher DNA binding ability and better release of plasmid in comparison to both unmodified polyallylamine and to the 5% and 20% counterparts.

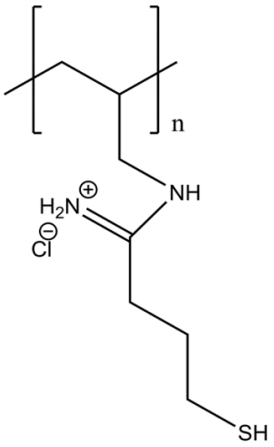

Figure 1.14 Proposed structure of PAAm thiolated with Traut's reagent

Also using thiolated polymers as a non-viral delivery vector for DNA, Kommareddy and Amiji (2005) thiolated gelatin nanoparticles using Traut's reagent. Thiolation, in this case, was used as a means of stabilising the nanoparticle; the disulphide bonds formed due to oxidation in the body strengthened the gelatin's structure. Upon entering a reducing environment, these disulphide bonds would break and the drug would be delivered. Because of this theory, the delivery of the model drug, fluorescein isothiocyanate dextran, was tested in the presence of the reducing agent glutathione. Additionally to this, plasmid DNA was inserted into the nanoparticles. The encapsulation efficiency and stability were measured as well as transfection into murine fibroblast cells. In cytotoxicity testing, the gelatin nanoparticles were shown to be non-toxic with up to 92% cell viability seen at high concentrations; it was observed that the higher levels of thiolation resulted in higher levels of cytotoxicity. Varying levels of thiolation were analysed for both drug release and plasmid DNA encapsulation and they were compared to an unmodified gelatin nanoparticle. Additionally to this, nanoparticles were either crosslinked or non-crosslinked. In the case of loading efficiency of fluorescein isothiocyanate dextran, the thiolated nanoparticles were seen to have a higher loading capacity than the unmodified counterparts. Release was measured over a 5 hour period and rates varied depending on the amount of glutathione added, with higher amounts of glutathione resulting in enhanced release when compared to release in PBS. Thiolation levels affected the percentage release, depending on the concentration of glutathione added, with unmodified particles releasing the drug much quicker. Similarly, crosslinking of the

particles resulted in slowing the release of the drug in comparison to non-crosslinked particles, regardless of the amount of glutathione added. Thiolation levels did, however, affect the loading of plasmid DNA into the nanoparticles; unmodified gelatin particles had a loading capacity of 99% but this figure fell to 90% with thiolated nanoparticles of thiol content 16.41 mM/g gelatin. The plasmid DNA did remain stable within the thiolated particles and were comparable to the unmodified particles and also to naked plasmid DNA.

1.10 Drug Delivery

The preferable route of drug administration for both patients and doctors is by mouth. Oral delivery is the easiest route for administration i.e. does not need professional administration and is most likely to have higher patient compliance (Patel *et al.*, 2011). Absorption is through the small intestine. However, many problems are associated with this route, with low bioavailability, systemic side effects, acidic conditions in the stomach and enzymatic degradation of the active ingredient being leading concerns. The latter is a major concern with the delivery of protein/DNA based therapeutics (Gamboa and Leong, 2013; Patel *et al.*, 2011).

Mucoadhesive drug delivery as a route of administration has the ability of increasing the residence time of the dosage form at the site of action which may allow for greater bioavailability (Khutoryanskiy, 2011).

1.10.1 Oral Mucosa

The oral mucus is easily accessible for drug delivery and devices, such as implants or inserts, can be easily removed, if necessary. Although the surface area is relatively small, the mouth is highly vascularised leading to rapid drug absorption. The buccal (the cheek tissue) and sublingual (under the tongue) regions are the main areas used in oral drug delivery. Both routes bypass GI tract degradation and hepatic first pass metabolism, both of which often lead to decreased drug plasma levels and are important considerations in drug delivery. The thickness of oral mucosa varies throughout the mouth. Sublingual mucosa is more permeable and is a thinner layer

with high blood flow compared to buccal mucus, but has a number of drawbacks (Madhav *et al.*, 2009). The sublingual area is constantly being washed by saliva, which may impede adhesion or wash the polymer tablet away. It has a far higher tolerance to allergens than any other mucosal surface in the body, which is advantageous with regards to potential irritation caused by the polymeric systems. However, it has been observed that involuntary swallowing and increased saliva production can decrease the quantity of drug absorbed.

1.10.2 Ocular Mucosa

The eye is a notoriously difficult target for drug delivery, the structure of which is shown in Figure 1.15. Not only has the eye structural barriers that impede the penetration of a drug, it has functional barriers, such as drug clearance by the conjunctival tissues, affecting the delivery of drug to the target tissue (Anderson *et al.*, 2010). Similar to the oral mucosa, ocular mucosa has the advantage of being easily accessible. However, there are a number of structure components of the eye that act as protective barriers, two of which are the blood-aqueous barrier and the blood-retinal barrier collectively known as the blood-ocular barriers (Arto, 2006). Acting in a similar fashion to the blood-brain barrier, these barriers consist of tightly packed cells which form tight junctions and limit penetration of substances from the blood into the eye. Although most compounds are incapable of passing through these barriers, extremely lipophilic compounds are seen to penetrate due to paracellular permeation (Arto, 2006). The ocular surface is covered by a thin layer called the conjunctiva. This highly vascularised layer covers up to 80% of the eye surface and secretes mucus from goblet cells, keeping the surface hydrated (Gukasyan *et al.*, 2008). The conjunctiva consists of two layers: an outer layer of epithelila, which created the protective barrier to the eye, and the stroma, which contains the blood supply, nerves and other cellular components (Hosoya *et al.*, 2005).

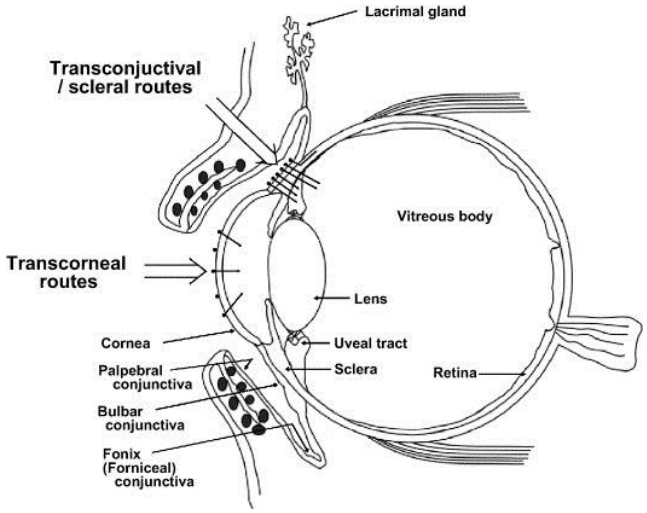

Figure 1.15 Structure of the eye (Hosoya *et al.*, 2005)

Topical preparations, such as eye drops, are the most convenient for patient use, however, they have a very short contact time on the eye and approximately 90% of the administered dosage is cleared within two minutes from blinking and lacrimation (Eljarrat-Binstock *et al.*, 2010). Of the small fraction that does reach the anterior chamber, it is quickly distributed into non-target tissues and is eliminated. Topical preparations require frequent administration and there is often a phase of over-dosage followed by under-dosage. Preparations can be improved with gel-like formulation, but this can cause clouding of vision and, therefore, suffer poor patient compliance. These treatments also only deliver to the surface of the eye and do not penetrate to the posterior segment, which is an area of great pharmacological interest. Systemic drug delivery for targeting the eye can result in serious side effects due to the high dosage needed to pass the blood-retinal barrier (BRB) and often the drug does not reach the eye at effective therapeutic drug concentrations (Eljarrat-Binstock *et al.*, 2010).

Posterior segment diseases of the eye are being treated using intraocular/intravitreal injections. This method delivers therapeutic doses and does bypass the problems associated with drug delivery to the eye but is highly invasive and the associated complications are numerous, including retinal detachment, endophthalmitis, cataracts or haemorrhage. Depending on the molecular size of the administered drug, elimination times from the vitreous can vary and due to the diffusion rates, higher MW compound can be retained in the vitreous for a number of weeks. This is in

contrast to small MW drugs (< 500 Da), which can be eliminated in a number of days and, therefore, need repeated administration (Thrimawithana *et al.*, 2011).

A number of components influence the creation of a drug delivery system to the eye including the half-life, MW and lipophilicity of the drug (the more lipophilic, the more likely to pass through the BRB). Anderson *et al.* (2010) described the penetration of substances into the ocular cavity as being inversely proportional to increasing MW and proportional to increasing lipophilicity. Thus, the smaller the substance, the more likely it is to pass into the eye. Nanoparticle based delivery systems are good at passing through the BRB, particularly for diseases which require chronic administration (Nagarwal *et al.*, 2009). Microspheres have been known to cause a temporary clouding of vision, possibly due to the particles sinking to the bottom of the vitreous (Anderson *et al.*, 2010). Both the lipophilicity and the MW of the drug will influence its half-life.

Small molecules are capable of escaping through the vitreous. The method of PEGylating these molecules to increase their size can remedy this problem (Anderson *et al.*, 2010). Hayashi *et al.* (2007) designed a PAA based polymeric delivery systems surrounding a PEGylated drug in order to slow down the release and the absorption of the drug into the tissue. The PAA adhered to the mucosal surface releasing the drug, and due to the PEGylation and, therefore, increased MW of the drug, it was slowly absorbed across the mucosal surface.

Ocular inserts are becoming increasingly more popular as a delivery system to the eye in order to combat the failings of the eye drop. Without proper adhesion, the insert can move around the eye and cause irritation, however, it is better than most liquid formulations with regards to the release of drugs and concentration levels (Hornof *et al.*, 2003). One potential problem facing ocular polymeric implants and inserts is the possibility of inflammation and the likelihood of experiencing the foreign body sensation. Hornof *et al.* (2003) developed a thiolated PAA-cysteine ocular insert (Figure 1.16 (A)) containing diclofenac samples and evaluated the inserts in human volunteers. Inserts were positioned in the cul-de-sac of the eye. Various different physical attributes of the insert were analysed including sensation, blurring and irritation. Thiolated inserts were compared to unmodified insert controls. The controls dissolved within 15 min of administration whereas the

thiolated inserts were retained for up to 8 h. The drug release and drug removal patterns of the thiolated polymer (PAA-cysteine) insert, an unmodified insert and aqueous eye drops were all compared. Fluorescein, a fluorophotometric agent used as a diagnostic tool in ophthalmology, was used. The eye drop administration resulted in a high concentration of drug immediately after administration. This then decreased substantially after 2 h. For clarity, the drug release profiles of the unmodified PAA insert and the thiolated PAA insert as conducted by Hornof *et al.* are shown in Figure 1.16 (B). It was observed that the unmodified insert had a similar profile to that of eye drops, with an uncontrolled release of drug to the surface of the eye and fast removal. This was due to the lack of cohesion and quick disintegration of the insert. It was also in stark contrast to the thiolated polymer which had a more prolonged and controlled release of the fluorescein. Drug release from the thiolated polymer increased over 1 h but release then plateaued over 7 h.

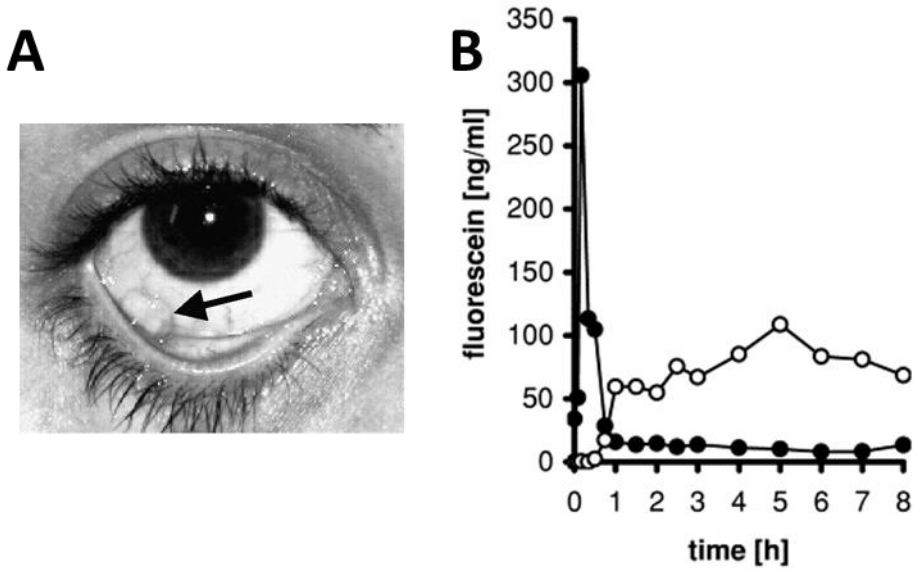

Figure 1.16 (A) Polymeric ocular insert and (B) drug release profile of thiolated PAA insert (open symbols) and unmodified PAA insert (closed symbols) (Hornof *et al.*, 2003)

Polymeric implants have a longer half-life in comparison to intravitreal injections. However, with regards to biodegradable polymer implants, if the drug has a longer half-life than that of the polymer carrier, the polymer may degrade quicker than the drug resulting in elimination of the drug from the target site. Non-biodegradable implants were first established for the treatment of cytomegalovirus retinitis, a

common opportunistic infection associated with AIDS. These implants were reservoir type carriers, surrounded by polyvinyl alcohol (PVA) and ethylene vinyl acetate (EVA) containing ganciclovir. They were a good device with regards to release kinetics, releasing a sustained therapeutic level of drug, but the device itself was very large and needed 4-5 mm sclerotomy for implantation. It also had to be surgically removed (Yasukawa *et al.*, 2004).

1.10.3 Nasal Mucosa

The small hairs and the mucosal layer of the nasal cavity are important protective features which trap foreign particles from inhaled air. The nasal cavity also heats the air on the way to the lungs. As much as two litres of nasal mucus is produced each day (Ugwoke *et al.*, 2005), which will gradually clear from the nasal cavity down the back of the throat, passing into the GI tract for elimination. The cilia of the nasal microvilli beat consistently and it is this action which causes the movement of the mucus. However, mucus turnover is as fast as every 15 - 20 min, which could cause complications with regards to drug delivery through this route. The microvilli in the nasal cavity increase the surface area and increase the area of absorption. The epithelium in the cavity is thin, porous and highly vascularised (Ugwoke *et al.*, 2005). Many factors, including environmental factors, such as temperature or inhaling cigarette smoke (Stanley *et al.*, 1986), and pathological factors, (such as allergies, having a cold or asthma), all affect the rate of elimination and of mucus production, i.e. lower temperatures decrease both the viscosity of mucus and its production. Similarly, inflammatory cytokines alter the mucosal production. This is important for drug delivery strategies. The nasal cavity is observed to have significant immunity against inhaled pathogens due to the production of the immunoglobulin, IgA, by both the mucosal cells and the adenoid tissue of the nose (Ugwoke *et al.*, 2005).

Drugs administered via this route are capable of passing through the olfactory region of the brain and, consequently, pass into the cerebrospinal fluid (CSF) for clearance which is renewed approximately 4 - 5 times a day. Absorbed substances are quickly circulated with little restriction and in certain cases, substances are capable of travelling directly into the central nervous system (CNS), bypassing the blood-brain

barrier (Ugwoke *et al.*, 2005). Enzymatic activity is lower via the nasal route in comparison to the GI tract and because of this, there is a higher bioavailability. In terms of mucoadhesion, initially the turn-over rate of nasal mucosa slows down with mucoadhesion. However, over time, this is reversed and the production and elimination rates return to normal (Ugwoke *et al.*, 2005). Nasal administration may not be a viable route for the delivery of large molecules, such as proteins, due to their size. Lipophilic drugs are absorbed well via the nasal cavity, and are thought to have a similar bioavailability to that of intravenous injection. Low permeability to larger and more polar molecules is the most challenging problem for nasal delivery. Permeable drugs utilise the transcellular routes like concentration gradients or receptor transport to pass through the membrane. A mucoadhesive tablet system for nasal administration will affect the mucociliary transport system (the elimination system). Mucoadhesion opposes this system and prolonged contact, which is key to the design of such a system, may cause irritation and even toxicity. This does not happen with the administration of an aerosol, as the contact time is minimal.

1.10.4 Gastrointestinal (GI) tract mucosa

The GI tract includes the mouth, stomach and small and large intestines. It has a large surface area, increased by the presence of microvilli within the intestine which also increases the total rate of absorption. Mucosal turnover rate is fast and mucosal density varies throughout the GI tract (Ensign *et al.*, 2012). In the stomach, the structure of which is shown in Figure 1.17, mucus is secreted to protect the cell lining from the acidic nature of the stomach. Within the mucosal layer of the stomach, bicarbonate ions are released and trapped which creates a pH gradient between the lumen (pH 1 – 2) and the mucosal layer (pH 6 – 7). Prostaglandins are also released locally which promote the excretion of both more mucus and bicarbonate (H.P. Rang *et al.*, 2003). Depending on the mobility in the GI tract, a specific area of the colon can be targeted for drug release either by coating the polymer with an enteric coating to prevent drug release in the upper GI due to low pH levels, or by increasing the crosslinking on the polymer matrix and thus slowing drug release from the matrix.

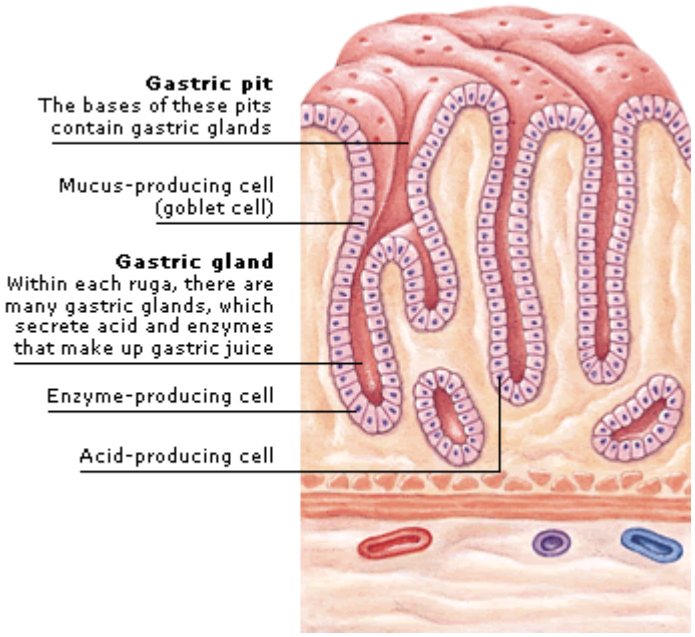

Figure 1.17 Structure of stomach lining (Aviva, 2015)

Chitosan and other polysaccharide based systems are good polymers to use when targeting the GI tract due to enzymatic degradation. Polysaccharides are stable in the stomach and small intestine. Enzymatic degradation begins in the colon, releasing the drug through surface erosion of the polymer matrix. Chitosan is seen to be pH sensitive, dissolving readily at low pH but remaining insoluble at high pH (Bhattarai *et al.*, 2010). Depending on the site of action, different conjugates can be added to the matrix in order to protect the drug. Examples of this include the addition of enzyme inhibitors for GI tract release, which is particularly useful when delivering peptide/protein based drugs to the GI tract (Bernkop-Schnürch *et al.*, 2004b). To avoid adhesion in the mouth or the oesophagus, chitosan tablets were coated with triglyceride (Bernkop-Schnürch *et al.*, 2004b). One reason why mucoadhesive polymeric delivery systems targeting the GI tract have not yet reached their full potential is due to the over-hydration of the matrix before reaching its target site (Kremser *et al.*, 2008). This over-hydration decreases the mucoadhesive abilities of the matrix as mucoadhesion is optimal when the polymer is completely dehydrated and swelling occurs due to the adhesion to the mucosal surface. In the GI tract, if the matrix reaches the target tissue in a hydrated state, it begins to lose its capacity to swell and does not adhere as strongly to the target tissue. To avoid this problem, the mucoadhesive polymeric matrix could be coated with an enteric coating, limiting the

possibility of the polymer becoming hydrated prior to adhesion but also to avoid the polymer adhering to an incorrect area of the GI tract.

To target a specific region in the intestine, a trigger is required so that the drug release will be to the target tissue, thereby ensuring drugs targeting the large intestine are not released in the small intestine. Hydrogels have been designed that are triggered to release the drug load by a change in pH e.g. acidic to basic conditions from the stomach to the small intestine. Gong *et al.* (2011) investigated a pH sensitive hydrogel in simulated GI tract conditions. The hydrogel was chitosan based, cross-linked with alginate and N-α-glutaric acid. The gel was loaded with bovine serum albumin (BSA) and release was measured in both acidic and more alkaline conditions, simulating the stomach (pH 1.2), intestine (pH 6.8) and colon (pH 7.4). The gel beads were initially placed into the acidic solution at pH 1.2 for 3 h and directly transferred into pH 7.4 solution for a further 4 h. It was observed that in the pH 1.2 solution, the hydrogel did not swell as much as in alkaline solution at pH 7.4, and release of the protein was much lower, with as little as 18% being released over the 3 h period. The remainder of the content was released in the alkaline solution.

1.11 Aims and objectives

The aim of this project is to synthesise and characterise three highly thiolated and potential mucoadhesive polymers for drug delivery. Two synthetic polymers, namely polyacrylic acid (PAA) and polyallylamine (PAAm), and a natural polymer, gelatin, will be modified by thiolation of the polymer backbone, thus creating highly thiolated and mucoadhesive polymers. PAA is a well-established mucoadhesive polymer and thiolation has increased its mucoadhesion by up to 140-fold, as discussed in sections 1.8.2 and 1.9.1.1. Therefore, a direct comparison of cohesive and mucoadhesive properties between a known mucoadhesive polymer and two novel thiolated polymers, the synthetic polymer (PAAm) and the natural polymer (gelatin), can be made.

Several reaction processes will be utilised to achieve the modification of the polymers. PAA will be thiolated using L-cysteine and EDC - this is a well-documented process and will be discussed in detail in chapter 2, focusing on a

method in which the levels of PAA thiolation are tightly regulated by controlling the pH (and, therefore, the concentration of EDC) of the reaction. Reproduction of the thiolation levels has been noted as a problem in the literature, and this is the first time, to the authors knowledge, that targeted levels of thiolation can be produced. The thiolation of gelatin will be conducted using a novel two-step reaction process, firstly aminating the polymer with ethylene diamine and EDC and secondly thiolating the polymer with 2-iminothiolane (Traut's reagent). The use of thiolated gelatin for mucoadhesive drug delivery has not been investigated previously, and the synthesis and characterisation of thiolation of gelatin will be discussed in chapters 3 and 4. PAAm will also be thiolated using Traut's reagent in a one-step direct method. Previous work on the thiolation of PAAm has been limited (Vigl *et al.*, 2009; Bacalocostantis *et al.*, 2012; Ibie *et al.*, 2015) and full mucoadhesive evaluation has not been conducted. Therefore, the thiolation and investigation of cohesive and mucoadhesive properties of PAAm will be discussed in chapters 5 and 6.

As thiolation is known to improve cohesion and mucoadhesion, all three thiolated polymers, thiolated PAA, thiolated gelatin and thiolated PAAm, will then be tested for cohesive properties using swelling studies and for mucoadhesive properties using porcine small intestinal tissue and the rotating cylinder method; the thiolated polymers will also be compared to unmodified and control samples of the same polymer. Rheological studies of the unmodified and thiolated polymers will give further insight into the viscoelastic properties and the mucoadhesive properties of the polymers once mixed with a mucin solution.Using chlorpheniramine maleate as the model drug, drug release studies will be conducted using the thiolated polymers and their unmodified counterparts. The thiolated polymers will be characterised using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC), as well as scanning electron microscopy (SEM). Analysis will be conducted on thiolated samples, control samples, and their drug incorporated counterparts. Antimicrobial analysis of thiolated PAAm will also be conducted and compared to the unmodified PAAm samples.

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Chapter 2 Controlling the thiolation of polyacrylic acid by monitoring reaction pH

2.1 Introduction

2.1.1 Thiolation of polyacrylic acid (PAA)

Polyacrylic acid (PAA) is a synthetic polymer commonly used in mucoadhesive polymeric delivery systems. PAA is thiolated with L-cysteine in the presence of a cross-linker 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and the reaction is shown in Figure 2.1.

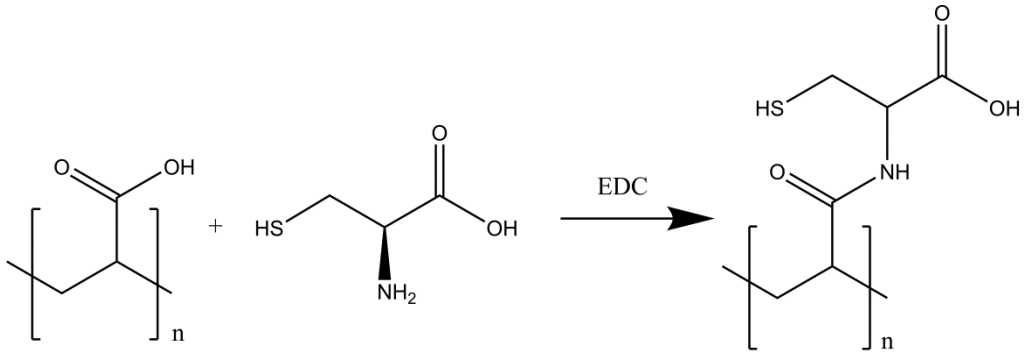


Figure 2.1 Thiolation of PAA with L-cysteine

2.1.1.1 pH and the thiolation reaction

pH plays an integral role in terms of mucoadhesion and the formation of disulphide bonds between the polymer and the mucosal layer. This has been discussed in the literature (Bernkop-Schnürch *et al.*, 2001) and will be addressed in section 2.1.1.2. pH, however, has not been discussed in terms of the thiolation reaction of PAA and the influence that pH may have on that reaction. The pKa of polyacrylic acid is between 4.5 - 5 (Jabbari and Nozari, 2000) and therefore, the pH of the reaction solution will have an effect on the protonation state of the carboxylate groups along the PAA backbone, with increased pH producing a polymer which is less protonated and vice versa. In the literature, the PAA thiolation reaction was conducted at pH 6 (Bernkop-Schnürch *et al.*, 1999; Hornof *et al.*, 2003; Marschütz and Bernkop-Schnürch, 2002). The reactivity of the carboxylate groups would be dependent on the form in which they exist in solution and this could have an effect on the reactivity of the polymer towards L-cysteine in the presence of EDC, and potentially the level of thiolation. Therefore, by monitoring the pH profile of the PAA-EDC solution, an investigation into the influence of pH will be conducted.

2.1.1.2 pH and disulphide bond formation

Disulphide bond formation can occur through a simple reversible oxidation reaction or through thiol-disulphide exchange reactions. The general thiol-disulphide exchange reaction is shown in the Equation 2.1 (Fernandes and Ramos, 2004; Shaked *et al.*, 1980):

Equation 2.1

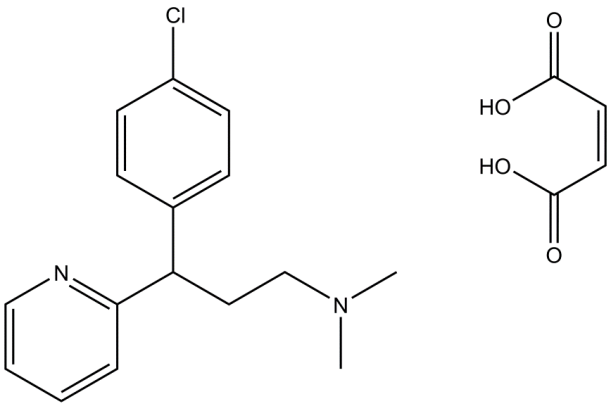
$$\text{R-SH} + \text{R'-SS-R'} \rightleftharpoons \text{R-SS-R'} + \text{R'-SH}$$

It is thought that the thiol-disulphide exchange is a S_N2 reaction in which the thiolate ion attacks the disulphide bond (Fernandes and Ramos, 2004). With this being the case, the concentration of the thiolate ion in the solution will have a major impact on the disulphide bond formation. Thus, the pH of the reaction solution is also of vital importance as this will affect the ionisation of the thiolate ions (Fernandes and Ramos, 2004; Gyarmati *et al.*, 2013), with lower pH values impeding the exchange, while higher pH values speed it up. The nucleophilicity, as well as the pH of the solution, will influence the thiol-disulphide exchange. As it is a thiolate anion which attacks the S-S bond, the pKa of that anion will impact the exchange, as will the pH of the solution, due to the equilibrium between the free thiol and the thiolate anion shifting with changing pH. Bernkop-Schnürch *et al.* (2001) investigated the effect the media pH had on the thiol-disulphide bond exchange. Both polycarbophil and sodium carboxylmethylcellulose were thiolated using cysteamine. The thiol content of each was measured using an iodometric titration. The thiolated polymers were then dissolved in a range of buffers at pH 5, 6 and 7 at 37 °C. Over a period of time, aliquots were taken and again the thiol content was measured using the iodometric titration. It was observed that the thiol groups remained stable and did not oxidise at pH 5 but at pH 6 and 7, there was a vast decrease in thiol groups, implying the formation of disulphide bonds. The greatest thiol decreases were seen in the pH 7 buffer, thus highlighting the importance of pH with respect to disulphide bond formation. In terms of mucoadhesion, at physiological pH, thiol groups will crosslink which will allow the polymer to remain cohesive, thereby keeping the

hydrogel together, but it will also allow disulphide bonds to form between the polymer and the mucosal surface, thus mirroring the natural bonds of mucins.

2.1.2 Chlorpheniramine

Histamine is found throughout the body, but is found in higher concentrations in the lungs, skin and gastrointestinal tract. It is stored in mast cells and released during inflammation or allergic reactions. Anti-histamine drugs act as antagonists to this release and can act on three main histamine receptors: $H_{1\text{-}3}$. The anti-histamine drug chlorpheniramine is a potent H_1 antagonist. In its salt form, chlorpheniramine maleate (Figure 2.2) is most often used in the treatment of hay fever and symptoms of colds and is well tolerated with few side effects (Fried *et al.*, 2002). It is usually administered in the form of an oral tablet and is taken every 4 – 6 h. In general, H_1 antagonist drugs are quickly and well absorbed and distributed throughout the body. Chlorpheniramine is known to have a longer plasma half-life than the majority of H_1 antagonist drugs, with a half-life of 23 h (H.P. Rang *et al.*, 2003). Due to its chiral centre, chlorpheniramine shows enantioselectivity in its pharmacological actions. It is generally administered in its racemate form, with the S-configuration exhibiting the antihistamine response and the R-configuration being responsible for the sedative effects associated with antihistamines (Stephani and Cesare, 1998).



Molecular Weight: 390.86

Figure 2.2 Structure of chlorpheniramine maleate (Hood and Cheung, 2003)

2.1.3 Aims and objectives

The aim of this study is to synthesise thiolated PAA samples with regulated and varied levels of thiolation using an alternative method of thiolation by controlling the pH of the reaction. The resulting thiolated samples will then be investigated for cohesive and mucoadhesive properties, rheological properties and drug release of the model drug, chlorpheniramine maleate. Both the thiolated samples and polymer-drug conjugates will also be characterised using thermal analysis. Investigating the thiolation levels and mucoadhesive properties of PAA allows for a direct comparison between a well-established and well characterised mucoadhesive polymer and a novel, natural polymer, namely gelatin (as discussed in Chapters 3 and 4) and a novel synthetic polymer, PAAm (as discussed in Chapters 5).

2.2 Materials and methods

2.2.1 Materials

Polyacylic acid (450 kDa), 12 kDa cellulose dialysis membrane, 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (Ellman's reagent), L-cysteine, sodium borohydride ($NaBH_4$), Type II porcine mucin and chlorpheniramine maleate (CPM) were all purchased from Sigma Aldrich, Ireland. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was acquired from Carbosynth, Berkshire UK. L-cysteine was obtained from SAFC, Arklow Ireland.

2.2.2 Thiolation of PAA with L-cysteine

0.5 g 450 kDa PAA was dissolved in 50 mL deionised water. Once dissolved, the solution had a pH of approximately 2.9. The pH of the solution was adjusted to pH 5.5 with 20% (m/v) NaOH. To this, 200 mM (1.95 g) EDC was added. The pH initially rose to 7.5 and then began to drop; the pH profile was monitored over time. At different pH points, namely pH 6.5, 6, 5.5 and 5, 1 g L-cysteine was added to the PAA-EDC solution. The reaction was left stirring at room temperature for 3 h before being transferred to 12 kDa dialysis tubing which had been boiled for one hour prior to dialysis. Dialysis was conducted in the dark against 0.2 mM HCl. Control samples

were created using the above method without the addition of EDC. Thiolation of a lower molecular weight PAA, 250 kDa size, was also conducted using the above method. All samples, once dialysed, were frozen and freeze dried using a VirTis benchtop K freeze dyer.

2.2.3 Ellman's Reagent assay

Ellman's reagent solution was used to determine the thiol content of the modified samples. Ellman's reagent was prepared by dissolving 15 mg Ellman's reagent; in 50 mL 0.5 M phosphate buffer pH 8. Standards were created by dissolving 24 mg L-cysteine in 10 mL D.I. water. This solution was then diluted 1 in 10 and from this stock, a set of standards ranging from 0 - 60 µg cysteine/mL (0 – 1000 µmol thiol/g polymer) were made. A 2 mg/mL solution of polyacrylic acid dissolved in D.I. water was also added to each standard to act as a control.

Samples were made up as 1 mg/mL solutions in D.I water. 500 µL of sample was added to 500 µL Ellman's reagent. Standards and samples were incubated at room temperature for 2 h and analysed using a UV-Vis spectrophotometer (Shimadzu UV-2401PC) at 475 nm.

2.2.3.1 Determination of disulphide bond formation

Samples were reduced using $NaBH_4$ to analyse the formation of disulphide bonds. 1.5 mg of polymer was dissolved in 350 µL of D.I. water and 650 µL of 50 mM Tris buffer, pH 7.6. 1 mL of 4% (m/v) $NaBH_4$ was added and the polymer solution was incubated at 37 °C with gentle stirring for 1 h, after which 200 µL 5 M HCl was added to quench the $NaBH_4$. The samples were then analysed, as in section 2.2.3, with the Ellman's regent.

2.2.4 Swelling tests

Swelling tests were performed according to Kast and Bernkop-Schnürch (2001). 30 mg of lyophilised PAA was compressed into a ~1 cm flat disc, using a compaction pressure of 150 bar for approximately 1 min. The discs were attached to a needle and placed in a beaker of 100 mM phosphate buffer, pH 6.7 at 37 °C. At specific time

points, the disc was removed from the buffer, the excess water was removed and the disc was weighed. The weight gain indicated the amount of fluid uptake by the disc. Percentage change in mass was calculated as detailed in Equation 2.2.

Equation 2.2

$$\textbf{Change in mass}\ (\%) = \frac{(\mathbf{Wt} - \mathbf{Wo})}{\mathbf{Wo}} \times \mathbf{100}$$

Where Wt is the weight of the swollen polymer at each specific time point and Wo is the initial, dry polymer weight at time 0.

2.2.5 Mucoadhesive testing

2.2.5.1 Tissue collection and preparation

Porcine small intestine samples were collected from the Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark, Fermoy, Co.Cork.

Tissue samples were taken from 7 - 8 week old pigs. Samples were taken from both the jejenum (the mid-section) and the ileum (the terminal section) of the small intestine. To be consistent, samples of terminal ileum were cut at the ileocecal junction, 15 cm in length. As there is no obvious difference between each section of the small intestine, one metre was measured up from the previous cutting point, to ensure the jejunum was isolated and a length of tissue was cut.

Samples were cut in half, with the external membrane labelled with a permanent marker. The internal membrane was gently washed with deionised water to remove any undigested waste, ensuring the mucosal layer of the tissue was not disrupted. Samples were stored at 4 °C or frozen at -20 °C until use.

2.2.5.2 Rotating cylinder method for mucoadhesive testing

The rotating cylinder method was conducted according to Bernkop-Schnurch (2005). Frozen tissue samples were allowed to thaw overnight at 4 °C. Small sections were cut using a scalpel into approximately 2 cm^2 pieces and glued on to the stirrers of a

dissolution bath with cyanoacrylate glue. The samples were submersed into the dissolution bath containing 100 mM phosphate buffer, pH 6.7, at 37 °C for 10 min in order to rehydrate and to equilibrate the tissue prior to testing.

30 mg samples were compressed into ~1 cm tablets, as previously described in section 2.2.3. The dry tablets were held against the rehydrated tissue samples for 2 min, allowing the tablets to swell and to ensure they had adhered to the tissue before both the tissue and tablet were submersed into the 37 °C phosphate buffer. The system was agitated at 50 rpm and the time taken for the tablets to dislodge or disintegrate was determined.

2.2.6 Drug release studies using chlorpheniramine maleate

2.2.6.1 Drug incorporation

24 mg of PAA was dissolved in 25 mL deionised water at 37 °C. Thiolated samples were slow to dissolve in comparison to unmodified and control samples. To the dissolved polymer, 6 mg chlorpheniramine maleate (CPM) was added and the solution was gently stirred for 1 h. The PAA-CPM solution was then frozen and freeze dried. 30 mg PAA-CPM was compressed into a tablet at 150 bar of pressure.

2.2.6.2 Drug release studies

Samples were analysed using an Agilent HPLC 1200 series. Analysis was conducted according to Chittrakarn *et al.* (2012). The column used was a YMC-Pack CN cyano column, 250 mm x 4.6 mm in length and 5 µm particle size. Flow rate was set at 1.5 mL/min, column temperature was 35 °C and injection volume was 5 µL. As chlorpheniramine maleate was used, 2 peaks were observed; the first peak was maleate and the second was the chlorpheniramine peak, this was confirmed by running a standard of maleic acid and a standard of dex-chlorpheniramine. A gradient method was set up using 10 mM potassium phosphate buffer, adjusted to pH 3 with ortho-phosphoric acid, and acetonitrile (ACN) with the following gradient elution settings shown in Table 2.1:

Table 2.1 Gradient method for HPLC analysis of CPM

Time (min)	% A	% B (ACN)
0	96	4
3.8	96	4
7	60	40
8.7	60	40
8.8	96	4

Total run time of the gradient method was 15 min.

A set of standards of CPM were made up in deionised water. A second set of standards were made in 100 mM phosphate buffer, pH 6.7 to ensure stability of CPM at that pH.

The PAA-CPM tablet was placed into 20 mL of 100 mM phosphate buffer, pH 6.7, at 37 °C and was agitated gently at 50 rpm. At specific time points, 1 mL of the solution was removed and replaced with 1 mL of phosphate buffer, maintaining sink conditions. The sample was filtered with a 45 µm nylon filter prior to HPLC analysis.

2.2.7 Polymer characterisation

2.2.7.1 Rheological properties

Rheological methods were conducted according to Marschütz *et al.* (2002). Polymer samples were made in 50 mM phosphate buffer, pH 6.8, at a concentration of 3% (m/v). A solution of Type II porcine mucin was created by dissolving 4 g of mucin powder in 25 mL of deionised water. Once dissolved, the pH was adjusted to pH 6.8 and the solution was diluted to 50 mL with 200 mM phosphate buffer, pH 6.8, giving an 8% (m/v) mucin solution. The mucin solution was mixed in equal parts with the 3% (m/v) polymer solutions. Dynamic oscillatory tests were performed on a TA instruments AR 1000 rheometer, using a 40 mm cone and plate geometry. Analysis was conducted at 37 °C on polymer samples alone, at a concentration of 1.5% (m/v), and with the addition of mucin solution within the linear viscoelastic region. Amplitude sweeps were conducted over a stress range of 0.01 – 60 Pa at constant

frequency of 1 Hz in which the storage modulus (G') and the loss modulus (G") were determined. Frequency sweeps were also conducted at 37 °C on polymer samples alone and with the addition of mucin, at a constant stress of 0.01 Pa and a frequency range of 0.1 – 20 Hz.

2.2.7.2 Scanning electron microscopy (SEM)

SEM imaging was conducted using a Hitashti S-2460N scanning electron microscope. Samples were mounted onto a 10 mm diameter disc using carbon tape and sputter coated with gold under vacuum in an argon atmosphere. The surface morphology of the PAA samples and PAA-CPM conjugates samples were analysed at various magnifications with a voltage of 25 kV.

2.2.7.3 Thermogravimetric analysis (TGA)

Thermogravimetric analysis was conducted using a heating ramp of 10 °C/min to 500 °C on a TA instruments TGA Q50 series with nitrogen at a flow rate of 50 mL/min as the carrier gas to obtain the decomposition temperature of the polymer; the differential weight loss in relation to temperature was also obtained.

2.2.7.4 Differential scanning calorimetry (DSC)

DSC analysis was conducted using TA instruments DSC Q2000 series using nitrogen as a carrier gas, again at a flow rate of 50 mL/min. Accurately weighed samples were placed into Tzero hermetically sealed pans and were pin-holed to allow the release of water from the sample. Samples were equilibrated at 0 °C, heated to 180 °C with a ramp rate of 10 °C/min and cooled again to 0 °C, creating one cycle.

2.2.7.5 Modulated DSC (MDSC)

MDSC was also conducted on unmodified PAA and thiolated PAA samples. The MDSC method is outlined in Table 2.2. Prior to sample analysis, calibration was

conducted using a sapphire standard. The hermetically sealed pans were again pin-holed to allow for the release of water vapour.

Table 2.2 MDSC settings

Modulated amplitude	±0.75 °C
Modulation	60 s
Ramp rate	1 °C/min
Start temperature	-10 °C
Final temperature	200 °C

2.3 Results and discussion

2.3.1 Thiolation of PAA

Two things of importance with regards to the thiolation reaction of PAA were observed in this study: firstly, it was observed that the PAA thiolation reaction was strongly dependent upon the pH at which the cysteine was added. Secondly, depending on the pH of the PAA-EDC solution upon the addition of cysteine, products of differing thiol content could be formed. In the majority of the literature for the thiolation of PAA, the addition of cysteine to the PAA-EDC solution was based on time, e.g. the carboxylates on the polymer were activated with EDC for a certain length of time prior to the addition of cysteine. Length of time differed between papers and activation times varied from 20 min (Marschütz and Bernkop-Schnürch, 2002) to 45 min (Bernkop-Schnürch *et al.*, 1999). Marschütz and Bernkop-Schnürch (2002) incubated the PAA-EDC solution for 20 min prior to cysteine addition. To ensure higher thiol content was obtained between two separate PAA samples, the concentration of EDC added was increased. Addition of 150 mM EDC resulted in a sample of 90.5 µmol/g thiol content and addition of 200 mM EDC resulted in a sample of thiol content 511.6 µmol/g. The mass of cysteine added to both sample batches was 1 g and it was concluded that the amount of covalently attached cysteine was dependent upon the concentration of EDC added (Marschütz and Bernkop-Schnürch, 2002). The concentration of EDC used in this thiolation

reaction has a marked influence on the resulting thiol content, as was observed by both Marschütz and Bernkop-Schürnch (2002) and Palmberger *et al.* (2007), due to the amount of EDC available for coupling within the reaction. However, reproducible degrees of thiolation using a higher concentration of EDC appear not to be have been attained. Using a concentration of 200 mM EDC resulted in a thiol content of 656 ± 79 µmol/g as conducted by Iqbal *et al.* (2011). Using the same method, Wang *et al.* (2012) achieved a thiol content of 747 µmol/g while Grabovac *et al.* (2005) achieved a thiol content of 498.8 ± 47 µmol/g and Marschütz and Bernkop-Schürnch (2002) obtained a thiol content of 511.6 ± 52 µmol/g. Similarly, using a concentration of 150 mM, Palmberger *et al.* (2007) achieved a thiol content of 549.1 ± 4.2 µmol/g, whereas Marschütz and Bernkop-Schürnch (2002) attained 90.5 µmol/g thiol content using the same concentration. This demonstrates the variability in the resulting degree of thiolation using the same method and concentration of EDC in the PAA thiolation reaction and the lack of reproducibility achieved.

In this study, four separate reactions were conducted and the same amount of EDC (200 mM) was added to each reaction. Once the EDC was added to the PAA solution, the pH rose to approximately pH 7 – 7.5. Over a 40 min period, the pH of the PAA-EDC solution fell, resulting in a typical pH profile shown in Figure 2.3.

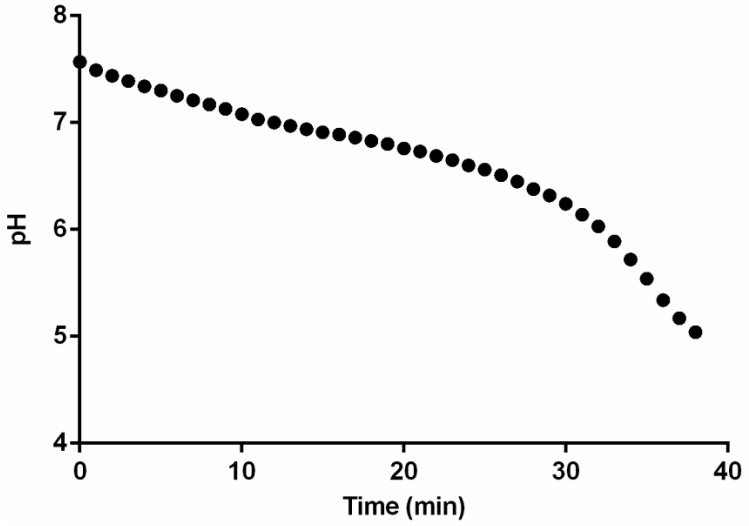


Figure 2.3 pH profile of PAA-EDC reaction

2.3.1.1 Thiol content of thiolated PAA

As the pH of the PAA-EDC solution fell, and at specific pH values, namely pH 5, 5.5, 6 and 6.5, L-cysteine was added to the reaction solution; the pH of the reactant solution was not altered, the change in pH was due to EDC hydrolysis, which will be discussed in more detail in section 2.3.1.3. By adding cysteine at specified pH values, the level of thiolation in the products could be reproducibly controlled and, at higher pH values, the addition of cysteine resulted in a product with increased thiol content. As a result, a set of thiolated PAA polymers with increasing thiol content was produced; thiol levels measured: 400.6 ± 61.3 µmol/g, 594.0 ± 6.1 µmol/g, 781.6 ± 37.2 µmol//g and 961.0 ± 83.8 µmol/g respectively, as illustrated in Figure 2.4. As the control sample did not contain EDC, the carboxylate groups on the polymer backbone could not be converted to thiols, therefore, it resulted in a thiol content similar to that of unmodified PAA, measuring 12.8 ± 2.3 µmol/g and 5.7 ± 1.6 µmol/g, respectively. This method of cysteine addition with regards to pH is in contrast to those described by others, where cysteine addition was based on time (Marschütz and Bernkop-Schnürch, 2002; Bernkop-Schnürch *et al.*, 1999; Bernkop-Schnürch and Steininger, 2000; Iqbal *et al.*, 2012).

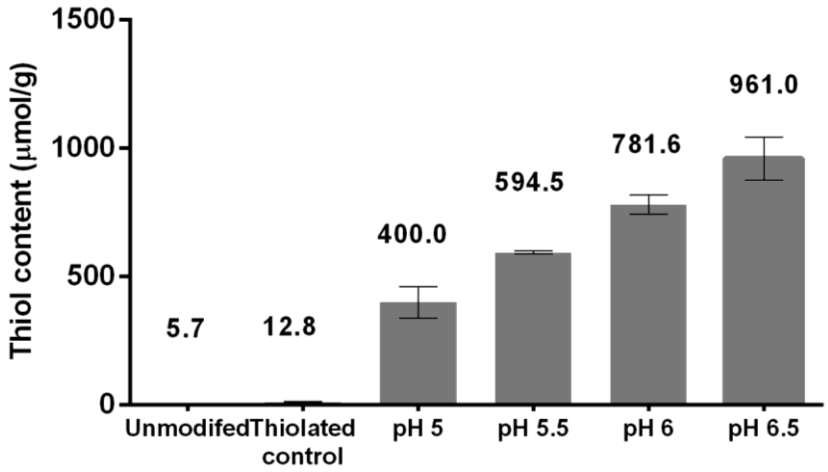


Figure 2.4 Thiol content of 450 kDa PAA: unmodified, thiolated control and samples thiolated at different pH levels (n=3)

At higher pH values, the resulting thiol content was also higher. The reactivity of EDC may limit this trend. EDC is most reactive in a pH range of 4 - 6 but can be

reactive up to pH 7.5; however, at this higher pH it could result in lower yields and will react slower (Hermanson, 2008). As this was the case, addition of cysteine to the PAA solution at a pH of 7 or higher will not necessarily result in a product with higher thiol content. There is also an optimum level of thiolation; if the thiol content is too high, mucoadhesion could be affected due to increased crosslinking of the polymer, limiting the concentration of free thiol groups available for disulphide bond formation with the mucin (Marschütz and Bernkop-Schnürch, 2002). Equally, if the thiol content is too low, mucoadhesion may occur, but cohesion may be compromised as the thiol groups will form intermolecular disulphide bonds with the mucins and not intramolecular bonds.

2.3.1.2 Disulphide bond formation

All thiolated samples were reduced with $NaBH_4$ and tested with Ellman's reagent to analyse the formation of disulphide bonds within the polymer matrix, as shown in Table 2.3. Interestingly, disulphide bond formation was highest in samples thiolated at lower pH; pH 5 and pH 5.5 had the highest levels of disulphide bond formation with 166.6 and 126.5 µmol/g disulphide bond formation, respectively. Both the pH 5 and pH 5.5 samples had lower free thiol content in comparison to the pH 6 and pH 6.5 samples. Bacalocostantis *et al.* (2012) thiolated polyallylamine with Traut's reagent with the aim to thiolate 5%, 13% and 20% of the total polymer backbone. Upon investigation into the disulphide bond formation content of the three samples, it was observed that disulphide bond formation was higher in the 5% thiolated sample, i.e. the sample with lowest thiol content. It was surmised that steric hindrance between polymer chains inhibited the formation of disulphide bonds in the samples with higher thiol content (13% and 20% samples) which in turn resulted in lower disulphide bond formation.

Table 2.3 Thiol content, disulphide bond content and total thiol content of the thiolated PAA samples

Sample	Thiol content (µmol/g)	Disulphide bond (µmol/g)	Total thiol content (µmol/g)
pH 5	400.6 ± 61.3	166.6 ± 15.0	567.2
pH 5.5	594.0 ± 6.1	126.5 ± 32.0	720.5
pH 6	781.6 ± 37.2	42.5 ± 23.0	824.1
pH 6.5	961.0 ± 83.8	74.1 ± 72.6	1035.1

2.3.1.3 Thiolation reaction of PAA

The reaction mechanism of PAA and L-cysteine in the presence of EDC is shown in Figure 2.5. The carboxylic acid of PAA (a) is deprotonated by EDC (b) which acts as a base in the solution. The EDC and carboxylate bond together to form the highly reactive, but unstable, o-acylisourea intermediate (c). This intermediate is in turn attacked by the amine group on L-cysteine (d), thus forming the isourea by-product (e) and the amide bond of the thiolated PAA product (f).

pH will have an effect on multiple factors in the PAA-EDC reaction, specifically on both PAA itself and on EDC. The pKa of polyacrylic acid is between 4.5 - 5 (Jabbari and Nozari, 2000). As the pH of the reaction increases, the PAA backbone becomes less protonated and, therefore, more reactive. In this study, upon the addition of EDC, the pH of the reaction vessel rose to ~ 7.5 and gradually fell, as illustrated by the pH profile in Figure 2.3. This pH change in the PAA-EDC solution was due to the hydrolysis of EDC, the mechanism of which is shown in Figure 2.6. When EDC was added to the PAA solution, it acted as a base, accepting protons, and increased the pH of the solution. The pH then began to fall as water molecules attacked the carbocation of the unstable O-acylisourea intermediate, forming the isourea by-product in the process. Similarly, if L-cysteine is not present in solution for further reaction with this intermediate, the intermediate will form the unreactive isourea by-product, thus releasing the unmodified PAA polymer in the process. This will then result in a decreased concentration of polymer within the solution available for modification by thiolation, which will, in turn, lower the resulting thiol content of the product. Additionally, as the more acidic polymer is released back into the solution, this will also contribute to the observed drop in pH of the PAA-EDC

solution. At pH 6.5 and 6, the carboxyl groups along the PAA backbone were deprotonated. They reacted with the EDC crosslinker and were highly reactive towards cysteine once it was added to the vessel, thus resulting in a thiolated product with high thiol content. As the pH dropped further, the pH became closer to the pKa of PAA and the carboxyl groups became more protonated; they were, therefore, activated to a lesser extent with EDC than at a higher pH and were, in turn, less reactive towards cysteine upon its addition to the reaction vessel. This can explain the increase in thiol content of the thiolation reaction when conducted at pH 6 in comparison to pH 5.

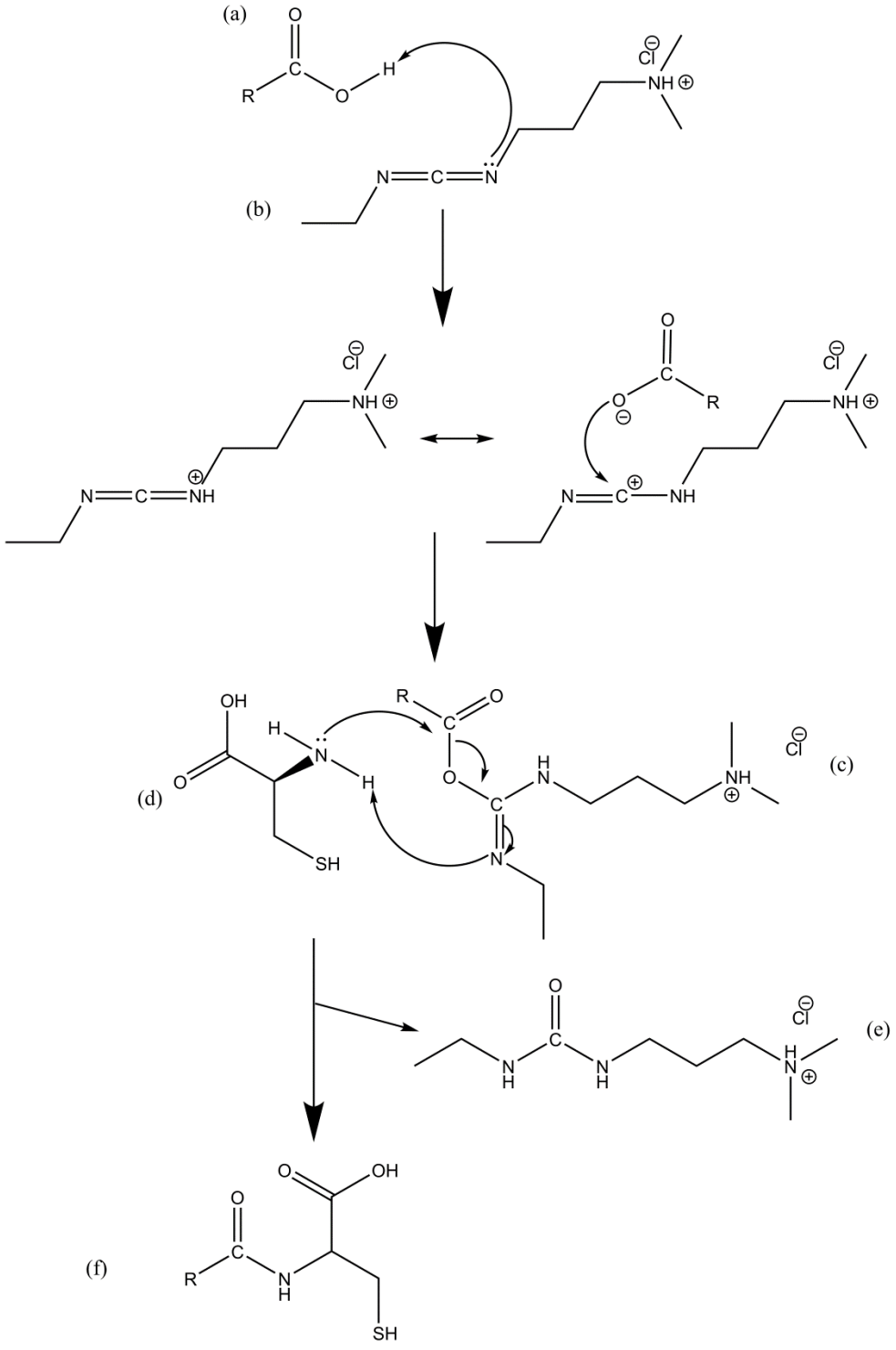


Figure 2.5 Reaction mechanism of PAA-EDC with L-cysteine

The reactivity of EDC at a given pH is also important. Nakajima and Ikada (1995) investigated the formation of amide bonds by EDC with both polyacrylic acid and maleic acid hydrogels. Reactions were conducted in a range of pH solutions. EDC was unstable at low pH and lost activity. However, the amide bond was formed in

one of two ways: in a narrow range of pH between 3.5 – 4.5, a stable intermediate of carboxylic anhydride was formed prior to the formation of the amide bond with the addition of an amine compound, at pH 4 - 6 an amide bond was formed without the formation of an intermediate. Madison and Carnali (2013) discussed the optimisation of pH in the EDC reaction. In this paper, the EDC reaction was classified as a two-step reaction, the first step being the activation of the carboxylate and formation of the O-acylisourea intermediate and the second step being the formation of the amide bond by attack of an amine group. It was noted that the activation of carboxylates by EDC was enabled by the ionisation of the carboxylate and the protonation of the nitrogen on the carbodiimide which reduced the electron density on the central carbon, allowing for the nucleophilic attack of the carboxylate. A specific optimised pH range for this step was 4.5 – 4.75. However, also noted was the importance of the protonation state of the attacking amine group, which needed to be unprotonated. Therefore, because of this, and the instability of the O-acylisourea intermediate formed in the EDC reaction, it was important to conduct the reaction at a pH of 5.5 – 8. Therefore, to conduct this EDC reaction in one-step, it was the pH for the nucleophilic attack of the amine that was important.

In this study, L-cysteine was added to the PAA-EDC reaction at different pH values, and thiolated products with increasing thiol content were formed, depending on the pH at which the cysteine was added. However, although the reaction pH will influence the protonation state of the PAA backbone which potentially changes the reactivity of the polymer and also influences the protonation state of the added amine group, the concentration of EDC will also have a large effect on the reaction. As mentioned above, EDC itself was hydrolysed, releasing the unmodified acidic polymer, which resulted in the drop in pH of the reaction vessel; the O-acylisourea intermediate formed is unstable and in the absence of an amine group to react with, the carboxylate can be reformed and the isourea by-product can also form in excess. Therefore, as the pH of the reaction solution fell and the EDC hydrolysed, in theory, there was also a lower concentration of reactive EDC in the vessel, e.g. the unstable O-acylisourea intermediate, which, once the L-cysteine was added to the solution, may also explain the resulting range of thiol content observed. This method of thiolation of PAA using EDC and L-cysteine is well documented and the effect altering the concentration of EDC has on the reaction has been discussed (Marschütz

and Bernkop-Schnürch, 2002; Palmberger *et al.*, 2007). Palmberger *et al.* investigated the influence the concentration of EDC had on the thiolation reaction of PAA. Using EDC concentrations ranging from 50 mM to 200 mM, the resulting thiol content increased with increasing EDC concentration. However, interestingly, within this paper, two PAA samples were thiolated with 50 mM EDC and 1 g of cysteine; one sample resulted in a thiol content of 53 ± 1.8 µmol/g while the second resulted in a thiol content of 113.4 ± 1.6 µmol/g. This difference in thiol content was not discussed in the paper and it again highlights the variability of achievable thiol content within the PAA-EDC reaction, as was discussed above.

The alternative method of thiolation described in this study, in which PAA is thiolated based on the pH of the reaction, produced thiolated samples with controlled and varied levels of thiolation which was, more importantly, extremely reproducible. As the thiol content of the samples varied, the cohesive and mucoadhesive properties may also vary due to the difference in thiol content; both swelling ability and mucoadhesive testing will be discussed in sections 2.3.2 and 2.3.3. Similarly, if swelling ability between the thiolated samples differs, a specific system with varying rates of drug release could be achieved. This will be discussed in section 2.3.4.

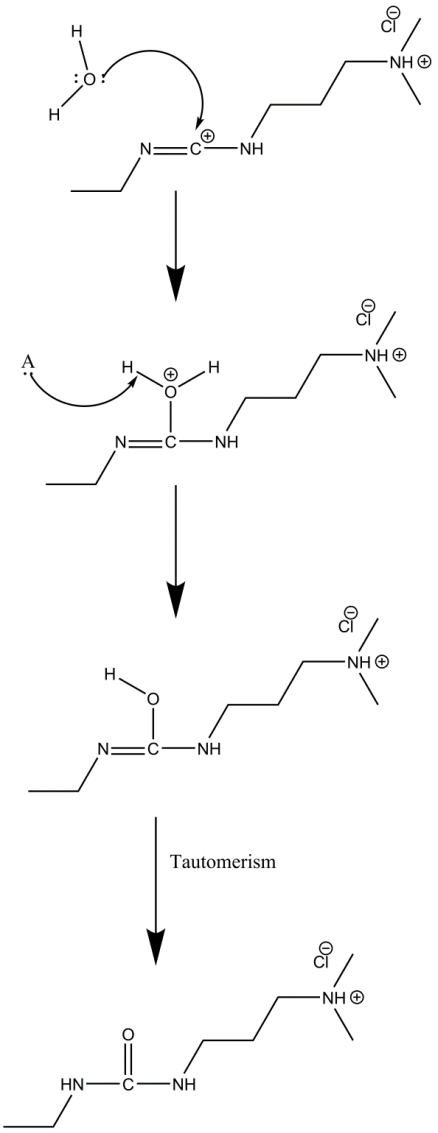


Figure 2.6 Hydrolysis reaction of EDC

2.3.2 Swelling studies

Swelling studies were conducted in a pH 6.7 phosphate buffer at 37 °C. Thiol groups are analogous to alcohol compounds. However, the S-H bond is weaker than the O-H bond and the thiolate ion $R\text{-}S^-$ is easily formed with treatment of a base. The pKa is lower in the thiol compound than their corresponding alcohol, e.g. pKa of thiophenol is 7.8 whereas pKa of phenol is 10 (Wade, 2003). Thiol groups are also easily oxidised to the disulphide bond, which is in equilibrium and again, are easily reduced back to the thiol. As this equilibrium is easily shifted, the presence of excess cysteine or unbound thiol groups will disrupt it, which will be discussed in more detail in chapter 4, section, 4.3.2. At pH 6.7, the thiol groups within the polymer

were likely to crosslink as the thiol group is more reactive at higher pH (Shaked *et al.*, 1980), thus producing disulphide bonds. As was discussed in the introduction to this chapter, disulphide bond formation in thiolated polycarbophil increased in a buffered solution of pH 6 or pH 7 in comparison to a solution of pH 5 (Bernkop-Schnürch *et al.*, 2001). Disulphide bonds will help keep the polymer cohesive, allowing the polymer to swell as opposed to disintegrate or dissolve in the buffer. The ability of a mucoadhesive tablet to swell is advantageous for a number of reasons, including: increased surface area which increases the ability of the polymer to bind to the mucosal surface thus producing a potentially stronger bond (Andrews *et al.*, 2009), and also, allowing for controlled drug release which occurs as the polymer slowly swells, releasing the drug as it does. The higher the degree of crosslinking results in a slower and more controlled rate of drug release (Andrews *et al.*, 2009). Crosslinking is required for the polymer matrix to retain its cohesion when hydrated. However, the process must be controlled, as high swelling capacity may lead to the over-swelling of the polymer, creating an over-hydrated polymer which loses its shape thus leading to decreased mucoadhesive ability. Over-hydration of the polymer will also lead to the fast release of drug, as the polymer will swell to its full capacity quickly and, therefore, release the incorporated drug (Andrews *et al.*, 2009).

Initial swelling studies were conducted on thiolated PAA samples of differing MW: 250 kDa and 450 kDa. Both thiolated PAA samples had similar thiol contents, 437.5 ± 3.1 µmol/g and 472.4 ± 28.3 µmol/g, respectively. Swelling study results of the two thiolated polymers are shown in Figure 2.7. The difference between the two MW compounds was noteworthy, with PAA 450 kDa swelling to approximately 1500% of its starting mass over a period of 80 min, while PAA 250 kDa increased in mass by 40% and began to disintegrate within 4 min. This result is in contrast to the results of the literature. Leitner *et al.* (2003) examined the cohesive and mucoadhesive properties of thiolated PAA conjugates of varying MW. Five PAA samples ranged from 2 – 450 kDa, all of which had comparable thiol contents of approximately 400 µmol/g; therefore, it was solely the influence of MW which was investigated. A thiolated polycarbophil sample of MW 750 – 3000 kDa was also evaluated. In both swelling and mucoadhesive tests, the lowest MW samples of the five samples tested, 2 kDa and 45 kDa dissolved quickly and were disregarded. In

relation to the cohesive properties (the mucoadhesive properties will be discussed in more detail in section 2.3.3), the thiolated PAA 250 kDa had comparable swelling abilities to the thiolated PAA 450 kDa. Leitner *et al*. stated the thiol contents of the samples as 404.1 ± 65.5 µmol/g and the results are, therefore, directly comparable to thiolated 250 kDa and 450 kDa PAA samples used in this study. However, the swelling abilities of PAA 250 kDa in this study compared to those conducted by Leitner *et al.* were vastly different. In this study, the shorter chain length of PAA 250 kDa may have affected its ability to crosslink, therefore reducing its cohesive properties.

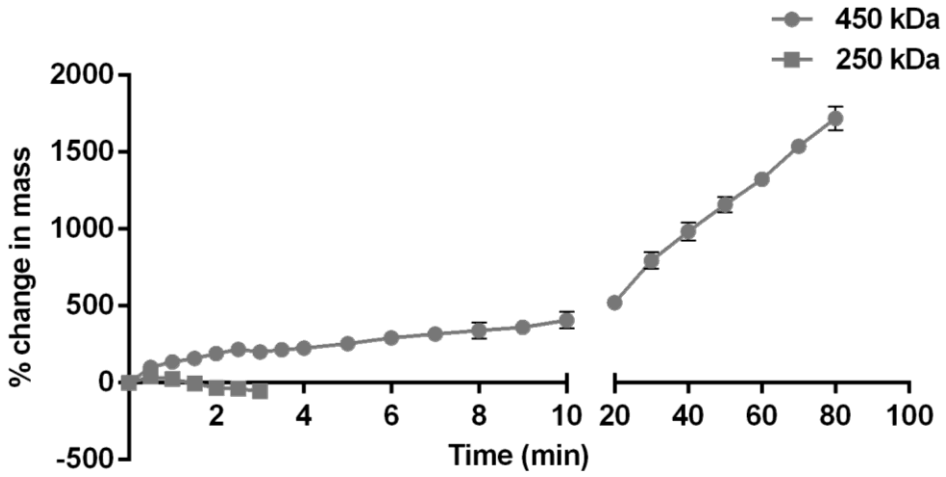


Figure 2.7 Swelling tests on thiolated PAA of two molecular weights, 250 kDa and 450 kDa (n=2). Both samples had comparable thiol content measuring 437.5 ± 3.1 µmol/g and 472.4 ± 28.2 µmol/g, respectively.

Swelling abilities of the thiolated polymer samples, which had thiol contents ranging from 400 – 1000 µmol/g, were compared to unmodified PAA samples (5.7 µmol/g thiol content) and control samples (12.8 µmol/g thiol content). Control samples and unmodified PAA samples had similar swelling patterns, as shown in Figure 2.8. Visually, both samples did not appear to swell and both had a similar adhesive texture once submerged in the buffer. The unmodified and control samples did show natural cohesion, swelling to approximately 100% of their initial mass, showing the cohesive nature of unmodified PAA, but both samples dissolved slowly over time.

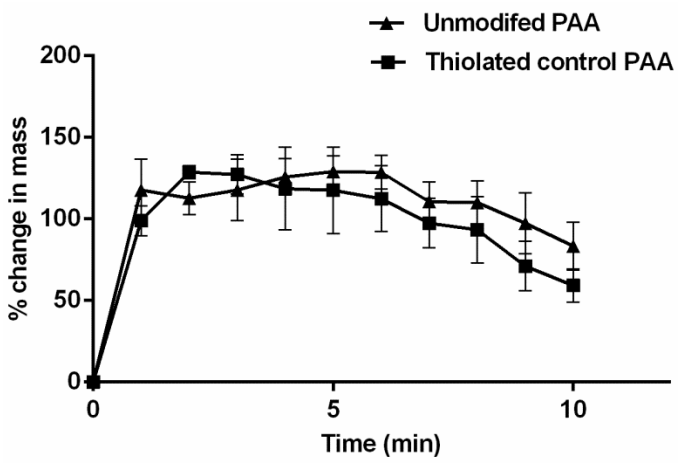


Figure 2.8 Swelling studies of unmodified PAA vs thiolated control PAA (n=3)

Swelling studies were conducted on thiolated PAA samples of differing thiol content, as shown in Figure 2.9. Unlike the unmodified and control samples, the thiolated PAA samples became a clear gel-like structure during the swelling studies and had a similar appearance to a teardrop as it took on more liquid into the matrix. As the pH of the thiolation reaction increased, the resulting thiol content also increased. In the swelling studies, with increasing thiol content, the swelling ability also increased and higher levels of swelling occurred in the pH 6.5 sample in a faster time, particularly in the initial minute. This seems to contradict theory, as a higher thiol content should have higher degrees of crosslinking in a faster time due to the increased number of thiol groups (Marschütz and Bernkop-Schnürch, 2002). If crosslinking occurs at a faster rate in polymers with a higher thiol content, then there should be a decrease in the swelling ability in comparison to a polymer with lower thiol content. Figure 2.9 shows that the swelling ability of the pH 6.5 sample within the first minute of the sample submersion in the buffer was greater than either of the other samples which had lower thiol content. The disulphide bond formation of the pH 6.5 sample was lower than the other thiolated PAA samples, as shown in Table 2.3, suggesting that crosslinking had occurred within the polymer matrix of the pH 5, pH 5.5 and pH 6 samples prior to the swelling tests. The cohesive properties of the pH 5 and pH 5.5 samples may already be greater than the pH 6.5 sample. If the increased swelling rate of the pH 6.5 sample was too great, crosslinking of the thiol groups may have been impeded – the increased rate of swelling may have created a barrier for the thiol groups to form disulphide bonds as the distance between the thiol

groups was then too great. This may not have influenced the pH 5 or pH 5.5 samples, as disulphide bond content was higher, slowing the swelling rates of those samples.

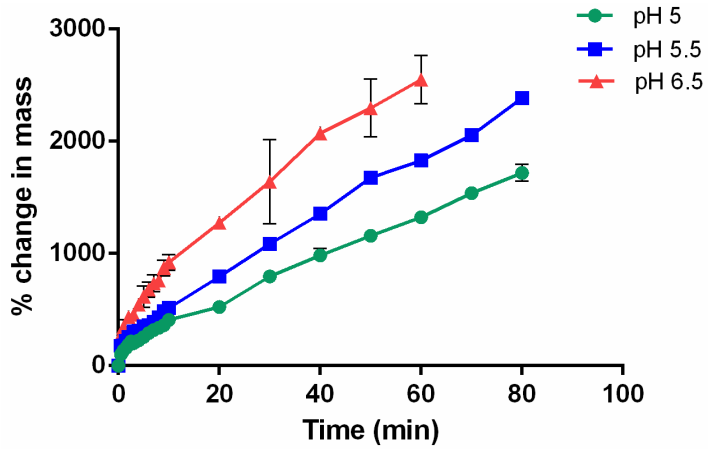


Figure 2.9 Swelling tests on thiolated 450kDa PAA of varying thiol contents (n=2 or 3)

The influence pH has on both cohesive and mucoadhesive levels in PAA has been conducted (Guggi *et al.*, 2004), in which thiolated PAA samples of unstated thiol content were dissolved and dialysed. The pH was then adjusted to between pH 3 – 8 and the samples were then freeze dried. Thiol content measurements showed that the samples adjusted to pH 3 had a higher thiol content (332 µmol/g) than samples adjusted to pH 8 (162 µmol/g). However, the adjustment of pH was conducted on pre-thiolated samples and it did not address the influence pH had on the initial thiolation reaction; the initial thiol contents of the thiolated PAA samples prior to pH adjustment were not stated. Improved swelling ability and mucoadhesive properties were observed in samples with higher thiol content, i.e. pH 3 and 4 in comparison to samples adjusted to between pH 5 – 8. Therefore, similar to this study, pH 6 and pH 7 samples swelled more and at a faster rate than samples at lower pHs. Guggi *et al.* suggested the higher degree of swelling observed by the samples of higher pH was due to the improved hydratability of the sample because of higher ionic substructures within the more basic polymer. However, the thiol content of the samples investigated by Guggi *et al.* were markedly lower than those in this study, measuring 241 µmol/g in the sample adjusted to pH 6 in comparison to the thiol content in this study of 781.6 µmol//g and 961.0 µmol/g for the pH 6 and 6.5 samples.

Swelling behaviour of thiolated PAA of differing thiol contents, one of 90.5 µmol/g and the second of 511.6 µmol/g, were examined in comparison to a control sample (Marschütz and Bernkop-Schnürch, 2002). Again, the higher thiol content modification was controlled by the addition of more EDC as opposed to controlled by pH, as is the case in this study. The swelling behaviour of the control sample matched the results observed in this study, with the tablet beginning to disintegrate within 10 min. The swelling abilities of the two thiolated samples differed greatly, however, as does the thiol content. The tablet with a thiol content of 90.5 µmol/g showed a 3-fold swelling ability but began to disintegrate within 20 min. This is in contrast to the 511 µmol/g sample which showed 28-fold increase in mass due to swelling. This result observed by Marschutz *et al*. is in agreement with results observed in this study.

2.3.3 Mucoadhesive testing: rotating cylinder method

Variability between samples is often observed in mucoadhesive testing in the literature (Grabovac *et al.,* 2005; V. M. Leitner *et al.,* 2003). Focusing on the gastrointestinal tissue samples, both morphology and mucosal density and strength can differ greatly between portions of the gastrointestinal (GI) tract of the same animal, let alone between different animals or different species (Sigurdsson *et al.*, 2013; Varum *et al.*, 2010). This variability could have a significant impact on the mucoadhesive nature of tablets, as areas of greater mucosal density may allow for longer and more efficient adhesion. Varum *et al.* (2010) investigated how the mucoadhesive properties of Carbopol (PAA) were affected by the thickness and the site of the mucosal layer using porcine tissue. The mucosal thickness was measured throughout the length of the GI tract using histological methods. Mucosal thickness variations were observed within similar regions of the tract. The stomach had the thickest layer of mucus (67.9 ± 54.7 µm) followed by the rectum (40.8 ± 12.5 µm), with slight variation between the upper and the lower small intestine (25.9 ± 11.8 µm in the duodenum to 31.0 ± 15.7 µm in the ileum). Mucoadhesive properties of PAA were then analysed on different regions along the GI tract including the stomach, jejunum, ileum, and ascending colon. Tests were performed by measuring the tensile strength of the polymer-mucus bond by means of a texture analyser. Mucoadhesive bonding strength was observed to be higher in areas of thicker mucosal surface, i.e.

the stomach and the ascending colon. It was thought this was due to the greater ability for polymer chain interdiffusion into the mucosal layer in areas which were thicker. This was also acknowledged by Liu *et al*. (2005) where the mucoadhesive properties of modified pectin were tested on tissue samples taken from the GI tract, starting from the mouth to the large intestine. Mucoadhesion was highest in the large intestine due to the increase in goblet cell production and the higher levels of mucus produced. Because of this, mucoadhesive testing in this study was performed using a section of tissue large enough for all samples of similar thiol content to adhere to, therefore, reducing the likelihood of variability between similar samples due to mucosal density and structure.

Initial mucoadhesive testing was conducted on PAA of different MWs: 250 kDa and 450kDa; both samples had comparable thiol contents at 437.5 ± 3.1 µmol/g and 472.4 ± 28.2 µmol/g for the 250 kDa and 450 kDa samples, respectively. In swelling studies, PAA 450 kDa swelled to approximately 1500% while PAA 250 kDa disintegrated quickly (Figure 2.7). This was mirrored in the mucoadhesive testing; PAA 250 kDa adhered to the porcine tissue for an average of 1.13 ± 0.82 h whereas PAA 450 kDa adhered for greater than 24 h as shown in Figure 2.10. A large standard deviation in the 250 kDa sample was observed, which, as stated previously, may be due to variations in the mucosal tissues used as well as variations between polymer tablets.

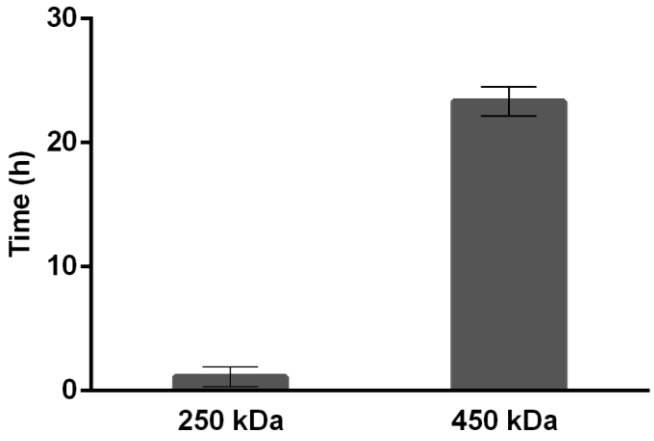


Figure 2.10 Mucoadhesive testing on thiolated PAA samples of comparable thiol content (~ 450 µmol/g) but differing MW, 250 kDa and 450 kDa (n=3)

This result reflects the work conducted by Leitner *et al.* (2003), which was discussed in terms of cohesion in section 2.3.2. As was mentioned above, Leitner *et al.* observed that PAA 250 kDa, and a polycarbophil sample of MW 750 – 3000 kDa, had comparable levels of swelling to PAA 450 kDa, which was in contrast to results observed in this study. Leitner *et al.* did observe poorer mucoadhesive properties in PAA 250 kDa in comparison to PAA 450 kDa (as was also observed with the high MW polycarbophil) but to a smaller extent than observed in this study; adhesion times of up to 14 h for PAA 250 kDa sample and 22 h for PAA 450 kDa were observed, which is in contrast to mucoadhesive levels measured in this study for PAA 250 kDa. In this study, both the cohesive properties and mucoadhesive properties of PAA 250 kDa were considerably poorer than PAA 450 kDa, as is shown in both Figure 2.7 and Figure 2.10. The shorter chain length of PAA 250 kDa may have affected the interpenetration into the mucosal layer of the tissue, therefore reducing its mucoadhesive properties. MW is of great importance to mucoadhesive and cohesive properties and the results shown in this study again indicates there is an optimum MW to be used in mucoadhesion; this is due to the chain flexibility, chain length and its overall ability to penetrate into the mucosal layer. In the case of PAA, Leitner *et al* (2003) concluded that PAA of 450 kDa is the optimal MW for mucoadhesion and cohesion and that again is highlighted here when comparing the swelling studies (Figure 2.7) and mucoadhesive studies (Figure 2.10) of PAA 250 kDa and 450 kDa.

In Figure 2.11, the mucoadhesive testing of unmodified PAA, thiolated control PAA and thiolated PAA with varying thiol content can be observed. Both the unmodified and control samples, like in the swelling tests, had similar mucoadhesive properties - this again highlights the mucoadhesive abilities of unmodified PAA, as it adhered for greater than 1 h. The thiol content, again like the swelling properties, affected the mucoadhesive properties, improving the mucoadhesive nature of all the thiolated polymers in comparison to the unmodified and control samples. The pH 5 PAA sample, which had the lowest levels of thiolation, 400 µmol/g, displayed the highest levels of mucoadhesion and the error bars of this sample were small. The other three thiolated samples, pH 5.5, pH 6 and pH 6.5, all of which had higher thiol content than the pH 5 sample, displayed lower, but comparable, levels of mucoadhesion. The error on all these three samples was also markedly higher than the pH 5 sample and

this may be due to the tissue used and the mucosal layer of that tissue. As mentioned above, the density of the mucosal layer will vary between animals and this may be a reason why such variability was observed in the thiolated samples; secondly the tissue used was frozen and defrosted prior to use. This may have changed the composition of the mucosal layer. All pH 5.5, pH 6 and pH 6.5 samples dislodged from the tissue during the mucoadhesive testing as a cohesive gel-like, clear material, suggesting that the density of the mucosal layer may have indeed been a major factor in the mucoadhesive properties of the samples.

The pH 6.5 sample which had the greatest thiol content and also lowest disulphide bond content, although in theory should have greater mucoadhesive properties and adhere for longer, in fact appeared to adhere for a shorter time than the pH 5 sample. As was the case in the swelling studies, it is possible that an increase in swelling once the polymer had adhered to the mucosal surface, inhibited the binding and interpenetration of the material into the mucosal tissue, as over-swelling of the polymeric matrix can lead to a decrease in mucoadhesion (Mortazavi and Smart, 1993). Similar to the swelling studies, Guggi *et al.* (2004) also observed inferior mucoadhesive properties with thiolated samples of higher pH. Guggi *et al*. surmised that the inferior mucoadhesive properties were due to the quicker uptake of liquid into the polymer matrix, and therefore, a stronger adhesive bond was required to allow the polymer to bond to the same degree as the samples which had a lower rate of swelling. Similarly, Guggi *et al.* discussed how the premature oxidation of the thiol groups within the polymer would be prevented by the lower pH, i.e. below pH 6, within the structural matrix of the polymer, therefore, potentially allowing for a greater number of free thiol groups to bond to the mucosal surface. This may be a reason as to why the mucoadhesive properties of the pH 6.5 sample are lower than the pH 5 sample, as shown in Figure 2.11. However, there was great variability in the adhesion times of the pH 6.5 sample (as well as in the pH 5.5 and pH 6 samples) which was not observed in the pH 5 sample. Although it is possible premature oxidation of the pH 6.5 sample lessened the mucoadhesive properties, it may be that the mucoadhesion of the pH 6.5 sample may not be as dissimilar as appears due to the variability observed in this study.

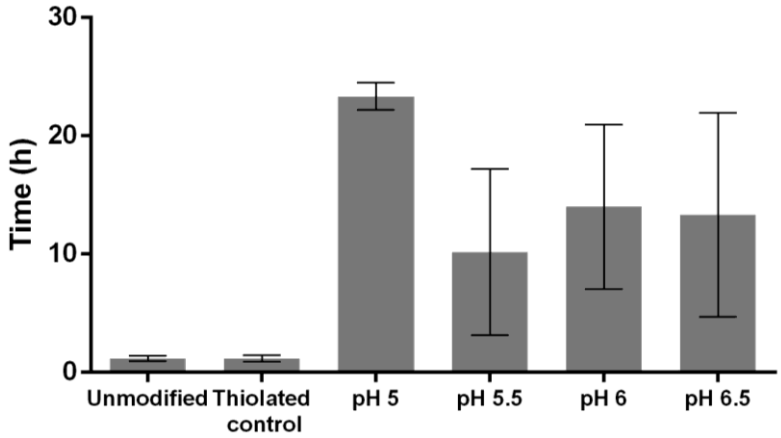


Figure 2.11 Mucoadhesive testing of unmodified PAA, thiolated controls and thiolated samples of varying thiol content (n=3)

Palmberger *et al.* (2007) conducted mucoadhesive studies on thiolated PAA samples of varying thiol contents, again thiol content was controlled by the addition of varying concentrations of EDC, as opposed to controlled by pH, resulting in a range of thiol contents. For clarity, Palmberger's results are shown in Figure 2.12. Levels of thiolation ranged from 53 µmol/g to 767 µmol/g and a control sample was also examined. The control sample, as was observed in this study, adhered for approximately 1 h. As thiol content increased, so did the mucoadhesive properties. Samples with a thiol content of 549 µmol/g adhered for approximately 30 h, which is in line with results observed in this study with the pH 5 sample. A sample with thiol content of 767 µmol/g was also assessed and adhered for more than 50 h. This sample had comparable thiol content to the pH 6 sample in this study, however, the length of adhesion was far greater than the adhesion times observed in this study.

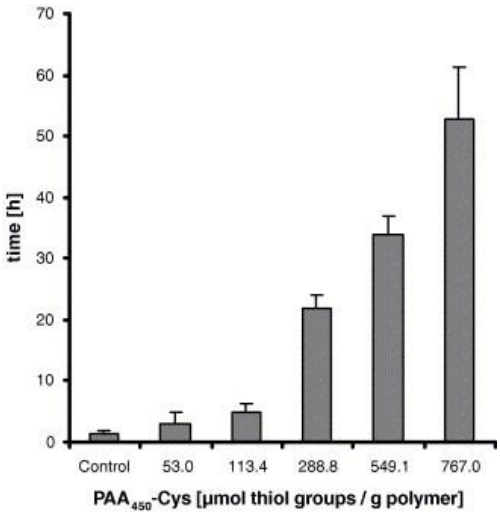


Figure 2.12 Mucoadhesive properties of PAA with varying thiol content, as conducted by Palmberger *et al.* (2007)

2.3.4 Drug release studies

Drug release studies of chloropheniramine (CPM) were conducted on unmodified PAA and thiolated PAA samples, samples pH 5.5 and 6.5. According to The Merck Index (2006), the solubility of CPM in water at 25 °C is 160 mg/mL. To maintain sink conditions during the drug release studies, the solubility of CPM needed to remain at least three times greater than the maximum concentration within the dissolution medium. In this study, 6 mg of CPM was added to the polymer tablets and the volume of drug release was 20 mL. Therefore, at maximum release, there was 6 mg/20 mL of phosphate buffer. Assuming that the phosphate buffer concentration and the temperature did not affect the solubility of CPM, sink conditions were maintained throughout the experiment. Similar to the swelling studies, the unmodified PAA sample fully disintegrated during the course of the drug release studies. This is in contrast to the thiolated samples which did not disintegrate in the buffer and remained cohesive for a number of days. The increased thiol content of the pH 5.5 sample resulted in a slower and controlled release of CPM over an 8 h period.

CPM was incorporated into thiolated PAA samples, pH 5.5 and 6.5, which had differing thiol content, 600 µmol/g and 1000 µmol/g respectively. A typical chromatogram for the drug release of CPM from the thiolated PAAm sample is shown in Figure 2.13 (A), displaying the maleate peak eluting at 3.2 min and the chlorpheniramine peak eluting at 8.7 min. In both swelling studies and

mucoadhesive testing, the abilities of the pH 6.5 sample were often less favourable than that of the pH 5.5 sample. The same was true in drug release studies of CPM as shown in Figure 2.13 (B); percentage drug release values shown in Figure 2.13 (B) were calculated with regards to complete theoretical percentage release. For the initial 3 h, both tablets have similar drug release profiles; this then changed and drug was released more quickly from the pH 6.5 tablet. The increased drug release from this sample was to be expected as the swelling studies showed the sample swelling to a much larger extent than the pH 5.5 sample and in a faster time (Figure 2.9). With the increased ability to swell, the drug will be released quicker. Faster drug release was also observed by Kast *et al.* (2002) with the release of clotrimazole from thiolated chitosan tablets. Two tablets of differing thiol content, 160 and 280 µmol/g, were tested for swelling, disintegration and mucoadhesive properties as well as drug release of clotrimazole, and these were compared to unmodified chitosan tablets. The sample with the higher thiol content showed improved mucoadhesion and disintegration in comparison to the lower thiol content sample and unmodified sample. However, the higher thiol content sample swelled to a much higher capacity than the unmodified and lower thiol content samples and in addition, drug release was seen to be quicker from the higher thiol sample.

With a thiolated PAA tablet of 450.1 µmol/g thiol content, Hornof *et al.* (2003) analysed the release of diclofenac salts; both sodium diclofenac and tris-diclofenac were investigated which both have different solubility in water. It was observed that the sodium diclofenac, which is more soluble in water than its tris-diclofenac counterpart, was released over 8 h. The less soluble tris-diclofenac showed a significantly lower release pattern which allowed for zero order release. A control of unmodified PAA sample, however, was not tested and a comparison cannot be made. The release of sodium diclofenac is similar to the CPM released from the pH 5.5 sample in this study, shown in Figure 2.13 (B). Both CPM and sodium diclofenac are water soluble drugs (Wishart *et al.*, 2008), while tris-diclofenac is less soluble and showed a more controlled drug release pattern. By changing the drug which is incorporated into the polymer matrix to a less water soluble drug, a slower release pattern may be achieved, as was shown by Hornof *et al.* (2003).

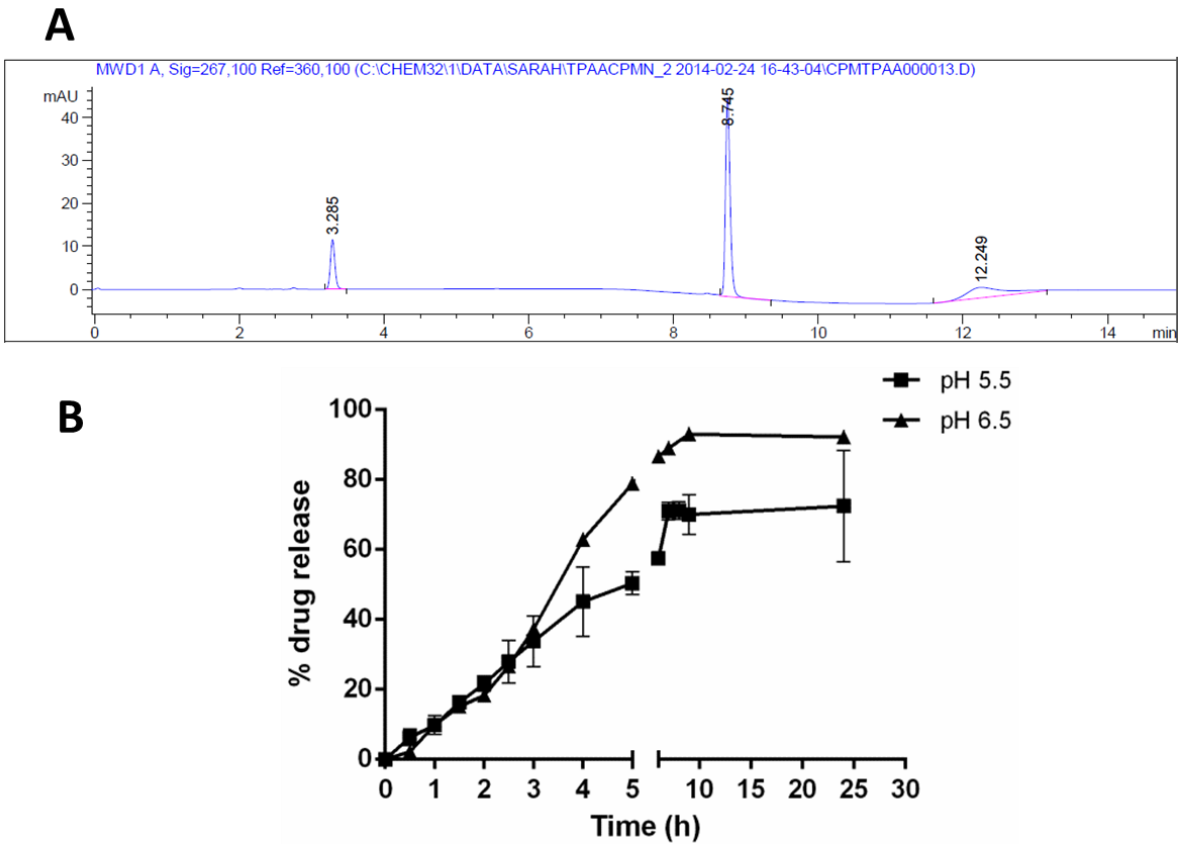


Figure 2.13 (A) Typical chromatogram of CPM drug release from thiolated PAA sample showing maleate peak at 3.2 min and chlorpheniramine peak at 8.7 min and (B) drug release of CPM from thiolated PAA tablets of differing thiol content, sample pH 5.5 (600 µmol/g) and sample pH 6.5 (1000 µmol/g) (n=3)

2.3.5 Polymer characterisation

2.3.5.1 Rheological properties

Rheology can examine the viscoelastic properties of a material, determining the storage modulus (G') and the loss modulus (G"). G' represents the elastic portion of the material, measuring the energy stored when a stress is applied and G" represents the viscous portion, measuring the energy lost when a stress is applied. Rheology was conducted on thiolated PAA samples to examine the viscoelastic properties of the polymer and results were compared to unmodified PAA. The viscoelastic properties of thiolated and unmodified PAA samples were also compared to polymer samples mixed with an 8% mucin solution. Rheological studies of polymer/mucin interactions is commonly accepted as a representative model of the *in vivo* behaviour of a mucoadhesive polymer (Andrews *et al.*, 2009). Once a mucin solution is mixed with the polymer, the polymer/mucin interactions, and strength of those interactions,

can be investigated, thus giving further information about the mucoadhesive properties of the polymer. The glycoproteins within mucin bond together to form a weak viscoelastic gel (Madsen *et al.*, 1998); once a thiolated polymer interacts with mucin, the intermolecular disulphide bonds formed between the polymer and the mucin would be much stronger, which increases the observed rheological response compared to the response of the polymer or the mucin separately. In this study, a commercially available mucin was used in the rheological testing, which is known to give more reproducible results in comparison to fresh, native mucin (V. M. Leitner *et al.*, 2003).

2.3.5.1.1 Dynamic amplitude tests

Dynamic amplitude tests were conducted in which a stress of 0.01 – 60 Pa was applied to the samples and G' and G" were determined. All samples showed a similar response to increasing stress. At lower stress (the linear viscoelastic region) G' values were greater than G", indicating a predominantly solid-like response. Both G' and G" were independent of stress up to a certain strain, at which point both G' and G" began to decrease. The decrease of G' and G" indicated the onset of structural breakdown within the sample. A G'/G" crossover occurred in all samples indicating the transition to a predominantly liquid-like response (Figure 2.14 (A)).

The overall response to stress was similar among all samples. However, there was a marked difference in G', with an approximately 1000-fold increase between the thiolated samples and the unmodified sample, which is shown in Figure 2.14 (B). Visually, the thiolated samples became a gel during the experiment in comparison to the unmodified sample which remained a liquid. As the polymer solutions were made in phosphate buffer at pH 6.8, crosslinking of the thiol groups would occur, and the strong intramolecular disulphide bond formation within the thiolated samples would have an influence on G' values in comparison to the weaker electrostatic bonding within the unmodified sample. Secondly, the samples which had been thiolated at a higher pH, and therefore resulted in higher thiol content, had higher G' values. This trend of increasing G' with the increase in thiol content was due to the swelling ability of the samples. As shown in Figure 2.9, the swelling ability of the thiolated samples also increased with increasing thiol content. As the gel swells and

liquid is taken up by the polymer matrix, the structure and the gel strength of the polymer changed, therefore, changing the response of the polymer to stress.

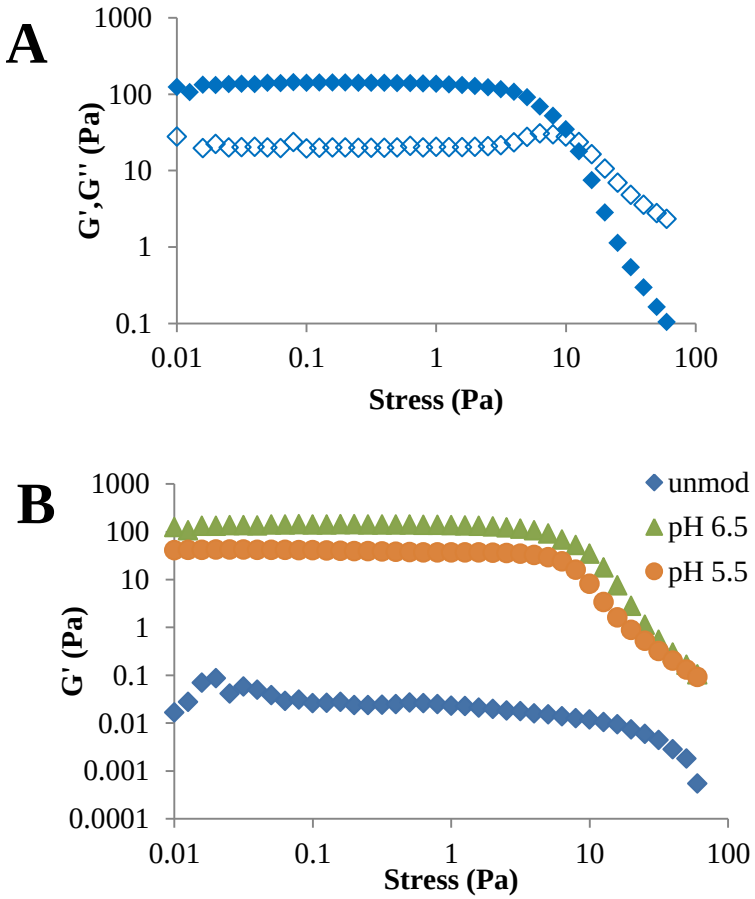


Figure 2.14 (A) Stress tests of thiolated sample pH 6.5 displaying G′ (closed symbols) and G″ (open symbols) values, (B) Stress tests of unmodified PAA and thiolated PAA samples, indicating G′ values

The G' values of the unmodified PAA observed in this study are comparable to both Marschutz *et al.* (2002) and Leitner *et al.* (2003). The G' values of the thiolated PAA samples do, however, vary. Leitner *et al.* (2003) observed G' values of approximately 3 Pa for a thiolated PAA sample of thiol content 712 µmol/g. This is in contrast to the pH 6 sample in this study, which has a thiol content of 781.6 µmol/g, but displayed a G' value of approximately 70 Pa. Similarly, Marschutz *et al.* (2002) examined a thiolated PAA sample with thiol content of 511.6 µmol/g. G' values were measured at 3.07 Pa for this sample; comparing it to the pH 5.5 sample in this study, with thiol content of 594.0 µmol/g, G' values were, again, much higher at 41 Pa. Marschutz *et al.* thiolated the PAA sample with 200 mM EDC, at pH 6

with an EDC activation time of 20 min. The difference in G' values between the pH 5.5 sample in this study and the thiolated PAA sample as conducted by Marschutz *et al.* may be due to the difference in swelling ability of the samples; as shown in Figure 2.9, the pH 5.5 sample increased by 2000% in 80 min. As discussed by Marschutz *et al.*, the thiolated PAA sample, with thiol content of 511.6 µmol/g, increased by 28.55-fold over a 90 min period. There was a substantial increase in mass due to swelling observed in this study with all thiolated samples, and it may be this increase in swelling ability which has increased the observed G' values.

An 8% mucin solution was mixed with the polymer solutions to examine the potential mucoadhesive interactions. As shown in Figure 2.15 (A), once mucin was added to the polymer solutions, there was a significant increase in G' and G" values in comparison to polymer samples alone; this increase in G' and G" occurred in both the unmodified and thiolated PAA samples, however, to a greater extent in the thiolated samples. The viscoelastic properties of the mucoadhesive polymer/mucin mixture should be in a large excess when compared to the properties of the mucin or the polymer properties alone (Riley *et al.*, 2001); Figure 2.15 (B) shows that the G' response of the polymer/mucin solution was considerably greater than either the polymer alone or the 8% mucin solution alone. This indicates that stronger interactions and stronger intermolecular bonds have formed in the polymer/mucin mix in comparison to the intramolecular bonds within either the polymer or within mucin. The increase in G' and G" values upon the addition of mucin to PAA samples have been observed by Marschutz *et al* (2002) and Hagerstrom *et al.* (2000); the observed increase in values indicates interactions, either chemical or physical, have occurred between the polymer and the mucin which in turn indictates mucoadhesive properties (Hägerström *et al.*, 2000).

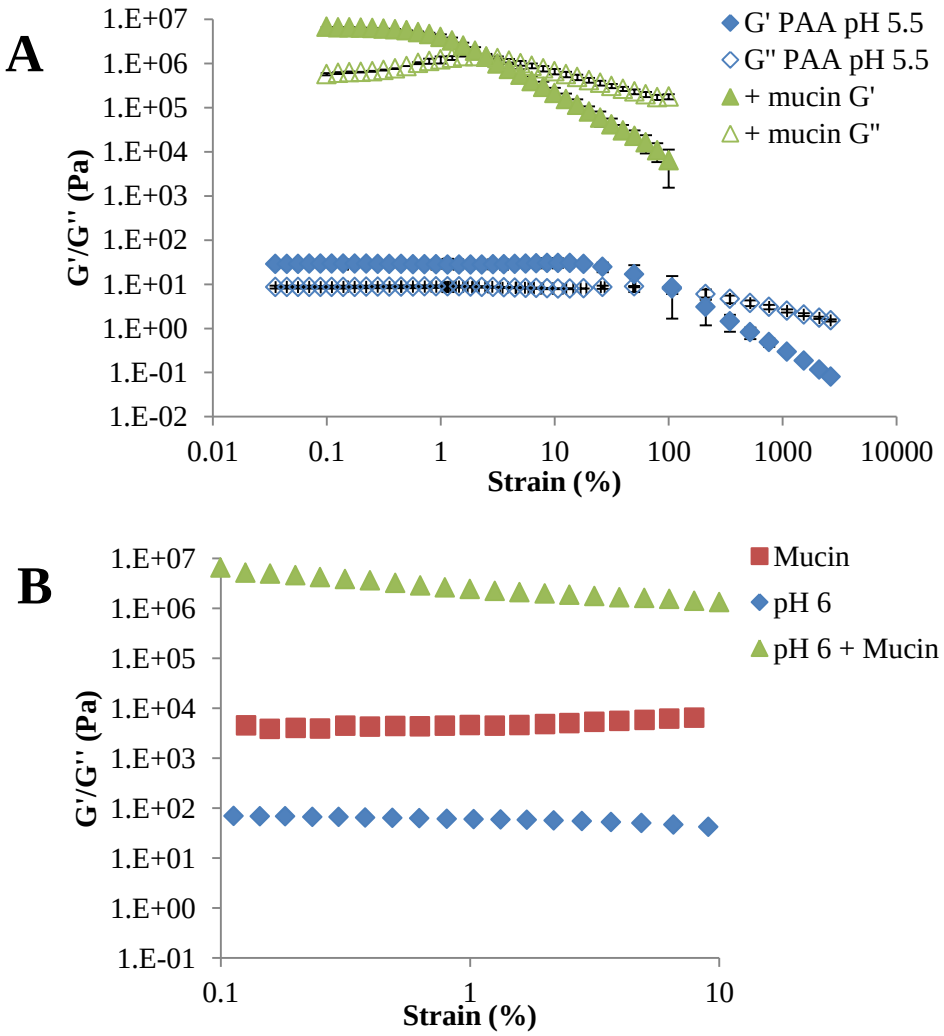


Figure 2.15 Strain sweeps displaying (A) G' and G'' values of PAA pH 5.5 with and without the addition of mucin and (B) G' of PAA pH 6, PAA pH 6 with mucin and mucin alone (n=2)

Similar to the polymers samples themselves, there was a crossover of G' and G" in the mucin samples, after which G" became higher than the G' value indicating the structural breakdown of the samples. The crossover point upon mucin addition was at a lower strain than that of the polymer solutions alone, as is shown in Table 2.4. As the crossover points in the mucin samples are lower, this signifies that the transition to a predominantly liquid-like response occurred at a lower strain in the mucin samples than in the polymer samples themselves. The bonds and interactions within the polymer/mucin mix may be different than within the polymer; although disulphide bonds would have formed within the polymer/mucin mix, the secondary bonding (hydrogen and electrostatic bonding) and entanglement interactions may be

weaker within the polymer/mucin mix in comparison to the polymer itself, resulting in the crossover occurring at a lower strain.

Table 2.4 Strain at which G' and G'' crossover occurred of thiolated PAA samples with and without mucin addition

Sample	Crossover points of G' and G'' (Strain %)	
	Without mucin	With mucin
pH 5.5	107.31	2.5
pH 6	62.25	25.07
pH 6.5	45.48	15.85

2.3.5.1.2 Frequency sweeps

Frequency sweeps were conducted on the unmodified and thiolated PAA samples within the viscoelastic region. All samples displayed a linear response across the frequency range of 0.1 – 20 Hz, as shown with the thiolated pH 6 sample in Figure 2.16. Similar to the stress tests, G' in the frequency tests was higher than that of G" over the entire frequency range, again, indicating a viscoelastic solid material. There was no crossover between G' and G" in the frequency tests, which is characteristic of a crosslinked system (Riley *et al.*, 2001). As was observed in the stress sweeps, G' and G" were higher in the thiolated samples than in the unmodified sample. G' also increased with increasing pH and, therefore, thiolation levels in the thiolated samples. The slopes of the unmodified and thiolated samples were notably different. The slope of the unmodified G' curve was 0.08 in comparison to the slopes of thiolated sample curves which ranged from 3.59 – 4.28. The differences in slope are suggestive of different structural networks present, which again may be due to the formation of disulphide bonds within the thiolated samples in comparison to the weaker electrostatic interactions within the unmodified PAA sample.

Similar to the stress tests, once the 8% mucin solution was mixed with each of the polymers, both G' and G" substantially increased, as shown in Figure 2.16, which is indicative of a mucoadhesive polymer. Similar to the polymer samples alone, the frequency response upon mucin addition was also linear, again characteristic of a

crosslinked system (Mortazavi *et al.*, 1993). All polymer samples, both thiolated and unmodified, displayed an increase in G' values upon the addition of mucin, as highlighted in Table 2.5. The increase in G' values upon the addition of mucin to the unmodified PAA sample again demonstrates the inherent mucoadhesive properties possessed by PAA. However, as shown in Table 2.5, the increase in G' in the unmodified sample was not as large in comparison to the thiolated samples and these results mirror the rotating cylinder mucoadhesive test in section 2.3.3.

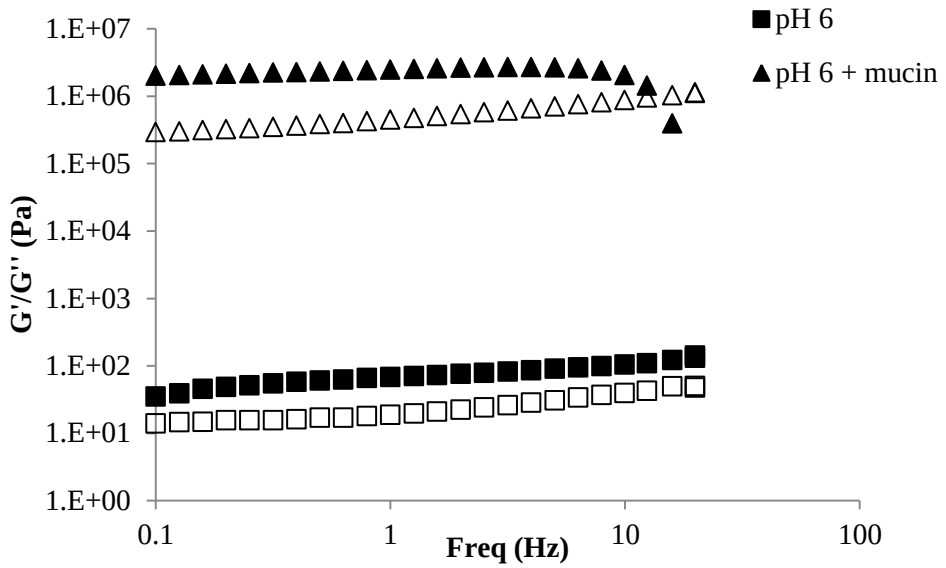


Figure 2.16 Frequency tests of thiolated sample pH 6 displaying G′ (close symbols) and G″ (open symbols), with and without the addition of mucin (n = 2).

Table 2.5 G′ values of Unmodified and thiolated PAA at 0.1 Hz, with and without the addition of mucin

Sample	G' at 0.1 Hz (Pa)	
	Without mucin	**With mucin**
Unmodified	0.826717	1223
pH 5	0.5617	299650
pH 5.5	22.565	5745500
pH 6	34.68	2000000
pH 6.5	66.325	

2.3.5.2 Scanning electron microscopy (SEM)

SEM images of thiolated PAA and thiolated PAA-CPM conjugates are shown in Figure 2.17. The samples had been subjected to similar conditions during both reaction processes and analysis and the SEM images of the samples were taken after freeze drying. The thiolated PAA and thiolated PAA-CPM samples, once removed from the freeze dryer, had obvious visual differences between them, and this can also be observed in the SEM images. The thiolated polymer had a cotton wool-like texture and looked quite porous, which can be seen in images A and B below. This is in comparison to the thiolated CPM conjugates which were more rodlike in appearance post freeze drying and, looking at the SEM images C and D, had a sharper and more sheet like appearance in comparison to the thiolated PAA itself, implying that incorporation of the drug has altered the polymeric material. This implies there are interactions between the polymer and the drug occurring, therefore changing the morphology of the polymer. This phenomenon was also observed with the incorporation of ciprofloxin into polyvinyl alcohol (PVA) scaffolds, in which the pore and morphology differed with incorporation of the drug (Mabrouk *et al.*, 2014).

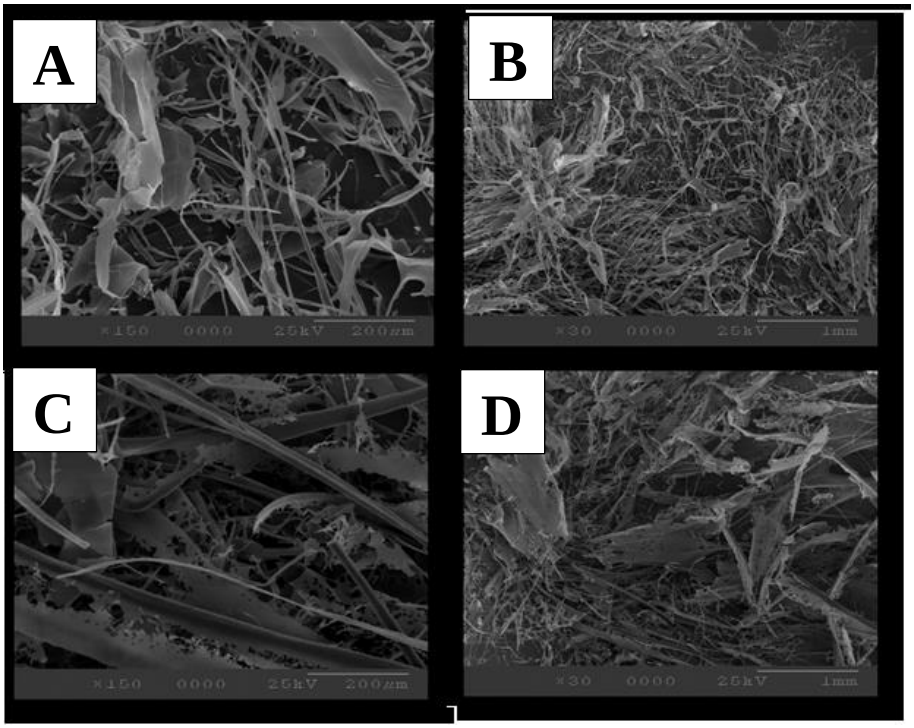


Figure 2.17 SEM images of thiolated PAA (A and B) and thiolated PAA-CPM conjugate (C and D) at magnifications of 150 on the left and 30 on the right

2.3.5.3 Thermogravimetric analysis (TGA)

TGA was conducted on unmodified PAA and thiolated PAA samples and the thermograms are shown in Figure 2.18 (A). There was water loss from both the unmodified and thiolated samples below 100 °C; although the samples had been freeze dried, moisture uptake had occurred. The degradation pattern of unmodified PAA was in agreement with the literature (Dubolazov *et al.*, 2004; Teresa Garay *et al.*, 1997), with a two-step degradation pattern being observed. The initial degradation step, seen at approximately 210 °C, was due to the formation of anhydride groups with final degradation of the polymer then seen from 310 °C (Teresa Garay *et al.*, 1997; Dubolazov *et al.*, 2004). The thiolation of PAA changed the degradation pattern, shifting the initial stage of degradation from 210 °C in the unmodified sample to 175 °C in the thiolated sample. This may be due to the loss of thiol groups from the polymer backbone, which has shifted the initial degradation to lower temperature. The intra- and inter-molecular bonding in unmodified and thiolated PAA would be quite dissimilar. Hydrogen bonding would occur within the unmodified PAA; Thiolation of the PAA backbone would potentially interrupt the hydrogen bonding, and, as shown in Table 2.3, disulphide bonds had formed within the thiolated PAA sample, which may have changed the degradation pattern of the material. The second and final degradation event occurs in both the unmodified and thiolated samples.

Figure 2.18 (B) illustrates the TGA of unmodified PAA-CPM and thiolated PAA-CPM, while Figure 2.18 (C) shows the TGA of CPM itself. The evaporation of CPM began at 147 °C. Comparing both (A) and (B) of Figure 2.18 shows that incorporation of CPM into the polymer matrix changed the degradation pattern in both the unmodified and thiolated samples, lowering the initial degradation temperature from 210 °C to 155 °C in unmodified PAA and from 175 °C to 164 °C in the thiolated sample. The degradation patterns of both samples displayed in Figure 2.18 (B) were quite similar, with slight shifts in weight loss between the unmodified and thiolated samples. Therefore, the incorporation of CPM in the matrix of unmodified PAA and thiolated PAA has caused comparable changes to the polymer matrix, implying bonding, such as hydrogen bonding, must occur in a similar manner between the drug and the polymer in both unmodified and thiolated PAA samples. The thiolation of PAA changed the degradation pattern in a more significant manner

than the incorporation of the drug. This was due to the modification of the polymer backbone in the thiolated sample converting carboxyl groups to amides which contain thiols, as opposed to the bonding of drug to the polymer.

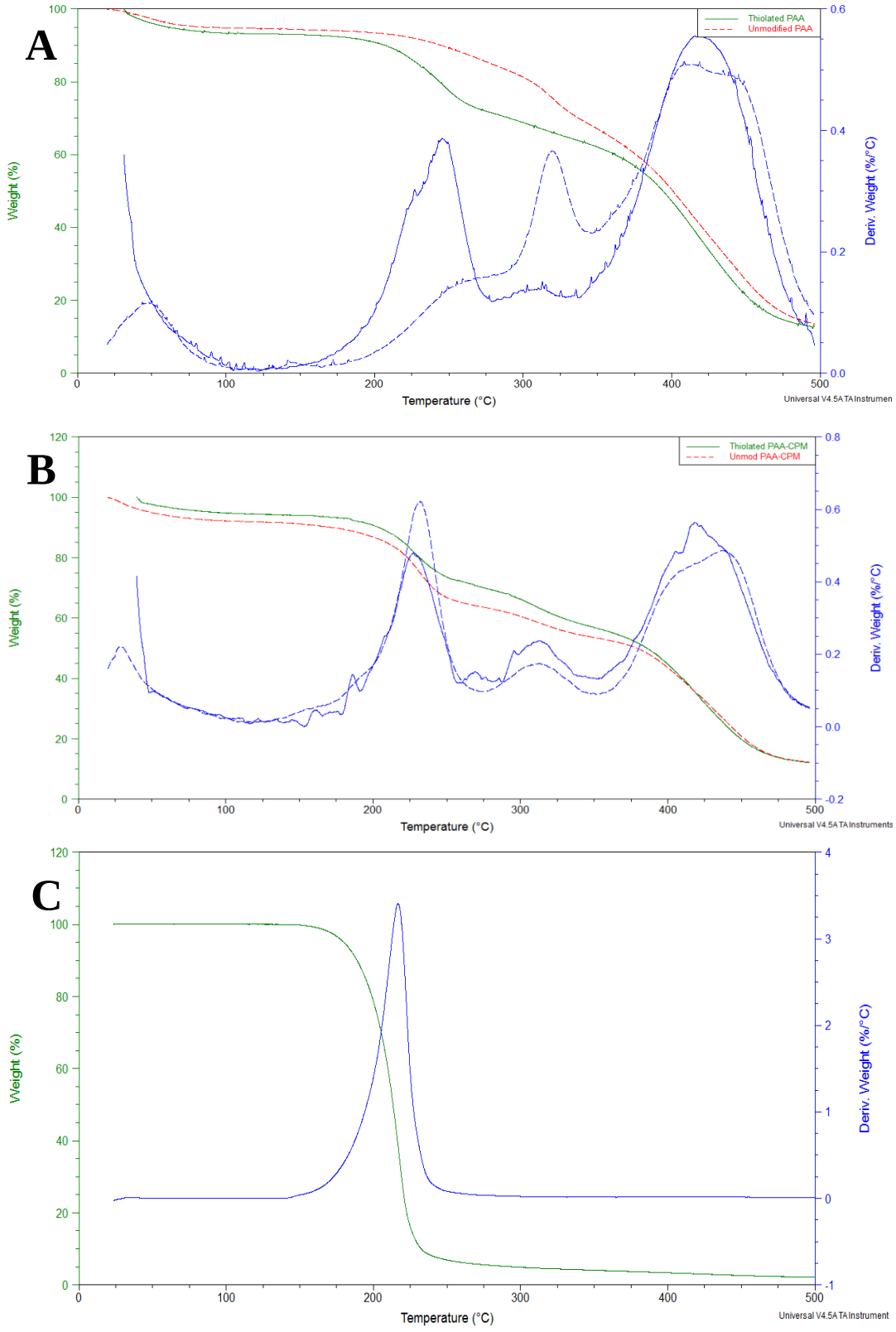


Figure 2.18 TGA analysis of (A) unmodified PAA and thiolated PAA samples, (B) unmodified PAA-CPM and thiolated PAA-CPM conjugates and (C) CPM

2.3.5.4 Differential scanning calorimetry (DSC)

Unmodified (freeze dried) PAA, thiolated control (thiolated without the addition of EDC), thiolated PAA samples and PAA-CPM conjugates were all analysed by DSC. All samples were run on a heat-cool-heat cycle; in the first heating cycle, a broad endothermic peak was observed which correlated to water loss from the sample. This can mask the glass transition (T_g) of the sample – the point at which the material becomes more mobile and flexible and is observed as a endothermic step transition. The DSC heat-cool-heat cycle of thiolated control PAA is shown in Figure 2.19, and a broad endothermic water peak at approximately 90 °C can be seen in the 1st heating cycle which masked the T_g; the T_g was then observed in the 2nd heat cycle at 135.52 °C. This demonstrated the importance of running the sample on a heat-cool-heat cycle, as the T_g can be easily seen in the 2nd heating cycle.

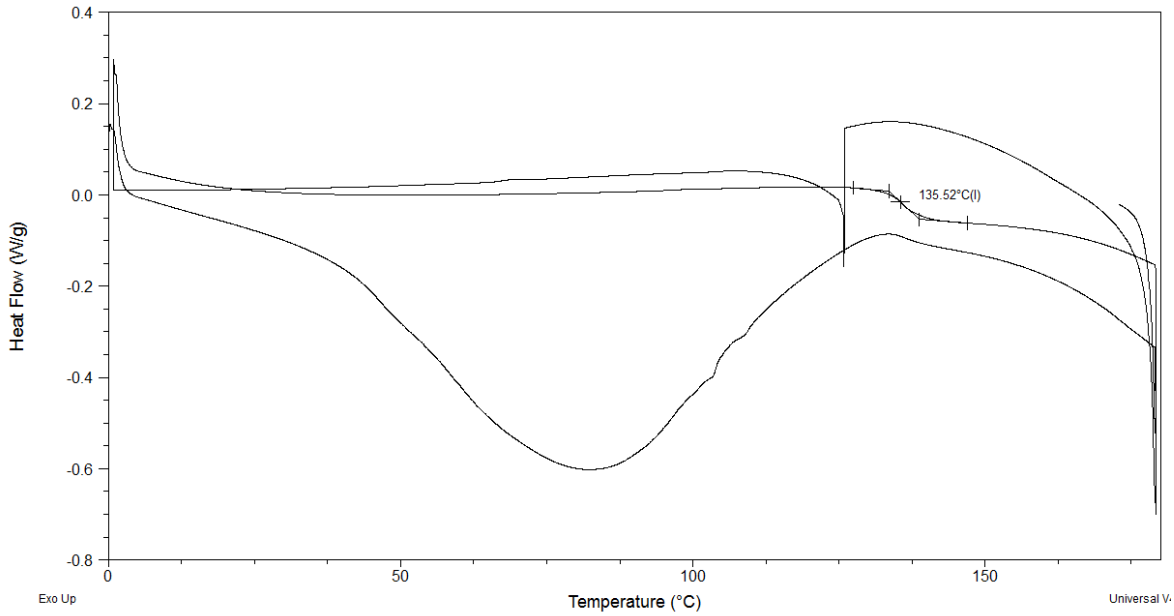


Figure 2.19 DSC curve showing heat-cool-heat cycles of thiolated control PAA

The thermogram displaying the 2nd heating cycle of unmodified and thiolated control PAA samples is shown in Figure 2.20, with highlighted T_gs. The T_g increased marginally in the control sample (135 °C) in comparison to the unmodified sample (130 °C). This may be due to the presence of any unbound thiol which was not dialysed from the thiolated control sample. In the unmodified sample, there was a

small endothermic dip at the end of the T_g transition, which was classified as an endothermic relaxation peak. This in turn may be altering the true T_g value of the unmodified sample, therefore, the T_g temperature values of both the unmodified and control samples may in fact not be as dissimilar as they appear. Heating the unmodified sample more slowly through the T_g or faster cooling may remove any annealing effect, thus removing the endothermic relaxation peak which may allow for a clearer and more precise measure of the T_g (Gabbott, 2008). The T_g measured for unmodified PAA is higher in this study than in the literature, with Dubolazov *et al.* (2004) stating a T_g of 106 °C for PAA, however, the PAA used was 230 kDa, not 450 kDa as was used in this study. The higher T_g observed may be due to the higher MW polymer used. Chan and Chu (2001) also used a 450 kDa PAA and measured the T_g as 129 °C, similar to the T_g observed in this study, at 130 °C.

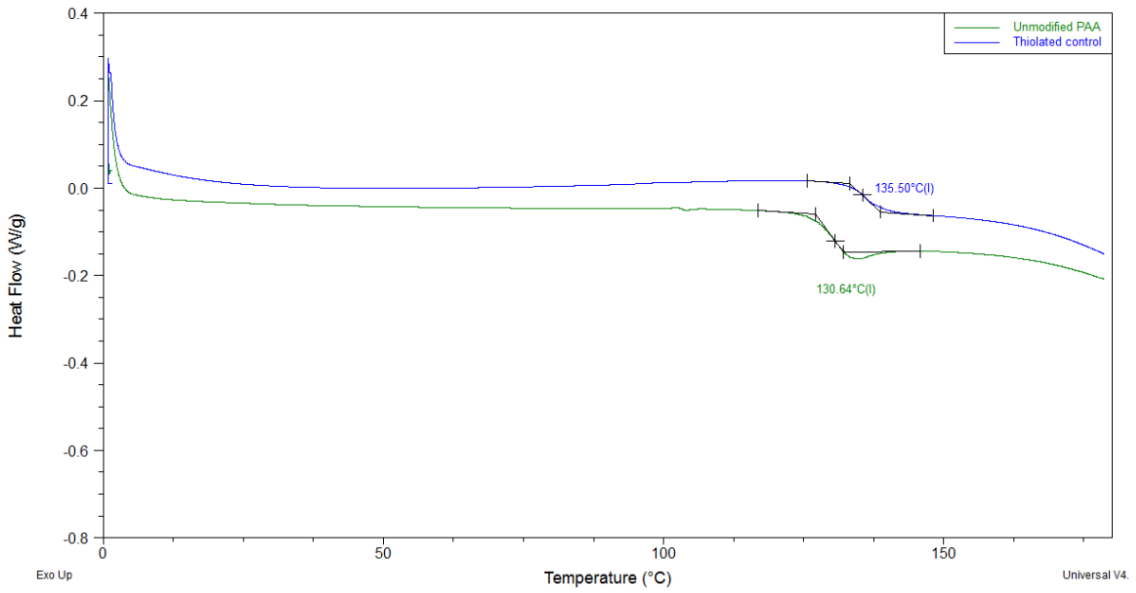


Figure 2.20 DSC curve showing the 2nd heat cycle of unmodified and thiolated control PAA. Tg values are labelled.

Figure 2.21 (A) shows the DSC curve of chlorpheniramine maleate (CPM). There was one sharp endothermic peak observed at 134 °C indicating the melting point of the compound. The 2nd heat cycle of thiolated control PAA, thiolated PAA and the thiolated PAA-CPM conjugate is shown in Figure 2.21 (B). The T_g of the thiolated samples increased to 156 °C in comparison to the thiolated control sample which

measured 135 °C. This may be due to the potential crosslinking within the thiolated samples. The step transition itself was much less steep in the thiolated sample in comparison to the control. The T_g was not visible in the PAA-CPM conjugate.

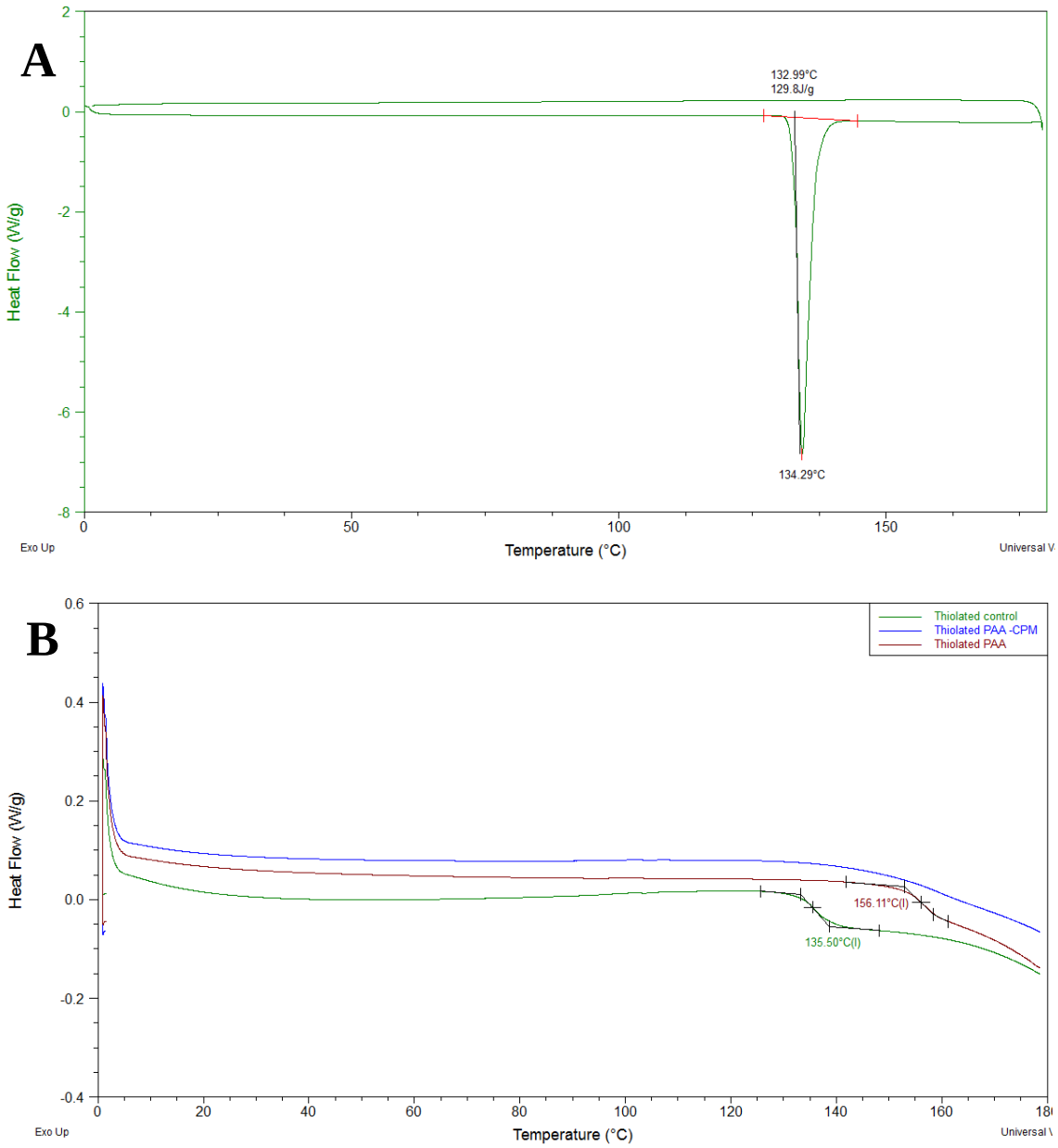


Figure 2.21 DSC curve showing (A) melting point of CPM and (B) the 2nd heat cycle of thiolated control PAA, thiolated PAA and thiolated PAA-CPM conjugate, highlighting the Tg where possible.

The change in T_g due to polymer modification or incorporation of another compound is commonly observed in the literature. Chan and Chu (2001) incorporated varying

levels of silica into PAA through a sol-gel process and noted a shift in T_g, both increasing and decreasing the T_g depending on the ratio of PAA:silica; with ratios of 60:40 and 40:60 PAA:silica, two T_g were observed. In the case of the samples displaying two T_g, it was concluded that one was from the silica resulting in the lower T_g and the second higher T_g was due to the PAA. In the sample of 80:20 PAA:silica, there was only a low T_g at -7.5 °C observed. Once the ratio of PAA:silica had increased to 20:80, the lower T_g was no longer observed, and a T_g comparable to unmodified PAA was measured, although the T_g was broader. The reasoning behind the shifts in T_g between samples was concluded as the effect of hydrogen bonding. Hydrogen bonding is stronger between carboxyl and carbonyl groups in comparison to carbonyl to silianol groups, and it was this that changed the T_g in the samples.

Chitosan, thiolated with N-acetyl-D,L-homocysteine thiolactone, was analysed by DSC and compared to unmodified chitosan (Ferris *et al.*, 2014). The T_g of the unmodified chitosan was measured at 40 °C whereas the thiolated chitosan sample displayed a T_g of 38 °C. Although the change in T_g was small, it was concluded that the decrease in T_g temperature was due to the addition of the bulky side chains upon thiolation and, similarly, due to the ability of the thiolated chitosan to incorporate water into the matrix in comparison to the unmodified sample. The thiolation of a tamarind seed polysaccharide using thioglycolic acid was also noted to initiate changes in DSC analysis in comparison to its unmodified counterpart (Kaur *et al.*, 2012). Although a T_g was not measured for either the unmodified or the thiolated tamarind seed polysaccharide samples, there were shifts in endothermic peaks. The unmodified sample displayed two broad endothermic peaks at 85 °C and 270 °C. An exothermic peak at 318 °C was also measured in the unmodified sample. Upon thiolation, the first endothermic peak shifted from 85 °C to 81 °C while the second endothermic peak became sharper and also shifted, from 270 °C to 145 °C. The exothermic peak observed in the unmodified sample was not visible in the thiolated sample. The shift and disappearance of the endo- and exothermic peaks were concluded as indications of modification of the polymer due to thiolation.

2.3.5.5 Modulated DSC (MDSC)

The T_g in the PAA-CPM sample was not visual using convectional DSC methods (Figure 2.21 (B)). Because of this, modulated DSC (MDSC) was conducted on PAA-CPM conjugates, and also on unmodified PAA and thiolated PAA samples. In MDSC, the total heat flow and the reversing and non-reversing heat flows can be isolated. As the T_g is a reversible process, it will be displayed on the reversing heat flow axis. Analysis of the PAA-CPM conjugates by MDSC may allow for T_g measurement and give a better understanding of the samples and possible changes occurring due to drug incorporation.

Shown in Figure 2.22 are the MDSC thermograms of unmodified PAA, thiolated PAA and a thiolated PAA-CPM conjugate, both of which were thiolated at pH 6. A clear T_g in the unmodified PAA sample was observed in the reversing heat flow. However, the T_g values of the thiolated sample and thiolated PAA-CPM conjugate were less clear but were measurable. The T_g values of all samples as measured by MDSC are displayed in Table 2.6.

Table 2.6 Glass transition values of samples analysed by MDSC

Sample	T_g (°C)	T_g with CPM addition (°C)
Unmodified	130	134
pH 5	152	123
pH 5.5	159	159
pH 6	158	157
pH 6.5	158	145

The T_g of the PAA-CPM conjugates did shift in all samples, increasing in the case of the unmodified PAA sample but decreasing in all thiolated samples, implying that CPM displays plasticising effects. The decrease in T_g is in agreement with the literature. Zhu *et al.* (2002) investigated the effect the incorporation of different concentrations of CPM and of triethyl citrate (TEC) had on Eudragit® RS PO, a copolymer of ethyl acrylate, methyl methacrylate and methacrylic acid ester. Using MDSC, the T_g of Eudragit® RS PO, of Eudragit® RS PO with TEC incorporation

and Eudragit® RS PO with CPM incorporation were measured. Both CPM and TEC were described as plasticisers: CPM as a solid state and TEC as a liquid state. Zhu *et al.* observed that the T_g decreased as the concentration of both TEC or CPM increased, with the decrease being greater in the TEC samples, having approximately double the plasticising effect on Eudragit® RS PO in comparison to CPM. The T_g of Eudragit® RS PO alone was measured as 67.4 °C; this decreased by 2.7 °C for every 1% of TEC added and by 1.31 °C for every 1% of CPM added. The plasticising effects of CPM was also observed by Wu and McGinity (1999), who again saw that increasing concentrations of CPM decreased the T_g of Eudragit® RS 30 D, a copolymer of ethyl acrylate and methyl methacrylate. Therefore, the decrease in T_g observed in the thiolated PAA-CPM conjugate samples in this study was due to the to the plasticising effects of the drug, CPM.

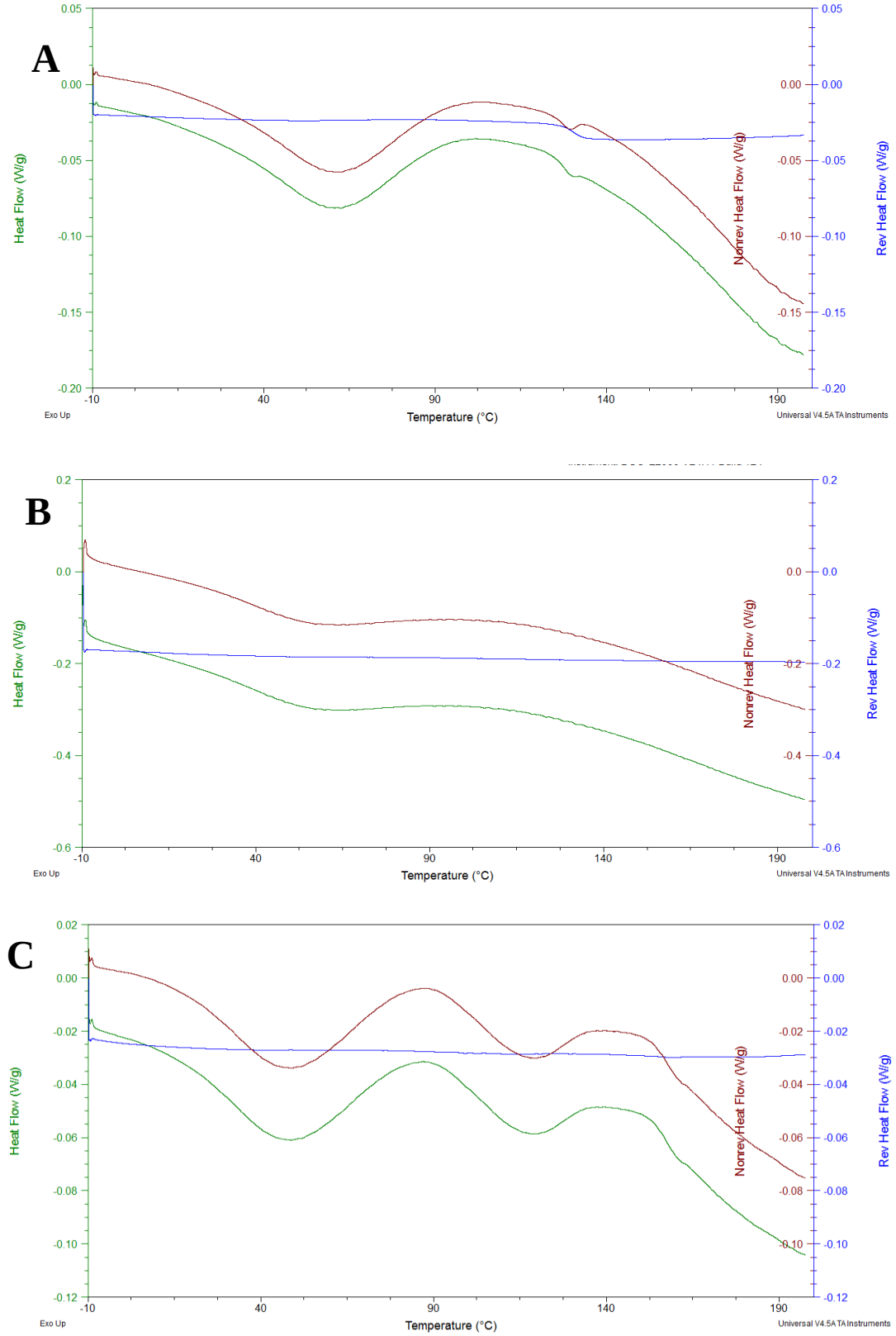


Figure 2.22 MDSC of (A) unmodified PAA, (B) thiolated PAA at pH 6 and (C) thiolated pH 6 PAA-CPM conjugate

2.4 Conclusion

PAA is a well-established polymer in terms of both thiolation and mucoadhesion, with significant amounts of work having already been conducted using this polymer (Bernkop-Schnürch *et al.*, 1999; Marschütz and Bernkop-Schnürch, 2002; V. M. Leitner *et al.*, 2003; Guggi *et al.*, 2004; Palmberger *et al.*, 2007; Iqbal *et al.*, 2012; Bonengel *et al.*, 2014). The thiol content of thiolated PAA has been shown to have a large effect on both swelling ability, cohesion and mucoadhesive properties (Palmberger *et al.*, 2007). The thiolation of PAA was conducted by activating the carboxyl groups on the PAA backbone with the crosslinker EDC, followed by the addition of L-cysteine, thus creating a thiolated PAA material through the formation of an amide bond. In the literature, the addition of L-cysteine was based on time of EDC activation (Marschütz and Bernkop-Schnürch, 2002) and on concentration of EDC added (Palmberger *et al.*, 2007), changing both time and EDC concentration in order to create polymers with varying thiol content. Reproducibility of thiol content proved difficult and the addition of 200 mM EDC resulted in a thiol content ranging from 498.8 µmol/g to 747 µmol/g (Grabovac *et al.*, 2005; Wang *et al.*, 2012). As was shown in this study, adding a constant concentration of EDC to the PAA solution and controlling the pH point at which L-cysteine was added resulted in highly thiolated products with specific and varied thiol content. The concentration of EDC in this reaction was very important and, due to the hydrolysis of EDC within the solution which resulted in the observed drop in pH, the concentration of EDC will not have remained constant between samples; however, a highly reproducible method for regulating the level of thiolation on the PAA backbone has been created. The ability to create a thiolated PAA material with specified thiol content may be extremely advantageous to mucoadhesive drug delivery, as the crosslinking, swelling and mucoadhesive properties of the material will also vary with increasing thiol content. When drugs with longer half-lives are incorporated into a drug delivery matrix, it is vital that the matrix does not degrade quicker than the half-life of that drug, as it could result in elimination of the drug from the target site and excretion of the drug. With this in mind, a tailor-made thiolated material designed for a specific drug can be created, and altering the swelling and cohesive properties of that thiolated matrix will allow for the precise release of that specific drug. More potent drugs need to be administered carefully due to the potential immune responses and

harmful side-effects that they can initiate and utilising a thiolated matrix which allows for a slower, controlled release of drug could lessen the potential side effects often associated with these more potent drugs.

The thiolated PAA samples with varying thiol content created were tested for swelling ability and mucoadhesion and were compared to both unmodified and thiolated control samples. The swelling abilities of the thiolated samples were greatly improved with an increase in mass of up to 1500% during swelling tests. The thiolated samples displayed far greater cohesive and mucoadhesive properties and mucoadhesion improved by over 20-fold in comparison to the unmodified and control samples. The importance of molecular weight was demonstrated and improved cohesion and mucoadhesion was observed using PAA of MW 450 kDa over PAA of 250 kDa.

An increase in thiol content showed an increase in swelling ability; as thiol content increased, the degree of crosslinking should also increase, therefore lowering the rate of swelling, yet this was not observed. However, with this increased swelling ability came improved cohesion as the tablets remained together regardless of the degree of swelling. Disulphide bond formation was highest in the pH 5 samples, which had the lowest thiol content, yet the rate and degree of swelling was also lowest in this sample. The lower levels of disulphide bonds formed within the pH 6.5 sample may have influenced the swelling ability of the samples. The pH 5 sample may have had higher levels of cohesion initially due to disulphide bond formation and the initial burst of swelling in the pH 6.5 sample may have impeded the tablet from crosslinking further as the distance between thiol groups may have been too large. Therefore, there was limited crosslinking to slow the tablet from swelling and so it continued to swell at a faster rate.

Thiolation improved the levels of mucoadhesion in comparison to unmodified and control samples. Similar to swelling studies, the pH 5 sample had the greatest levels of mucoadhesion on porcine intestinal tissue, adhering for approximately 24 h. The pH 5.5, 6 and 6.5 samples all displayed greater mucoadhesion than unmodified samples, and had comparable adhesion times of approximately 15 h; however, the intestinal tissue used, and the density of mucin on that tissue did influence the adhesion of the polymers greatly.

The drug release profile of the thiolated samples were also greatly improved in comparison to unmodified and control samples. The unmodified and control samples had decreased levels of cohesion and released CPM quickly in contrast to the thiolated samples. Drug release from the thiolated samples was achieved over an 8 h period. The faster rate of swelling of the pH 6.5 sample had a major effect on the material and the drug release from this sample was faster in comparison to the pH 5.5 sample.

The rheological analysis gave further insight into the mucoadhesive properties of the thiolated PAA samples. Viscoelastic properties, both the storage modulus (G') and the loss modulus (G"), of the thiolated polymers were measured, and these properties were compared to the unmodified PAA sample. In stress and frequency tests, G' was higher than G" in all samples, indicating a more solid-like response. Both G' and G" were considerably larger in the thiolated samples in comparison to the unmodified PAA sample, possibly due to the different bonds within the matrices of the unmodified and thiolated samples. This also reflected the improved swelling ability and cohesion properties of the thiolated samples. As the thiol content of the thiolated samples increased, so did the G' values, which reflected the swelling ability of the samples. Upon the addition of a mucin solution, there was a marked increase in both G' and G", indicative of a mucoadhesive polymer. G' and G" both increased in the unmodified/mucin sample, mirroring the mucoadhesive testing and again highlighting the inherent mucoadhesive properties of PAA.

SEM, TGA and DSC were conducted on samples, with and without the incorporation of the drug, chlorpheniramine maleate. Analysis indicated that incorporation of the CPM changed the properties of PAA, in both unmodified and thiolated samples, in terms of morphology and visual properties and also thermal analysis. Thiolation of PAA had a large effect on the decomposition properties in comparison to unmodified PAA which was observed by a quicker onset of decomposition in the thiolated sample in comparison to the unmodified sample. This may have been due to the different types of inter- and intra- molecular bonding which occurs within the thiolated and unmodified samples which in turn effected the degradation of the materials. The incorporation of CPM into both unmodified and thiolated PAA appeared to effect both samples in a similar fashion, implying bonding of the drug in unmodified and thiolated PAA occurred in a similar manner.

In DSC analysis, the T_g of the thiolated samples increased in comparison to unmodified PAA. Using conventional DSC, the T_g was not clear in the drug incorporated samples and, therefore, modulated DSC analysis was conducted. With the addition of CPM to the matrix, the T_g of the thiolated PAA-CPM conjugates decreased in comparison to non-drug incorporated samples, which was in agreement with the literature.

2.5 References

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Chapter 3 Synthesis of thiolated gelatin using a two-step reaction process

3.1 Introduction

Thiolated synthetic polymers, such as polyacrylic acid (PAA), have been shown to have improved cohesive and mucoadhesive properties, as discussed in chapter 2. Thiolated chitosan, a cationic polysaccharide, has demonstrated comparable mucoadhesive properties to thiolated PAA with the benefits of biocompatibility, biodegradability and non-toxic effects (Bernkop-Schnürch *et al.*, 2003; Roldo *et al.*, 2004; Schmitz *et al.*, 2008). Recently, further studies have been conducted into the thiolation and mucoadhesion of other natural polymers, such as the polysaccharides pectin (Sharma and Ahuja, 2011; Hintzen *et al.*, 2013) and alginate (Hauptstein *et al.*, 2015). In this study, the protein, gelatin, will be thiolated and its mucoadhesive properties will be examined. Gelatin is an FDA approved, natural polymer which is often utilised within the food and pharmaceutical industries. A novel two-step reaction process is used to create a highly thiolated and potentially mucoadhesive tablet.

3.1.1 Amination of native gelatin

The amination process, which is shown in Figure 3.1, is the first step in a two-step reaction which will create a highly thiolated product. Native gelatin has an approximate primary amine content of 300 µmol of amine per gram of polymer (PBgelatins, 2009). Native gelatin contains a high level of free carboxylate groups, approximately 1000 µmol/g (PBgelatins, 2009). The amination of gelatin will convert these carboxylate groups to amide bonds, which contain primary amines as side chains, by reaction of native gelatin with the amine, ethylene diamine, in the presence of a carbodiimide coupling reagent, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC).

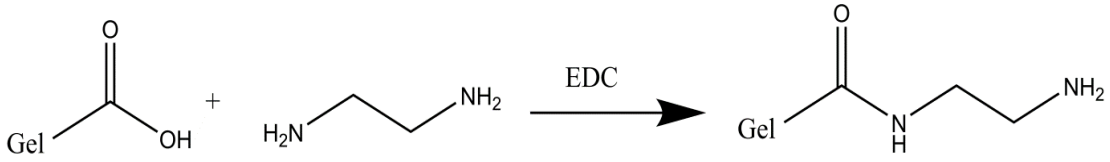

Figure 3.1 Amination reaction of gelatin with ethylene diamine in the presence of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) (Wang *et al.*, 2000)

EDC, the structure of which is shown in Figure 3.2, is a water-soluble compound which facilitates the amination reaction in the absence of an organic solvent (Hermanson, 2008). EDC and its isourea intermediate are both easily removed from the reaction media by dialysis. It is also classified as a zero-length crosslinker which allows for a direct linkage between the reaction components without the addition of a foreign structure into the polymer backbone (Kuijpers *et al.*, 2000). In the case of gelatin, this involves activating the carboxyl group to react with free amines producing an amide bond. Amide formation with EDC is highest at a pH range of 4 - 6.

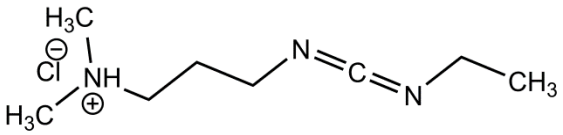

Figure 3.2 Structure of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

A commonly used amine in this type of reaction is ethylene diamine, due to the lack of hydrophobic interactions and minimal steric effects associated with it (Hermanson, 2008). Reaction of gelatin with an excess of ethylene diamine will ensure only one end of the amine will react with the carboxylate on the gelatin while also stopping the material from crosslinking (Hermanson, 2008). Kushibiki *et al.* (2006) conducted a study preparing gelatin hydrogels which were aminated using a variety of amines, including ethylene diamine. In this study, the hydrogels were inserted into mice and the inflammatory response initiated by the hydrogels was measured. This was done by enzyme-linked immunosorbent assay (ELISA) for interleukin-1β (IL-1β). It was observed that ethylene diamine did not initiate a significant inflammatory response in comparison to the control, saline. This was in contrast to the other three amines examined in the study (putrescine, spermidine and spermine) which initiated significantly higher levels of IL-1β than saline. In terms of drug delivery, the lack of inflammatory response by ethylene diamine is advantageous.

The amination process is, in theory, capable of increasing the free amine content to approximately 1300 μmol/g. Once thiolated, if 100% of the primary amines were

then converted to thiol groups, a highly thiolated product of 1300 µmol/g thiol content would be achieved. This is in comparison to a maximum thiol content of 300 µmol/g, which is achievable through the direct thiolation of native gelatin. Mucoadhesion improves with increased thiolation, as was observed with PAA and chitosan (Verena M. Leitner *et al.*, 2003; Palmberger *et al.*, 2007; Schmitz *et al.*, 2008; Dünnhaupt *et al.*, 2012; Iqbal *et al.*, 2012). Palmberger *et al.* (2007) showed that thiol content of the resulting thiolated PAA products influenced mucoadhesive properties and an approximate 2-fold increase in mucoadhesion was observed when thiol content was increased from 288.8 µmol/g to 767 µmol/g. Therefore, modification of gelatin, by first increasing the amine content of the polymer and then thiolating the aminated gelatin, could yield a highly thiolated product with the potential to have improved mucoadhesion.

To potentially improve the thiolation content further, gelatin will also be aminated with diethylene triamine, the structure of which is shown in Figure 3.3. The reaction of gelatin with diethylene triamine may result in an aminated product with higher amine content as the carboxyl groups could be converted to a compound containing two free amine groups as opposed to one, as is the case with ethylene diamine. This could potentially allow for double the thiol content once the aminated product has been reacted with Traut's reagent in the thiolation step.

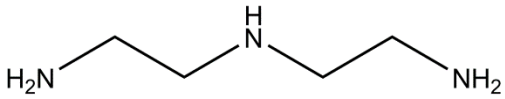

Figure 3.3 Structure of diethylene triamine

3.1.1.1 Thiolation using Traut's reagent

In the second part of the reaction process, thiolation is achieved by reacting the previously aminated gelatin with Traut's reagent (2-iminothiolane). Traut's reagent reacts with the amine group in a ring opening reaction forming the free thiol group (Hermanson, 2008), as shown in Figure 3.4.

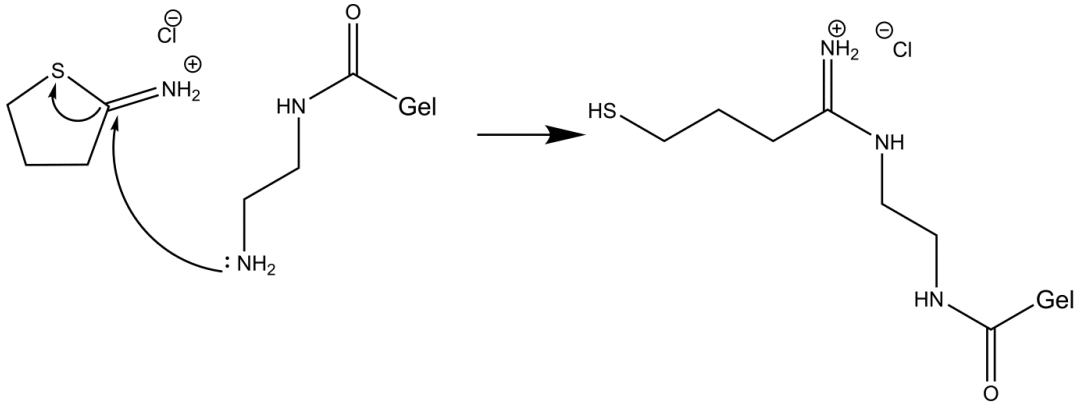

Figure 3.4 Reaction of aminated gelatin with Traut's reagent resulting in thiolated gelatin

Traut's reagent is most reactive with primary amines between pH 7 - 10. However, above pH 5 - 6, disulphide bond formation can occur. This could decrease the potential for mucoadhesion in the final product, as there will be fewer free thiols to bind to the cysteine rich domains of the mucin glycoproteins in the mucosal layer. Performing the reaction at pH 5 may ensure disulphide bond formation is kept to a minimum. However, Traut's reagent will be less reactive at this pH and the thiolation reaction may not go to completion. One way to combat this problem is to start the reaction at pH 7, where Traut's reagent will be most reactive, then after 20 min reaction time, lower the pH to approximately 5 to decrease the possibility of disulphide bond formation. As Traut's reagent is most efficient at pH 7 - 10, approximately 80% of the reagent will have reacted within 20 min (Hermanson, 2008). This method will ensure the reaction goes to completion while minimising the formation of disulphide bonds. Addition of a reducing agent may also combat the potential of S-S bond formation. A commonly used reducing agent in thiolation reactions is β-mercaptoethanol (Bernkop-Schnürch *et al.*, 2003). The addition of β-mercaptoethanol to the reaction solution will reduce any disulphide bonds which have formed during the thiolation reaction. Yoshitake *et al.* (1979) observed that the addition of EDTA during antibody reduction prevented the re-oxidation of sulfhydryls by chelating any metals present in the reaction which may initiate oxidation of the bonds. A 7% decrease in thiol groups was observed with the addition of EDTA in comparison to a decrease of 63 – 90% without EDTA addition. Additionally, dialysis of the thiolated product is conducted in acidic solution, as was the case with the dialysis of PAA in chapter 2. This will also minimise the formation of disulphide bonds during the dialysis process (Bernkop-Schnürch *et al.*, 1999).

Thiol content is measured by the Ellman's reagent method (Ellman, 1959). Ellman's reagent, (5,5'-dithio-*bis*-(2-nitrobenzoic acid), (Figure 3.5), reacts with free thiol groups on the gelatin backbone releasing a chromogenic compound, 5-thio-2-nitrobenzoic acid (Hermanson, 2008).

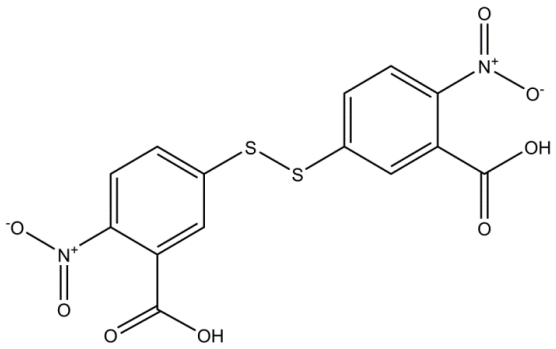

Figure 3.5 Structure of 5,5'-dithio-*bis*-(2-nitrobenzoic acid)

The downfall of this method is that it does not measure disulphide bonds and only measures free thiols as the reagent itself bonds to the free thiol groups creating a disulphide bond. The pH of the buffer required for the Ellman's assay is also relatively high at pH 8 and again, this can lead to unwanted oxidation reactions in the sample (Hansen *et al.*, 2007). So, as before, disulphide bonds which may have formed during the reaction process must be reduced in order to get an accurate measurement of the total thiol content of the gelatin product. In order to measure complete thiol content, a reducing agent, such as sodium borohydride, could be used to reduce any S-S bonds which may have formed throughout the reaction prior to analysis with Ellman's reagent. Alternatively, using other amine determining methods, ortho-phthalic dialdehyde (OPA) or 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) methods which are indirect methods, would give the amine content before and after thiolation; the difference indicating the level of amine thiolation (Vlierberghe *et al.*, 2011).

3.1.1.2 Aims and objectives

The overall aim of this study is to thiolate gelatin in order to increase its mucoadhesive properties. The thiolation reaction of gelatin is conducted in a two-

step process, the first step of which is aminating the native gelatin in order to increase the number of free amine groups on the gelatin backbone. The second stage of the process is thiolating this aminated gelatin, potentially producing a highly thiolated and mucoadhesive material in comparison to native, unmodified gelatin.

3.2 Materials and methods

3.2.1 Materials

Gelatin samples of varying molecular weights were kindly donated by Lapi Gelatine, Italy. Ethylene diamine was purchased from Fluka Analytical, Ireland. 2,4,6-trinitrobenzene sulfonic acid (TNBS), L-Alanine were purchased from Sigma Aldrich, Arklow, Ireland. 2-iminothiolane (Traut's reagent) was obtained from Soltec Ventures, USA.

3.2.2 Native gelatin

Gelatin gel strength is measured in terms of bloom strength, which also correlates to the molecular weight (MW) of the gelatin. Table 3.1 shows the list of gelatins used, their bloom strength and their corresponding MWs.

Table 3.1 Gelatin bloom strengths and corresponding molecular weights (MW)

Gelatin sample no.	Bloom strength (g)	Molecular weight (kDa)	Animal type
4567	210	40-50	Bovine
4568	278	50-100	Bovine
4569	108	20-25	Bovine
4571	299	50-100	Porcine

3.2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on all native gelatin samples, and also the aminated and thiolated gelatin samples of gelatin 4569, to ensure the bloom strengths of the native gelatin samples corresponded to true MW values.

A 15% separating gel was made by carefully mixing 1.875 M Tris-HCl (pH 8.8), stock acrylamide, D.I. water, 10% sodium dodecyl sulfate (SDS) and (10%) ammonium persulphate, as outlined in Table 3.2. The mixture was degassed and 5 µL of tetramethylethylenediamine (TEMED) was added to the solution with gentle stirring. The solution was added to a cassette and allowed to set. A 4% separating gel was also prepared by mixing and degassing 0.6 M Tris-HCl (pH 6.8), stock acrylamide, D.I. water, 10% SDS and (10%) ammonium persulphate, also outlined in Table 3.2. 5 µL of TEMED was also added with gentle stirring. The stacking gel was poured on top of separating gel and a comb was added to create wells in the gel. The solution was allowed to set.

Table 3.2 Components of separating gel and stacking gel for SDS-PAGE

	Separating gel (15%)	**Stacking gel (4%)**
1.875 M Tris-HCl, pH 8.8	2.0 mL	-
0.6 M Tris-HCl, pH 6.8	-	0.5 mL
Stock acrylamide	2.85 mL	0.68 mL
Water	5.0 mL	3.75 mL
10% SDS	100 µL	50 µL
Ammonium persulphate (10%)	50 µL	25 µL

Once the stacking gel had set, the comb was removed and the cassette was placed into an electrophoresis tank and covered by electrode buffer (0.05 M Tris, 0.348 M glycine, 0.1% SDS in 2 L water). A 5 mg/mL stock sample solution was made in sample buffer, components of which are shown in Table 3.3. The sample stock was diluted 1 in 2 and all samples, including a standard protein mix, were boiled for 5 min. Boiling of the samples ensured the unfolding of the protein strands and the break down of the secondary structure of the protein; this allowed for the complete

denaturation of the protein by SDS prior to analysis. The samples and standard protein mix were then loaded into the wells. Voltage was set at 200 V and electrophoresis ran for approximately 1.5 h.

Table 3.3 Components of the sample buffer, made up to 50 mL

	Sample buffer
0.6M Tris-HCI pH 6.8	5.0 mL
SDS	0.5 mL
Sucrose	5.0 g
Mercaptoethanol	0.25 mL
Bromophenol blue (0.5%)	5.0 mL

Once separated, the gels were stained whilst shaking gently for a minimum of 30 min using the Coomassie Brilliant Blue staining method (0.1% Coomassie brilliant blue dissolved in 50% methanol and 10% acetic acid). Once the staining was complete, destaining with 10% methanol, 7% glacial acetic acid was performed, again with shaking, for a minimum of 30 min or until the bands were clear.

3.2.4 Native gelatin amination

Aminated gelatin was prepared according to Seki et al. (2005). 1 g gelatin was dissolved with heating in 25 mL 0.1 M phosphate buffer (pH 5.02). Once dissolved, 3.14 mL ethylene diamine was added to the gelatin. The pH increased to approximately 13.5. The reaction was adjusted with HCl to pH 5. 0.5 g EDC was added. The reaction was left stirring for 24 h at room temperature. Control samples were carried out using the above method without the addition of EDC.

Samples were transferred to boiled 12 kDa dialysing tubing and were dialysed against distilled water in the dark, monitored by conductivity. Once dialysed, samples were frozen, powdered and freeze dried. Samples were stored at 4 °C, ready for analysis using the TNBS (2,4,6-trinitrobenzene sulfonic acid) method for amine quantitation (section 3.2.5) and further modification (section 3.2.6).

3.2.4.1 Investigation into excess concentration of ethylene diamine

Using gelatin 4567, the amination reaction was conducted as before, however, using the addition of 10%, 25% and 50% of the previously used volume of ethylene diamine. All samples were analysed using the TNBS method (section 3.2.5), and stored at 4 °C ready for thiolation (section 3.2.6).

3.2.4.2 Investigation into the influence of pH during the amination reaction

Using gelatin 4567, the amination reaction was conducted as before, with varying neutralisation pHs, namely pH 5, 5.5, 6 and 6.5. pH changes occurred prior to the addition of EDC. All samples were analysed by the TNBS method (section 3.2.5), prior to thiolation (section 3.2.6).

3.2.4.3 Investigation into unbound amine content

To investigate whether unbound amine remained within the samples after dialysis, a solution of aminated gelatin was filtered using 3 kDa centrifugal filters. In theory, the aminated gelatin would remain in the filter as the cut-off molecular weight is low, and any unbound amine would pass through the filter and remain in solution. Therefore, by testing the filtrate with the TNBS method, the quantity of unbound amine would be found. Solutions of 1 mg/mL were made up in water. The solutions were spun at 4000 rpm for 1 h and then the filtrate was analysed using the TNBS method (section 3.2.5).

3.2.4.4 Amination with diethylene triamine

Amination of gelatin 4569 was conducted at pH 5, replacing ethylene diamine with 5.34 mL of diethylene triamine in the presence of EDC.

3.2.5 2,4,6-trinitrobenzene sulfonic acid (TNBS) method

The TNBS method was followed according to Hermanson (2008) and Habeeb (1966). Standards of L-Alanine and gelatin samples were made up in 0.1 M sodium bicarbonate (pH 8.5). 250 µL 0.01% (w/v) TNBS was added to 500 µL

sample/standard and was incubated for 2 h at 37 °C, after which 250 µL 10% SDS and 125 µL 1 M HCl were added. Samples were analysed at 410 nm on the UV-Vis spectrophotometer (Shimadzu UV-2401PC).

3.2.6 Gelatin thiolation

Thiolated gelatin was prepared according to Kommareddy and Amiji (2005) and Bernkop-Schnürch *et al.* (2003). 200 mg of aminated gelatin/native gelatin was dissolved with heating in 20 mL D.I. water. A 2-fold molar excess of Traut's reagent was added to the solution. pH was adjusted with 1 M NaOH to pH 7 and the solution was left stirring gently at room temperature for 20 min. After 20 min, pH had fallen slightly to approximately 6.8. pH was adjusted to 5 and left for 24 h gently stirring at room temperature.

Thiolated samples were again dialysed in 12 kDa dialysis membranes, initially against 5 mM HCl, followed by 1 mM HCl and finally 5 mM HCl again. Samples were then frozen and freeze dried before thiol content analysis using Ellman's reagent. Samples were stored at 4 °C.

3.2.7 Ellman's Reagent solution

Thiol content determination was conducted using Ellman's reagent solution, as outlined in chapter 2, section 2.2.3. A 1 mg/mL solution of unmodified gelatin in D.I. water was prepared as a reference standard.

3.2.7.1 Determination of disulphide bond formation

Reduction of disulphide bonds was completed according to the method stated by Hermanson (2008). A 1 mg/mL solution of gelatin in water was made. The pH of the solution was changed to pH 8 to optimise the activity of $NaBH_4$. $NaBH_4$ was added slowly to create a final concentration of 0.1 M. The solution was incubated at room temperature with gentle stirring for 1 h. The pH was then changed to pH 4 with 1 M HCl to quench the $NaBH_4$ reduction and was further incubated for another 10 min at room temperature to ensure the complete removal of $NaBH_4$. The samples were then analysed using Ellman's reagent method.

3.3 Results and discussion

3.3.1 Gelatin amination

3.3.1.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Amination of gelatin was conducted on four samples of varying bloom strengths ranging from 108 – 299, correlating to a MW range of 20 – 100 kDa, as indicated in Table 3.1. The amination reaction was completed by reacting gelatin with ethylene diamine in the presence of EDC, after which the reaction solution was dialysed against distilled water. Initial amination experiments resulted in a large and variable loss of product after dialysis regardless of the MW of the gelatin used. Table 3.4 shows the percentage yields of amination reactions of gelatins 4567 and 4569 at pH 5, highlighting the low and variable yields achieved.

Table 3.4 Percentage yields of amination reactions of gelatin 4567 and 4569, both aminated at pH 5

Yield (%)	
Gelatin 4567	**Gelatin 4569**
23.6	41.0
22.8	8.5
19.6	12.8
28.7	19.5
37.6	18.6

Native gelatin samples have a water content of approximately 10 - 15% which would account for some loss after freeze drying. However, total yield losses were far greater with up to over 90% loss observed, as is indicated in the Table 3.4. A 12 kDa cellulose membrane was used for dialysis and all gelatin samples were above 20 kDa in size, so product loss should have been minimal. Because of the product loss observed during the reactions, SDS-PAGE was run on all native and aminated samples to ensure the stated MW of the gelatin samples matched their actual MW. An example of a SDS-PAGE gel of gelatin 4569 is shown in Figure 3.6. Gelatin 4569 has a MW of 20 - 25 kDa. Results showed large bands, suggesting the gelatin

contained a heterogeneous mix of MW, ranging from as low as approximately 14.4 kDa to above 97 kDa, as shown in Figure 3.6. This was observed in each gelatin bloom strength band. Aminated samples showed similar band streaks to the native gelatin, indicating no degradation of polymer after the amination reaction. As the MW varied over a wide range, and also close to the 12 kDa cut-off point of the membrane, it was possible that the gelatin product was dialysing through the membrane and was then removed from the reaction. This may explain why product loss was variable.

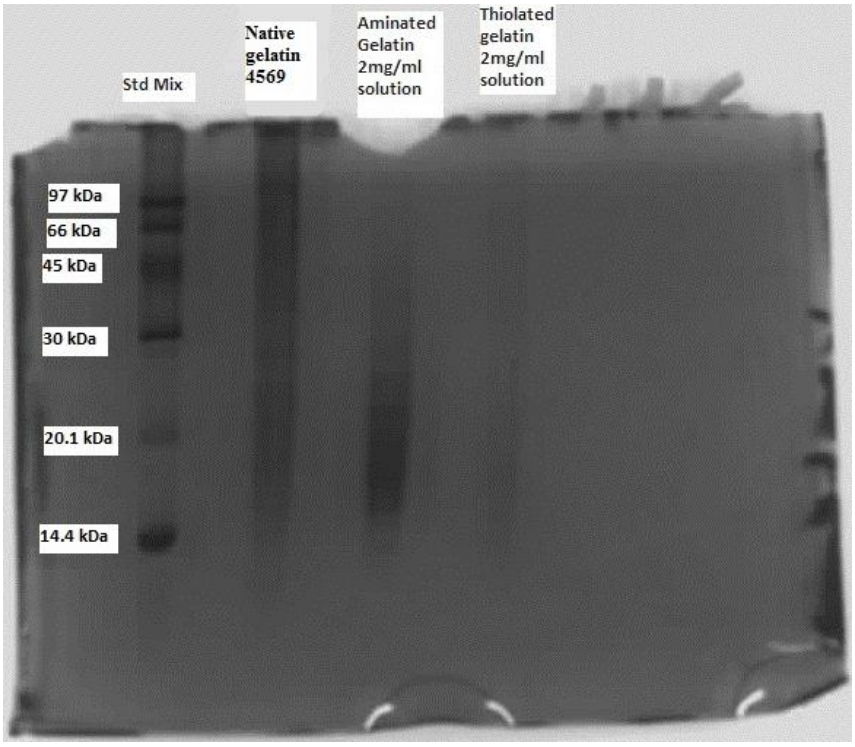

Figure 3.6 SDS-PAGE of native 4569, aminated 4569 and thiolated 4569

3.3.1.2 Conductivity

Dialysis of the aminated products was an integral part of this reaction process, occurring after both the amination and thiolation steps. In the amination reaction, the TNBS method was used to quantify the levels of amination but this method does not distinguish between bound and unbound amines; if unbound amines were present in the final aminated product, it could have negative effects on the thiolation process. Amine content was directly related to the amount of thiolating compound added, which was added in a 2-fold molar excess to ensure complete thiolation of the

polymer. Therefore, if any unbound amine remained in the sample, the thiol content would in turn be affected, as thiolation of both bound and unbound amine would occur. However, the unbound compound would then be removed by dialysis after thiolation. This then could mean insufficient quantities of Traut's reagent were added to ensure complete thiolation of the bound amines on the polymer backbone, therefore, reducing the thiol content of the final product. Similarly, any unbound free thiol groups remaining in the polymer matrix after dialysis could disrupt mucoadhesion (Verena M. Leitner *et al.*, 2003); this will be discussed in greater detail in chapter 4, section 4.3.2. To combat this and to ensure the complete removal of unbound amine, the dialysis solution of the aminated samples was monitored by conductivity. Figure 3.7 shows an example of the conductivity profile of the amination reaction, which was typical for all samples. After 24 h dialysis, the majority of unbound amine had been removed. The conductivity stabilised for 3 – 4 days and, after 5 days of dialysis, conductivity had reached a minimum. Morimoto has conducted a range of experiments aminating gelatin with ethylene diamine (Seki *et al.*, 2005; Wang *et al.*, 2000; Seki *et al.*, 2006). Throughout these papers, both reaction times and dialysis times were minimal, with dialysis times often being stated as 24 h. The amine content reported in the literature was also variable; although the same method was used, amine contents were often dissimilar ranging from 301 µmol/g (Wang *et al.*, 2001) to 1627 µmol/g (Wang *et al.*, 2002). As was observed in this study, dialysis times require a minimum of 5 days in total to ensure complete removal of unbound amine groups from the product; it may be that shorter dialysis times resulted in the varying amination results observed by Morimoto.

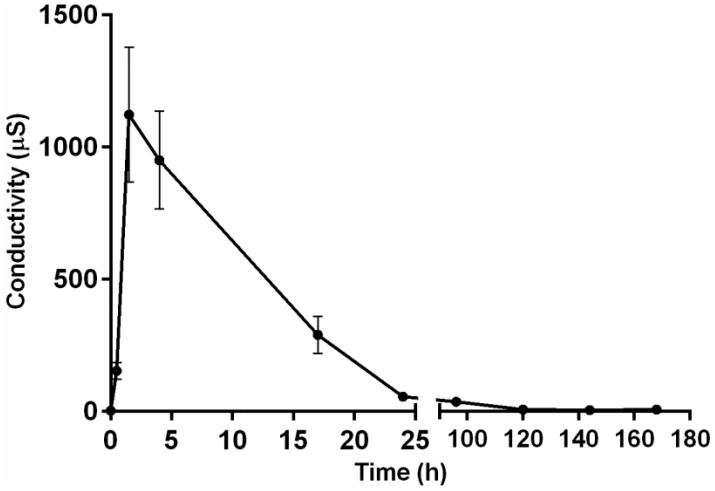

Figure 3.7 Measurement of conductivity during dialysis of aminated gelatin 4567, pH 5 (n=2)

3.3.1.3 Gelatin amine content

Following the literature (Seki *et al.*, 2005), initial amination was conducted using gelatin 4569 (see Table 3.1 for bloom strength and MW information). Using the same method, Seki *et al.* (2005) aminated two gelatin samples with MWs of 100 kDa and 5 kDa. The resulting amine content was higher in the 5 kDa sample, measuring 1020 µmol/g in comparison to 810 µmol/g in the 100 kDa. As increased amine content would be more advantageous in terms of final thiol content, the lowest MW gelatin was initially aminated in this study. During the amination reaction, gelatin was dissolved in a phosphate buffer at 50 °C; the increased temperature was required to dissolve the gelatin into solution. Van den Bosch and Gielens (2003) discussed the degradation patterns of gelatin at elevated temperatures using different buffered solutions; different salts had the potential to initiate the unfolding of the gelatin protein in solution and also enabled water to attack the bonds by influencing the hydrolytic nature of water. It was noted that both the concentration of the gelatin solution and the vessel used (both shape and material) had an impact on the degradation of gelatin. Degradation was also thought to occur when temperature was increased or with an increase in pH. Temperatures ranged from 30 – 70 °C and gelatin began to degrade more quickly at higher temperatures. Keeping this in mind, in this study, dissolving gelatin at 50 °C was necessary but it may have initiated degradation of the protein.

In the literature, gelatin was aminated at 37 °C (Wang *et al.*, 2000; Wang *et al.*, 2002; Seki *et al.*, 2005). Reaction times were 1 h and resulting amine content ranged

from approximately 800 - 1600 µmol/g. In this study, preliminary amination reactions using gelatin 4569 were conducted at two different temperatures: at room temperature (18 °C ± 2 °C) and at 37 °C. The amination reaction conducted at 37 °C resulted in a much lower amine content with approximately half the value of the room temperature sample, as shown in Figure 3.8. This contradicts the results obtained by Seki *et al.* and Wang *et al.*, both of which measured markedly higher amine content in the samples aminated at 37 °C (800 - 1600 µmol/g, respectively). Because of this, it was decided to conduct the amination reaction solely at room temperature for all gelatin MW samples. The yields of the samples aminated at 37 °C were comparable to those aminated at room temperature.

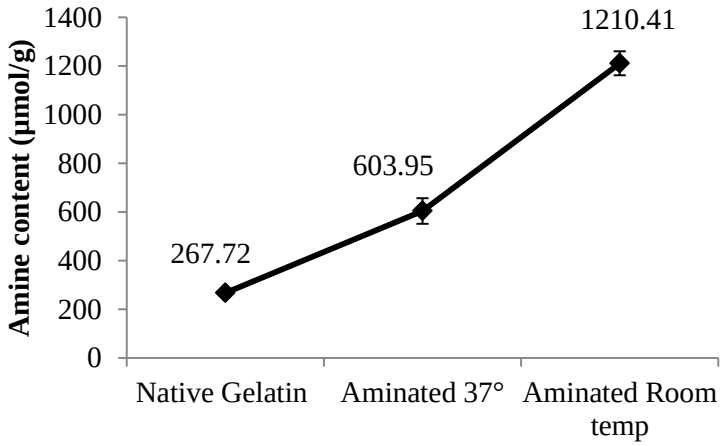

Figure 3.8 Amine content of gelatin 4569 in its native state and aminated at both 37 °C and room temperature (n=3)

Additionally to temperature changes, the reaction time was also investigated. The amination of gelatin 4569 reacted at room temperature at pH 5 was conducted for 2, 6, 18 and 24 h. As mentioned above, both Wang *et al.* and Seki *et al.* reacted gelatin with ethylene diamine for 1 h with resulting amine content of 800 – 1600 µmol/g. It was observed in this study that aminating gelatin for a minimum of 18 h was ideal, as the amine content of the samples aminated for 2 and 6 h were above the theoretical yield of amine content of 1300 µmol/g and measured between 1450 – 1700 µmol/g. It was thought that with reaction times of 2 and 6 h, the amination reaction had not gone to completion and unbound amine was also measured by the TNBS method. Dialysis times of these 2 h and 6 h reaction samples may have

needed to be increased to ensure complete removal of unbound amine. As this was the case, reactions were conducted for 24 h.

Amination of gelatin 4569 showed a high conversion rate of carboxylates in the aminated sample in comparison to both the control sample and native gelatin, as shown in Figure 3.9. The aminated sample had high levels of amination, with a 4.5-fold increase in amine content in comparison to native gelatin. However, product yields were variable and were often very low, with a loss of product after dialysis and freeze drying of up to 90% on more than one occasion using the lowest MW gelatin 4569, as was shown in Table 3.4.

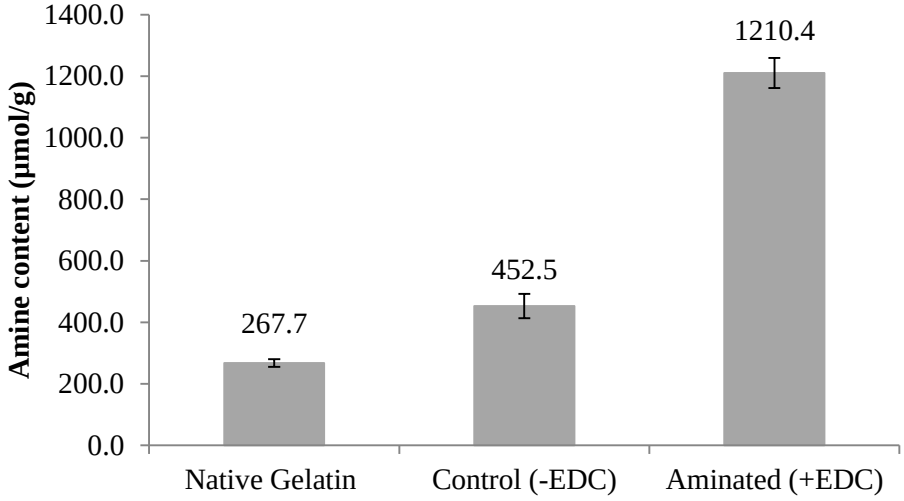

Figure 3.9 Amine contents of native (unmodified) gelatin 4569, and gelatin 4569 control and aminated samples, aminated at pH 5 with and without addition of EDC (n=3)

The control sample was conducted by reacting native gelatin with ethylene diamine without the addition of EDC. This meant the carboxyl groups of the gelatin were unable to react with the ethylene diamine, and thus amine levels should be similar to that of native gelatin. The control sample had slightly higher amine content measuring 452.5 µmol/g in comparison to the native sample (267.7 µmol/g), as shown in Figure 3.9, and it was thought that free, unbound amine was trapped within the gelatin matrix resulting in a higher amine content. The general structure of gelatin contains repeating units of glycine-X-Y, where X is often proline and Y is often hydroxyproline. It may be that electrostatic interactions, such as hydrogen bonding, between the amino acid residues of gelatin and the amine groups of the

unbound ethylene diamine trapped the unbound amine compound and caused the increase in amine content observed in the control sample. This was a trend which was seen across all control samples in this study; however, it has not been discussed in the literature of aminated gelatin as control samples were not included in the reactions (Wang *et al.*, 2002; Kushibiki *et al.*, 2006; Seki *et al.*, 2005).

As the lowest MW sample, gelatin 4569, was initially aminated, it was thought that a higher MW sample may be of more benefit for two reasons: firstly, from a product loss perspective as a higher MW gelatin would be less likely to dialyse through the 12 kDa membrane, and secondly, from a level of gelation, as higher bloom strength gelatin would be more viscous and more gel-like (PBgelatins, 2009) with greater potential for mucoadhesion once thiolated. Amination of all gelatin samples was conducted at pH 5; however, product loss was still high, with losses up to 50% despite the higher MW. As high levels of product loss was observed throughout the dialysis process, the dialysis solution was freezed dried in order to assess whether, and how much, gelatin had escaped from the 12 kDa dialysis membrane. Dialysis was conducted over a period of 7 days. The dialysis solution was freeze dried and gelatin residue was observed within the beaker after freeze drying. A mass balance experiment was conducted and it was concluded that over this time period, and together with the moisture content of native gelatin itself, the loss of gelatin product through the dialysis membrane was accounted for.

Although dialysis was monitored by conductivity in order to ensure unbound amine was removed, high and unrealistic levels of amine content were observed. Theoretical amination values are ~1300 µmol/g amine; however, values of 2000 - 4000 µmol/g were measured for gelatins 4567, 4568 and 4571, as shown in Figure 3.10. Gelatin 4569 amination was repeated resulting in an amine content of 2936.4 µmol/g (Figure 3.10), which was over 2 times that of the initial value which measured 1210.4 µmol/g (Figure 3.9). As the dialysis conductivity of these samples was monitored, it may be that the TNBS method of amine quantification was unreliable.

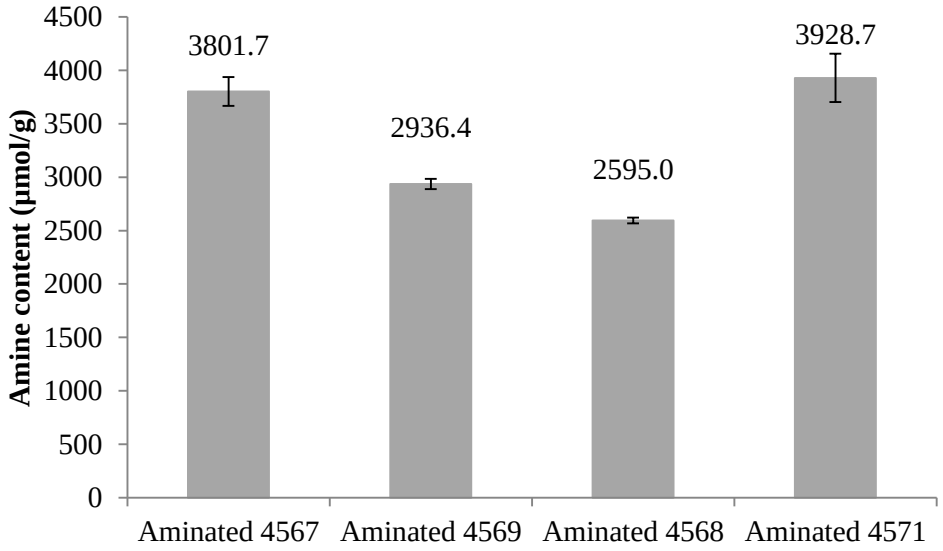

Figure 3.10 Amination of gelatin 4567, 4568, 4569 and 4571 (n=3)

Native samples were also analysed for amine content, results of which are shown in Table 3.5. The amine results were again higher than previously measured, with the amine content of the unmodified, native 4569 sample increasing from 267.7 µmol/g (Figure 3.9) to 314.4 µmol/g (Table 3.5). Again, the increase in amine content in the native samples highlights the problems associated with the TNBS method and indicates that the method of analysis may be the problem. Increased and varying levels of amine content in native gelatin samples have been observed in the literature with values ranging from 866 µmol/g measured by Wang *et al.* (2000) to 255 µmol/g measured by Seki *et al.* (2006). This suggests similar problems which were encountered in this study with the TNBS method were observed in both studies, however, it was not highlighted or discussed in either paper. Secondly to this, problems such as precipitation within the TNBS assay occurred. The TNBS solution was changed from a 0.01% solution to a 0.05% solution. TNBS concentrations varied in the literature, ranging from 0.001% w/v (Wang *et al.*, 2000) to 0.1% w/v (Seki *et al.*, 2005) and sometimes was not stated at all (Wang *et al.*, 2002). Again, the change in concentration was not discussed. Due to varying results obtained by the TNBS method, a second assay method for the determination of amines was tested in this study; the fluorescent assay using the reagent o-phthalaldehyde (OPA) was used (Go *et al.*, 2008). However, this method was also found to be unreliable. As both yields and amine content were inconsistent, a variety of investigations were

conducted in order to optimise the reaction process, details of which are discussed in the following sections. All reaction times remained at 24 h.

Table 3.5 Amine content of unmodified, native gelatin samples (n=2)

Sample	Amine content (μmol/g)
4567	316.7 ± 37.0
4568	314.4 ± 28.2
4569	352.9 ± 11.3
4571	334.2 ± 45.1

3.3.1.4 Ethylene diamine concentration

Seki *et al.* (2005) used a vast excess of ethylene diamine in the amination reaction. According to Hermanson *et al.* (2008), a 10-fold excess of ethylene diamine is required for this reaction to ensure that crosslinking does not occur and that only one end of the diamine couples to each carboxylate on the polymer. However, Seki *et al.* (2005) used up to 40 times the molar excess. It was thought that the loss of product during dialysis may be due to the degradation of gelatin caused by the large excess of ethylene diamine added to the reaction vessel. Using gelatin 4567, a mid-range bloom strength gelatin (see Table 3.1), the effect ethylene diamine concentration had on amination levels and on yields was investigated, results of which are shown in Figure 3.11. Percentage concentrations used in this experiment were relative to the concentration used by Seki *et al*. and the reaction was conducted with 50%, 25% and 10% of the initial ethylene diamine concentration.

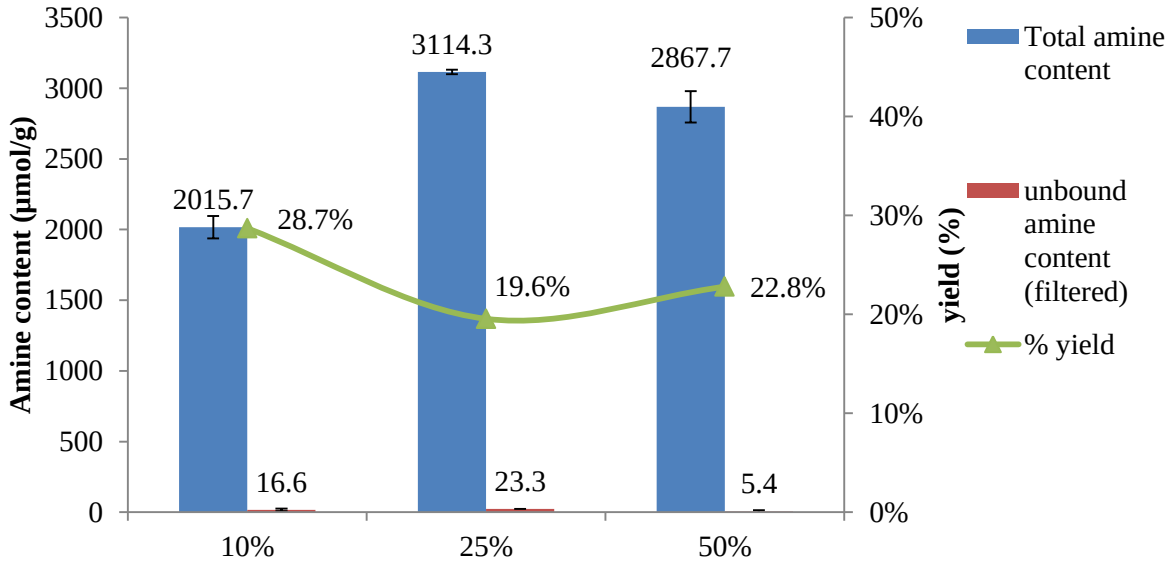

Figure 3.11 Gelatin 4567 samples aminated at different concentrations of ethylene diamine (n=2)

Again, amination levels were theoretically too high across all samples, measuring between 2000 – 3200 µmol/g. As amine content was higher than theoretical values, in addition to conductivity measurements during dialysis, the aminated samples were also centrifugally filtered using a 3 kDa centrifugal filter unit in order to measure any unbound amine remaining in each sample. As the unbound amine is a smaller compound, it passes through the 3 kDa centrifugal filter whereas the amine bound to the polymer will not. Therefore, by analysing the filtrate with the TNBS method the concentration of unbound amine can be measured thus confirming the action of dialysis. Measurements of unbound amine within the ethylene diamine experiment samples are illustrated in Figure 3.11. Low levels, between 5.38 – 23.32 µmol/g, of unbound amine content were measured in all samples which, therefore, does not explain the high levels of amine content in the samples, thus highlighting the issues with the TNBS assay. Yields across all samples were again low regardless of the concentration of ethylene diamine added and there was not a significant difference in yield between samples. This suggests that the ethylene diamine concentration was not initiating degradation of the protein. As mentioned previously, a 10-fold excess of ethylene diamine is necessary for this amination reaction to avoid intramolecular crosslinking (Hermanson, 2008), therefore, increasing the excess of ethylene diamine to 40-fold, as conducted by Seki *et al.* (2005) may not be required.

3.3.1.5 pH studies

As ethylene diamine concentration had no effect on the amination reaction or on the level of gelatin degradation, the pH at which the reaction was conducted and its influence on the reaction was investigated. pH will affect the protonation state of ethylene diamine. Hajós (2002) discussed the interchange between non-protonated, mono- and di-protonated species due to influences by pH. Below pH 6, ethylene diamine is largely in a di-protonated state however, between pH 6 - 8, it can exist as mono-protonated, di-protonated or a mixture of mono- and di-protonated which may have an influence on both the amination reaction and also the degradation of the gelatin. Additionally to this, EDC efficiency is highest at pH 4 - 6 but can be effective up to pH 7.5 (Hermanson, 2008). To investigate whether the pH of the final reaction had an influence on the amination yield and reaction efficiency, reactions were conducted over a range of pH levels; i.e the pH at which the reaction was neutralised prior to the addition of EDC. As shown in Figure 3.12, pH had little influence on amination levels but it did affect the yield. An increase in yield was observed at higher neutralisation pH's, with a 1.8-fold increase in yield between pH 5 and pH 6.5. These studies were conducted using 100% ethylene diamine concentration and the level of unbound amine was observed to be higher in these samples than in the samples aminated with varying concentrations of ethylene diamine, ranging from 77.0 µmol/g in the pH 6.5 sample to 248.7 µmol/g in the pH 5 sample.

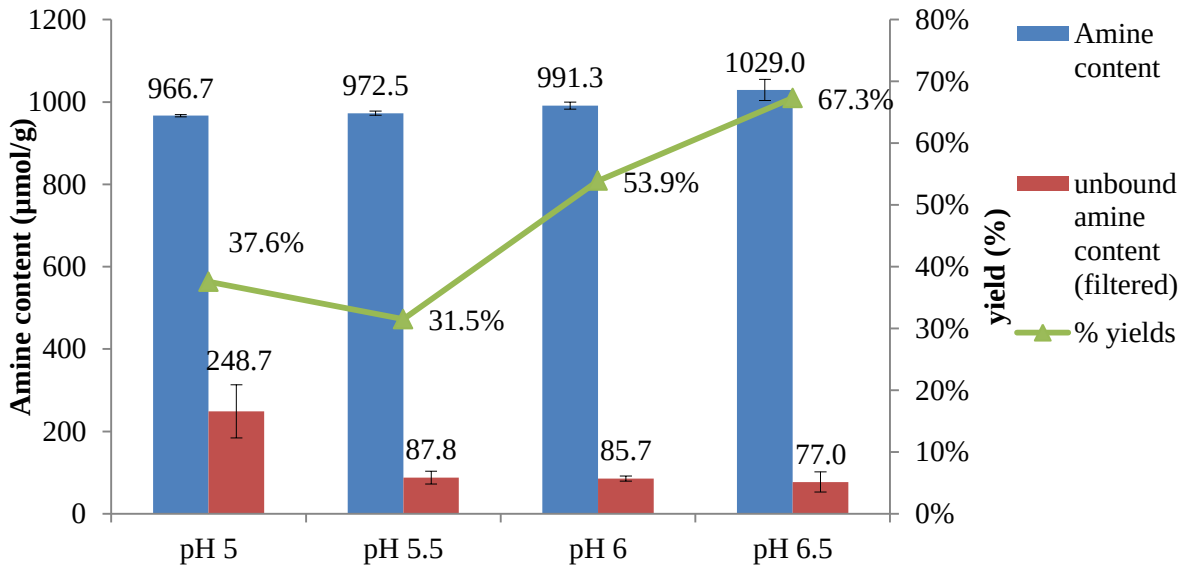

Figure 3.12 Gelatin 4567 aminated at varying pH levels (n=3)

MW has been highlighted as an important property in mucoadhesive polymers. Leitner *et al.* (2003) illustrated the importance of the MW had on the mucoadhesive and disintegration properties of thiolated PAA which had comparable thiol content but varying MW. A vast difference was observed in both the mucoadhesion and disintegration properties of each of the samples. Keeping this in mind and utilising the results obtained from the pH studies, gelatin 4569 was again employed as the gelatin of choice. Gelatin 4569 originally had promising results but poor yields (Figure 3.9), so by aminating at pH 6, which gave a good yield and good amination in gelatin 4567, instead of pH 5, there was potential to create a product with an equally high conversion rate of carboxylates to amine groups and a corresponding increase in yield. Amine content of gelatin 4569, aminated at pH 6 was high measuring 2680.94 µmolg/g, as shown in Figure 3.13. The amine content was again theoretically too high, and unbound amine content was low. Therefore, this does not explain the high levels observed. Although the amination reaction was conducted at pH 6, the percentage yield was again low, at 26.6%.

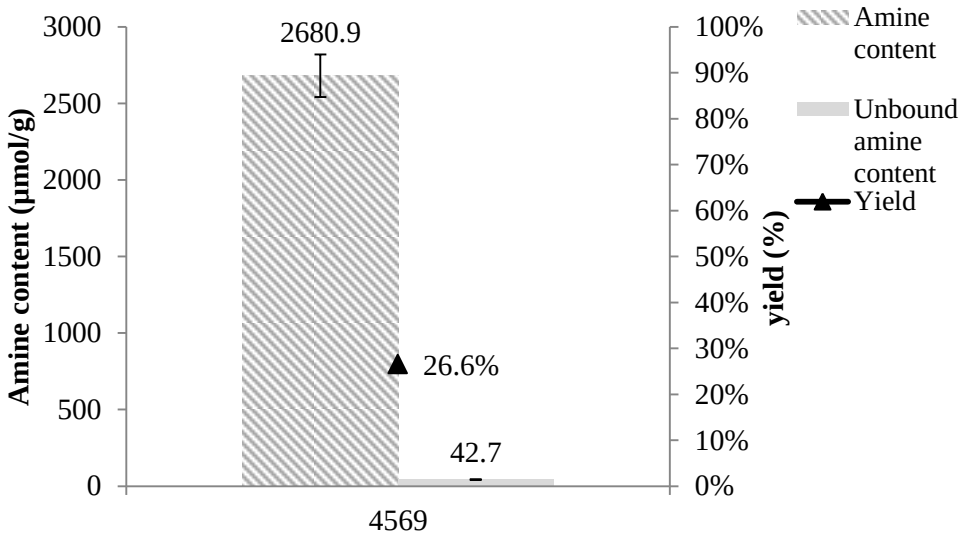

Figure 3.13 Amination of gelatin 4569 at pH 6 (n=2)

3.3.1.6 Amination with diethylene triamine

Amination of gelatin 4569 was conducted with diethylene triamine; the procedure was conducted as initially stated with ethylene diamine: at pH 5 and 100% compound added. The amination of gelatin with diethylene triamine could potentially increase the amine content of the product further as the carboxyl groups on gelatin could convert to an amide bond with two free amine groups attached in comparison to the amination reaction with ethylene diamine. The results of the aminated control and aminated sample are shown in Figure 3.14. The reaction with diethylene triamine resulted in an approximate 3-fold increase in amine levels, measuring 997.5 ± 273.0 µmol/g, in comparison to the control sample (355.1 µmol/g). As amination with diethylene triamine could form an amide bond which has two free primary amine groups, the amine content was unexpectedly low in comparison to the amine levels of the ethylene diamine reactions. The standard deviation was also higher with the triamine samples in comparison to the samples aminated with ethylene diamine. It is possible that steric hindrance influenced the amination reaction with the triamine; as the triamine compound is much bulkier than the diamine, the triamine may not have been capable of forming a bond with every carboxyl group along the polymer backbone due to space restrictions. This steric hindrance effect would not be seen in the ethylene diamine amination reaction and may explain why the amine content was higher.

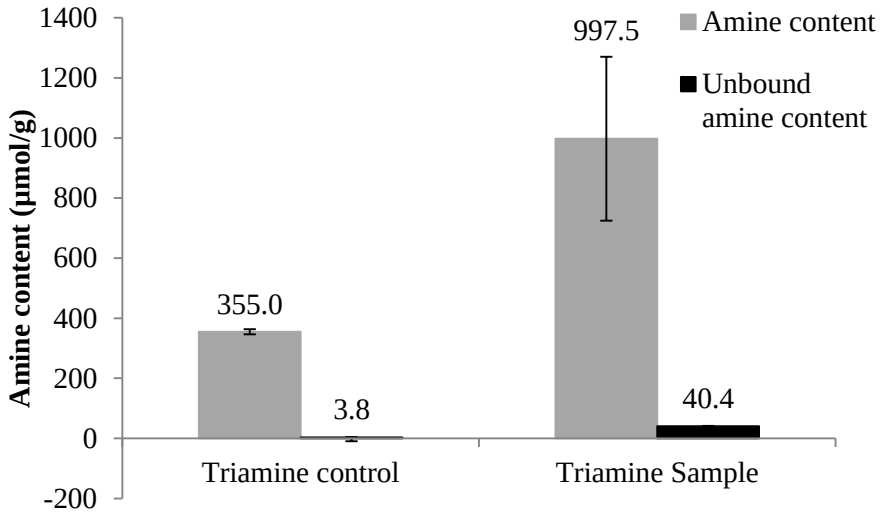

Figure 3.14 Amine content of gelatin 4569 aminated at pH 5 with diethylene triamine (n=2)

3.3.2 Gelatin thiolation

All samples were thiolated with Traut's reagent in a 2-fold molar excess. The same thiolation method was used for all samples regardless of the changes made during the amination reaction; therefore, a direct comparison can be made between samples.

Native gelatin, thiolated controls (aminated without EDC) and thiolated samples (aminated with EDC) of gelatin 4569 were compared and results are shown in Figure 3.15. As native gelatin contains amine groups along its backbone, it was also directly thiolated with Traut's reagent, designated as thiolated native, and resulted in a thiol content similar to that of the thiolated control sample. In the amination results, there was an increase in amine content in the control sample compared to the native sample (Figure 3.9). It was surmised this was free, unbound amine which was trapped within the gelatin matrix, therefore increasing the amine content. This did not transfer to or affect the thiol content and both the thiolated native and thiolated control samples had comparable thiol content, as shown in Figure 3.15.

When comparing the thiol content of the thiolated native/control samples to the thiolated sample which had undergone the two-step reaction process, a 10-fold increase in thiolation can be seen from approximately 60 µmol/g in the thiolated native/control samples to 663.2 µmol/g in the thiolated aminated sample; this reflects the initial results observed in the amination reaction step.

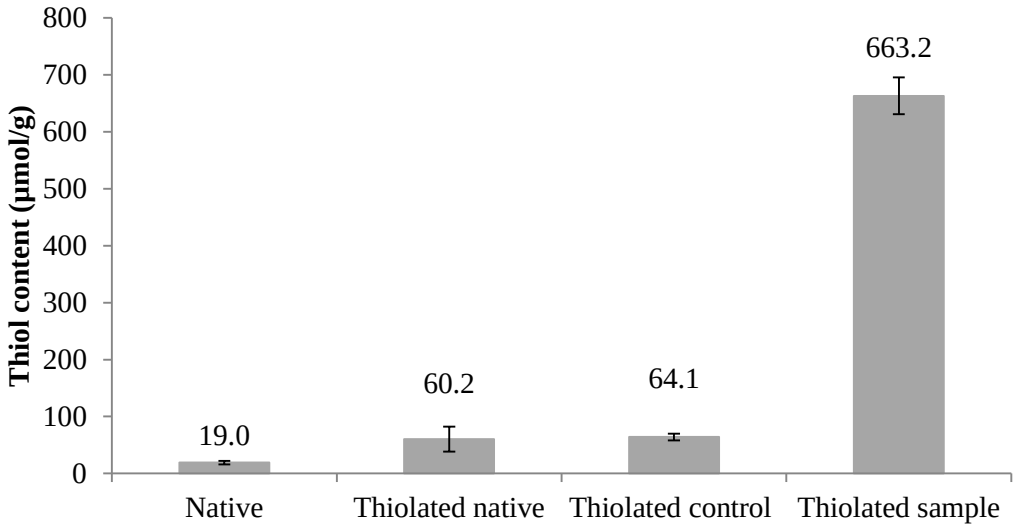

Figure 3.15 Gelatin 4569 thiolation, samples aminated at pH 5 (n=3)

The thiolation levels of gelatin 4569 aminated at pH 5 and thiolated were improved in comparison to controls samples; however, after thiolation, yields were low and variable which was similar to amination results, as illustrated in Table 3.6.

Table 3.6 Percentage yields of gelatin 4569 thiolation

Gelatin thiolation yield (%)
4.2
39
27.8
62
59

As gelatin with higher bloom strength form a stronger gel, are more viscous (PBgelatins, 2009; Rousselot, 2014) and could potentially be more mucoadhesive, other gelatin samples of higher MW were also thiolated. The high level of thiolation which was achieved using gelatin 4569, Figure 3.15, did not transfer to gelatins of differing and higher MW, all of which resulted in lower thiolation levels. The amination values for gelatin samples of varying MW were above the theoretical values, with levels measuring between 2000 - 4000 µmol/g. However, this did not

correlate to increased thiol levels, again suggesting the problems related to the TNBS method of amine detection. Figure 3.16 shows the thiol content of the two highest MW samples, gelatin 4568 and gelatin 4571 with MW of 50 – 100 kDa.

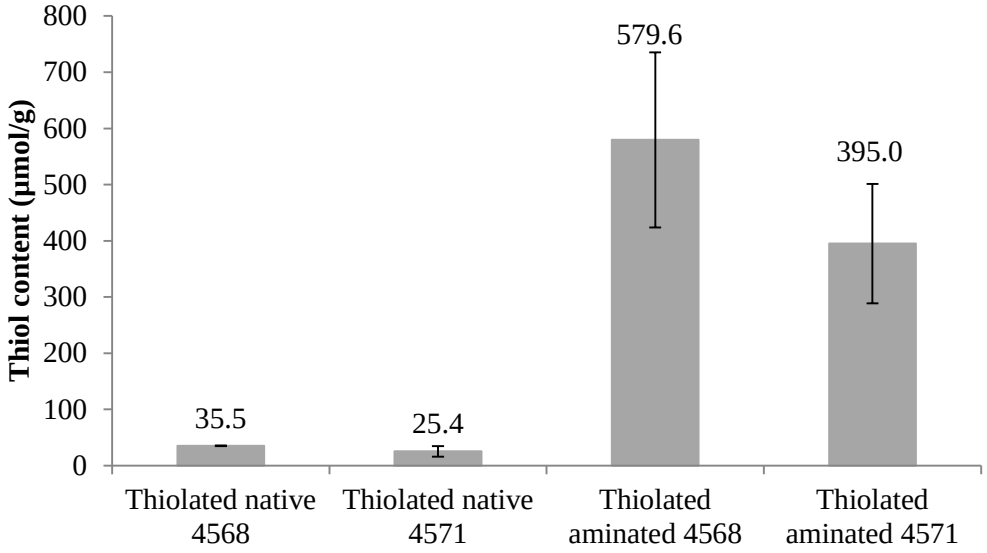

Figure 3.16 Thiol levels of gelatin samples 4568 and 4571 (n=3)

The thiolation levels of gelatin 4568 and 4571 showed improved thiolation after the two-step reaction process, with an approximate 10-fold increase in thiol content in comparison to their thiolated native counterparts, i.e. direct thiolation of native gelatin. The percentage yields of both the thiolated 4568 and 4571 were approximately 70%, which was an improvement in comparison to the low yields of the 4569 samples, as shown in Table 3.6. Gelatin 4569 showed excellent thiol content measuring 663.19 µmol/g (Figure 3.15). In comparison to this, there was a noticeable decrease in thiol content in both thiolated samples of 4568 (579.6 ± 155.9 µmol/g) and 4571 (395.0 ± 106.3 µmol/g), with up to half the measured thiol content in the case of gelatin 4571. This became a trend across all gelatin samples of differing MW. As was discussed in chapter 2, PAA has an optimal MW for mucoadhesion and cohesion; 450 kDa PAA displayed greater levels of thiolation and, in turn, improved mucoadhesion in comparison to PAA of lower or higher MW (V. M. Leitner *et al.*, 2003). In the case of gelatin, it may be that a lower MW gelatin - in this study, gelatin 4569 - is the more suitable material for use in mucoadhesion;

this will be discussed in more detail in chapter 4 where the mucoadhesive and cohesive properties of thiolated gelatin are examined.

3.3.2.1 Ethylene diamine concentration experiment samples

Gelatin 4567 was aminated using different concentrations of ethylene diamine and samples were then thiolated. As discussed previously, the amine content of these samples, shown in Table 3.7, was theoretically too high; however, similar to the thiol content of gelatin 4568 and 4571 samples (Figure 3.16), this did not translate into samples of high thiol content. Thiol content ranged from 273.7 µmol/g in the 10% sample to 515.1 µmol/g in the 50% sample, as shown in Table 3.7. Both sample yield and thiol content increased with increasing addition of ethylene diamine, suggesting conversion to amine groups was improved with the addition of higher ethylene diamine concentrations. Yields in this experiment were high, ranging from 57 – 72%.

Table 3.7 Thiolation of gelatin samples aminated with varying % ethylene diamine concentration (n=3)

Sample	Amination results		Thiolation results	
	Amine content (µmol/g)	yield (%)	Thiol content (µmol/g)	yield (%)
10%	2015.7	28.7	273.7	57.4
25%	3114.4	19.6	430.0	66.0
50%	2867.7	22.8	515.1	72.4

3.3.2.2 pH studies

Similar to the ethylene diamine concentration experiment, the gelatin 4567 samples aminated at different pH levels were also thiolated, results shown in Figure 3.17. The amine content of all four samples was comparable and, therefore, the same mass of Traut's reagent was added to each reaction vessel for thiolation. In theory, thiol contents should also be comparable, yet once thiolated, there was a marked difference in thiol content. Reacting at pH 6.5, the amine content was above 1000

µmol/g amine but once thiolated, the thiol content was low at 148.7 µmol/g thiol. Both reactions conducted at pH 5 and 5.5 had similar amine contents and once thiolated, had comparable thiol contents at 281.0 ± 13.8 µmol/g and 300.5 ± 12.3 µmol/g, respectively. Percentage yields across all four thiolated samples were comparable, at approximately 75%. The thiol levels of these samples were still lower than the initial reaction conducted with gelatin 4569 at pH 5 (663.2 µmol/g, Figure 3.2), with the pH 5 4567 sample having less than half the thiol content at 281.0 µmol/g. pH appears to have a strong effect on the amination and thiolation reaction results. After the amination reaction, the percentage yields of these samples would imply that a higher pH would be more advantageous. However, once thiolated, the thiol content of the lower pH samples was higher, thus amination at a lower pH of 5 or 5.5 appears to result in a thiolated material of higher thiol content and therefore, with more potential for a cohesive and mucoadhesive polymer. In the literature, gelatin was aminated in a phosphate buffer at pH 5, as described above (Wang *et al.*, 2000; Seki *et al.*, 2005; Seki *et al.*, 2006). No variations were made to pH and neither the pH of the reaction vessel nor product yields were discussed.

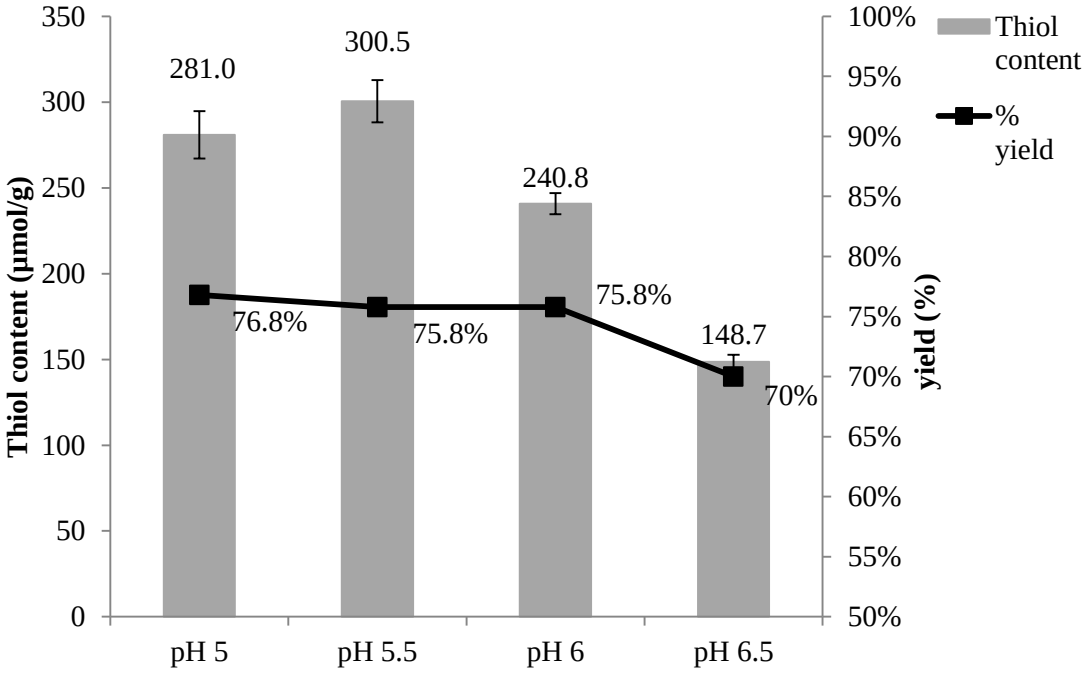

Figure 3.17 Thiolation levels of gelatin 4567 samples aminated at varying pH. (n=3)

Similar results were observed with the gelatin 4569 sample which was aminated at pH 6 (Figure 3.13) and was then thiolated, shown in Figure 3.18. The difference in thiol content between the thiolated control sample and the thiolated aminated sample is approximately 6-fold. A direct comparison of thiol levels between gelatin 4569 aminated at pH 5 and aminated at pH 6 can be made. Similar to the pH studies of gelatin 4567, the thiol content of this gelatin 4569 sample aminated at pH 6 (Figure 3.18) was again lower by approximately half in comparison to the sample aminated at pH 5 (Figure 3.15), measuring 257.8 µmol/g and 663.2 µmol/g, respectively. The amine content of the 4569 pH 6 sample was above theoretical values, measuring 2680.9 µmol/g amine. Although the conductivity of the sample was monitored, unbound amine may have been trapped within the polymer matrix. Therefore, when Traut's reagent was added for thiolation, it may have reacted with the unbound amine and have been dialysed from the reaction solution. This would affect the resulting thiol content of the product.

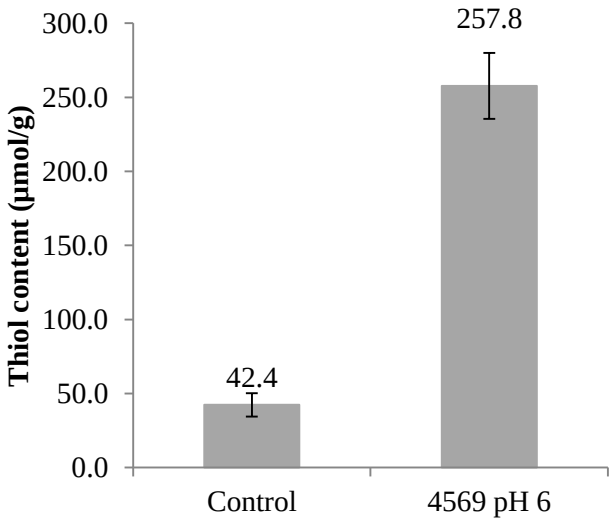

Figure 3.18 Thiolated Gelatin 4569 aminated at pH 6 and thiolated control (n=3)

3.3.2.3 Disulphide bond formation

In order to investigate the formation of disulphide bonds within the thiolated samples, the samples were reduced with $NaBH_4$ and analysed with Ellman's reagent. The difference in thiol content between the reduced and non-reduced samples indicates the disulphide bonds which may have formed in the sample during dialysis

and storage. The thiolation reaction was initially conducted at pH 7 but the pH was adjusted to pH 5 after 20 min in order to minimise disulphide bond formation. The efficiency of Traut's reagent is very fast (Jue *et al.*, 1978), and the reaction was not affected by this pH change. Secondly to this, the thiolated samples were dialysed in a 5 mM and 2 mM HCl solution, as conducted by Kommareddy and Amiji (2005), which again was to minimise oxidation of the thiol groups (Bernkop-Schnürch *et al.*, 1999).

The reduction of the thiolated samples in general revealed minimal disulphide bond formation across all samples. The thiolated pH 6 4569 sample (Figure 3.18) was reduced using $NaBH_4$, to measure disulphide bond formation within the sample. The disulphide bond content was low, measuring 7.1 ± 5.1 µmol/g. The occurrence of disulphide bond formation was minimal and, therefore, it can be concluded that oxidation of thiol bonds was not significantly occurring during the dialysis and storage period of the reaction process. Similarly, shown in Table 3.8, are the thiol content and disulphide bond content of gelatin 4567 samples, which had been aminated at varying pHs prior to thiolation. Disulphide bond formation was higher in these samples in comparison to the 4569 pH 6 samples, however, values were low, ranging from 37.6 to 87.6 µmol/g disulphide bond content. Although disulphide bond formation was low in all thiolated samples, both gelatins 4568 and 4571, the two highest MW samples, were observed to have lower levels of disulphide bond formation in comparison to gelatin 4569, with disulphide bond content measuring 7.1 ± 5.0 µmol/g in thiolated gelatin 4571. This may be due to the increase in chain length with the higher MW samples. Therefore, as the distance between thiol groups may have been greater, the potential for crosslinking within the sample may have been lessened.

The disulphide bond content of the thiolated gelatin samples were lower than that of the thiolated PAA samples in chapter 2, section 2.3.1.2. The thiolated PAA samples had disulphide bond contents ranging from 42.5 µmol/g to 166.6 µmol/g, with higher values measured in the pH 5 and pH 5.5 samples. As the thiol content was lower, this have resulted in a lower level of disulphide bond content, however, the thiolation reaction of gelatin was conducted at pH 7 for 20 min but the pH was then adjusted to pH 5 in order to minimise disulphide bond formation. This is in contrast to the

thiolation of PAA which was conducted at pH 5, pH 5.5, pH 6 or pH 6.5 throughout the reaction; no pH alteration was conducted in the PAA thiolation reaction.

Table 3.8 Thiol content and disulphide bond content of gelatin 4567 samples, aminated at varying pH and thiolated (n=3)

Sample	Thiol content (µmol/g)	Disulphide bond content (µmol/g)
pH 5	254.6 ± 73.7	61.9 ± 43.8
pH 5.5	340.3 ± 70.3	37.6 ± 26.6
pH 6	262.4 ± 79.4	87.6 ± 62.0
pH 6.5	257.2 ± 44.2	55.2 ± 39.1

3.3.2.4 Diethylene triamine

To investigate whether the thiolation levels of gelatin could be increased further, gelatin was aminated with diethylene triamine. The samples of gelatin 4569 which were aminated at pH 5 with diethylene triamine were then thiolated, the thiol content of which is shown in Figure 3.19, along with a sample which had been aminated at pH 5 with ethylene diamine and thiolated. As can be seen, the thiol content is lower in the sample aminated with diethylene triamine. As the amine content of the sample was lower than the sample aminated with ethylene diamine, it would follow on that the thiol content would be lower also. Again, steric hindrance may be a factor in this step of the reaction, as Traut's reagent is also a bulky compound and perhaps was incapable of forming bonds within close proximity to another along the backbone. However, the amine groups on the modified polymer would be at the end of a chain and would therefore be unhindered. It is possible that the reaction of Traut's reagent with diethylene triamine is less efficient than with ethylene diamine. Further analysis is required to investigate this by thiolation of both diamine and triamine compounds alone with Traut's reagent. As results were not promising using this ligand, it was decided that work would concentrate on sample aminated with ethylene diamine in the following chapter.

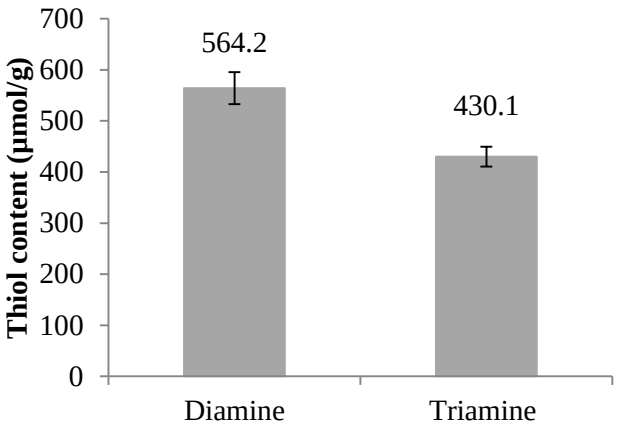

Figure 3.19 Thiolation levels of gelatin 4569 aminated with triethylene diamine, and a corresponding sample aminated with ethylene diamine (n=2)

3.4 Conclusions

In this study, a novel two-step reaction process was used in the synthesis of a thiolated gelatin product for mucoadhesive drug delivery. Four gelatin samples of differing MW were aminated and thiolated; their thiol content was measured and compared to the direct thiolation of their native gelatin counterparts. In general following the two-step reaction, thiol content was increased by up to 10-fold in comparison to thiolated native and control samples. The thiolated products were also reduced using $NaBH_4$ to investigate the disulphide bond formation, which was minimal in all thiolated samples.

Dialysis was an important step in the reaction process, occurring after both the amination and the thiolation steps of the reaction. Dialysis of the aminated samples was monitored by conductivity to ensure complete removal of unbound amine; the majority of unbound amine had dialysed from the reaction membrane after 24 h but a minimal conductivity value was not reached until day five. Therefore, after 24 h dialysis, unbound amine still remained within the polymer matrix. Gelatin amination within the literature showed amine content results that were varied and often higher than the results obtained in this study, however, both reaction times and dialysis times were shorter. The shorter dialysis times used in the literature could account for the higher amine content of the products in comparison to those synthesised in this study, as the unbound amine content would also be measured and included in the overall amine content value. As amine content in this study correlated to the amount

of Traut's reagent to be added for thiolation, it was vital that unbound amine was removed to ensure sufficient amounts of Traut's agent were available for thiolation of the polymer backbone.

When comparing the thiol content of thiolated PAA to thiolated gelatin, similar thiol levels were attained. However, when thiolating the synthetic polymer, a definite and reproducible range of thiol content was achieved, depending on the pH at which the reaction took place. Thiolation of gelatin resulted in often inconsistent thiol contents and low and variable product yields, a significant problem with natural polymers. Throughout this study, various investigations were conducted in an attempt to create a product with consistent thiol content and increase the product yield. The investigations into both the concentration of ethylene diamine added and the pH at which the amination reaction was conducted did not produce significant evidence for product loss nor did it lead to consistent thiol content. Aminating gelatin with a triamine compound also did not lead to an increase in amine content nor, consequentially, an increase in thiol content, however, this may have been due to steric hindrance along the polymer backbone reducing the ability of the triamine compound to react and bind to the polymer.

Polydispersity of MW is often observed within polymers, where there is a range of MW within a sample batch. However, when the samples were analysed by SDS-PAGE, it did show that the range of MW within the native gelatin samples was far greater than expected, ranging from approximately 15 kDa to 90 kDa in the 20 – 25 kDa sample (gelatin 4569). As MW has been shown to be important in mucoadhesion, demonstrated with PAA in chapter 2, this large polydisperity in MW of native gelatin may have a marked effect on the mucoadhesive properties of this protein. Proteins, such as gelatin, are likely to have inbuilt variability within them; the polymer chain contains a variety of different amino groups which are repeated throughout that chain. However, these units are not repeating moieties as they are, for example, in polysaccharides or indeed synthetic polymers which would have definite repeating units throughout the polymer chain. The amine content of the native gelatin samples showed variability when analysed with the TNBS method. Although amine levels were not significantly different between samples, there was a degree of change in the amine content measurement, which indeed may be due to the problems associated with the TNBS method or may be due to different

configurations of the amino acids in the four samples of gelatin resulting in varied levels of amine. The gelatin samples were aminated initially to convert carboxyl groups along the backbone to amide bonds which had free amine groups attached; these amine groups were then thiolated. With the inherent variability which may occur throughout the gelatin backbone, once thiolated, areas of high thiol content, or equally low thiol content, within the polymer matrix may have occurred. These pockets of thiol content could have an effect on both the cohesive and mucoadhesive properties of thiolated gelatin due to ability to form disulphide bonds; this will be discussed further in chapter 4.

Direct thiolation of gelatin using Traut's reagent has been conducted previously (Kommareddy and Amiji, 2005; Vlierberghe *et al.*, 2011). The thiolation of gelatin using a two-step approach has not been conducted before, nor have the mucoadhesive properties of thiolated gelatin been investigated prior to this study. A better understanding of the problems associated with natural polymers for mucoadhesion was also achieved.

Chapter 4 will discuss the cohesive and mucoadhesive properties of these novel thiolated gelatin products in comparison to native, aminated and thiolated native/control samples. As was seen in chapter 2, the increase in thiol content produced by the thiolation of a polymer has a significant and important effect on the cohesive and mucoadhesive properties in comparison to control samples. Although the yields and thiol contents were inconsistent, there was a significant increase of up to 10-fold in thiol content following the novel two-step reaction conducted in this study in comparison to the direct thiolation of native gelatin. The increase in degree of thiolation produced by the two-step reaction may have a marked influence on the cohesive and mucoadhesive properties of these novel thiolated aminated samples in comparison to direct thiolation samples. Polymers often have an optimal MW for mucoadhesion specific to that polymer; and as gelatin samples of varying MW were thiolated using this two-step reaction process, the impact MW has on the mucoadhesive properties of thiolated gelatin can also be investigated.

The thiolation of gelatin 4569 using the two-step reaction process resulted in a product with a thiol content of 663.2 µmol/g; this sample has the most potential for improved mucoadhesion and controlled drug delivery in comparison to its native

counterpart. This study has demonstrated an experimental process for the creation of a highly thiolated and potentially mucoadhesive gelatin tablet designed for the slow and controlled release of drug using a novel two-step amination/thiolation reaction; optimal levels of amination occurred at room temperature and at pH 5 and optimal thiolation levels occurred with a low MW gelatin, such as gelatin 4569.

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Chapter 4 Mucoadhesive properties and polymer characterisation of thiolated gelatin

4.1 Introduction

The cohesive and mucoadhesive properties and characterisation of native, aminated and thiolated gelatin will be discussed in this chapter. As mentioned in chapter 3, a natural polymer for use in mucoadhesive drug delivery has the benefit of biocompatibility, biodegradability and non-toxic effects. Using methods from chapter 2, direct comparisons between a well-established synthetic polymer used in mucoadhesive drug delivery, polyacrylic acid (PAA), and the natural polymer, gelatin, can be achieved. This will be of particular interest when comparing thiolated polymers of similar thiol content, as the properties of the synthetic and natural polymer may differ greatly. Similarly, the influence that molecular weight (MW) and thiol content has on the cohesive and mucoadhesive properties of gelatin will also be examined.

Both aminated gelatin and thiolated gelatin have separately been synthesised and used as drug delivery systems in the literature (Wang *et al.*, 2000; Seki *et al.*, 2005; Kommareddy and Amiji, 2005), all of which were discussed in detail in chapter 1, sections 1.8.5 and 1.9.1.2. However, little work has investigated the mucoadhesive nature of gelatin. Wang *et al.* (2001) explored the mucoadhesive properties of aminated gelatin microspheres. Gelatin was aminated with ethylene diamine in the presence of EDC, and the microspheres were then crosslinked with glutaraldehyde. The gelatin microspheres were tested for mucoadhesion using a mucin solution in which the extent of interactions between the mucin and the gelatin microspheres were measured by UV/Vis spectroscopy. Furthermore in an in-vitro experiment, the microspheres were also fluorescently labelled, suspended in a simulated gastric fluid and added into the excised stomach of a rat. The stomach containing the microspheres was incubated for 30 min at 37 °C, after which it was flushed with simulated gastric fluid for a further 30 min at a steady flow rate. Any remaining fluorescently labelled microspheres attached to the lining of the stomach were deemed mucoadhesive. Interactions between the mucin solution and the aminated microspheres were increased in comparison to native gelatin samples. Mucoadhesion was seen to increase by approximately 30% after amination. In-vivo experiments were also performed by feeding rats with a capsule of fluorescently labelled aminated microspheres. Two hours after ingestion, the rats were sacrificed, the stomachs were removed and as with the in-vitro experiment, the stomachs were

washed with buffer. Again, the remaining adhered microspheres indicated mucoadhesion, which was increased by up to 60% over unmodified gelatin.

Both Kommareddy and Amiji (2005) and Vlierberghe *et al.* (2011) discussed the synthesis of thiolated gelatin using Traut's reagent; the former investigated the thiolation of gelatin nanoparticles as a drug delivery system for DNA while the latter explored the potential of thiolated gelatin as a hydrogel material, both of which have been discussed at length in chapter 1, section 1.9.1.2. Both papers thiolated gelatin with Traut's reagent, however, neither paper used the two-step process of amination and thiolation. Mucoadhesion was also not discussed by either paper.

4.1.1 Aims and objectives

The aim of this study is to investigate the cohesive and mucoadhesive properties of thiolated gelatin samples of varying molecular weights and to compare them to both unmodified and thiolated control gelatin samples. Additionally, the cohesive and mucoadhesive properties of this natural polymer, which had undergone a two-step reaction process creating the thiolated material, will be compared to the synthetic and highly mucoadhesive polymer, PAA. Drug release studies of chlorpheniramine maleate (CPM) from the thiolated gelatin matrix and polymer characterisation will also be conducted.

4.2 Methods

4.2.1 Swelling studies

Swelling studies were conducted on native gelatin, aminated, control and thiolated gelatin samples according to the method outlined in chapter 2, section 2.2.4. Swelling studies were also conducted on aminated and thiolated gelatin samples aminated with varying ethylene diamine concentrations and at varying pH values.

4.2.2 Mucoadhesion testing

Mucoadhesive testing was conducted on native, aminated, control and thiolated samples using porcine gastrointestinal tissue according to the method outlined in chapter 2, section 2.2.5.

4.2.3 Drug release studies

Drug release studies were conducted on thiolated gelatin 4569 samples and thiolated control samples which had been aminated at pH 6. Studies were conducted as described in chapter 2, section 2.2.6.

4.2.4 Polymer characterisation

4.2.4.1 Scanning electron microscopy (SEM)

SEM imaging was conducted on aminated gelatin, thiolated gelatin and thiolated gelatin-CPM conjugates according to the method outlined in chapter 2, section 2.2.7.2.

4.2.4.2 Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) analysis

Samples were analysed using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) according to the method outlined in chapter 2, sections 2.2.7.3 and 2.2.7.4. All samples were analysed by TGA prior to DSC analysis; this gave the degradation temperature of the sample and the differential weight loss in relation to temperature. Samples for DSC were hermetically sealed and pin-holed to release water vapour. All samples were run on a heat-cool-heat-cool cycle, so that any water present would be released during the initial heating cycle. The samples were equilibrated at 0 °C, heated to 250 °C at a ramp rate of 10 °C/min, and then cooled to 0 °C, creating one cycle.

4.2.4.3 Isoelectric point (IEP) analysis

Isoelectric point titrations were conducted on all gelatin samples: native, aminated and thiolated. 1 mg/mL solutions were made in deionised water. The samples were titrated against 0.1 M NaOH and against 0.1 M HCl and the pH of the solutions was monitored. pKa 1 and pKa 2 were measured and the IEP of the gelatin samples was determined using Equation 4.1.

Equation 4.1

$$IEP = \frac{pKa\,1 + pKa\,2}{2}$$

4.3 Results and discussion

As discussed in chapter 3, various modifications to the amination reaction were conducted to investigate the effect that the concentration of ethylene diamine and the pH of the reaction had on the overall amine content, and subsequently, the thiol content. Gelatin gel strength is described in terms of bloom strength, with higher bloom strengths relating to higher MW but also to stronger gels. Gelatins of differing molecular weight (MW), as outlined in Table 4.1, were also analysed. The thiolation reaction was not altered and the same method was used, regardless of the modification to the amination reaction. Therefore, a direct comparison of cohesive and mucoadhesive properties can be made between samples.

Table 4.1 Gelatin bloom strength and molecular weight

Gelatin sample no.	Bloom strength	Molecular weight (kDa)
4567	210	40 - 50
4568	278	50 - 100
4569	108	20 - 25
4571	299	50 - 100

4.3.1 Swelling studies

Similar to chapter 2, section 2.2.4, the swelling studies were conducted in a 100 mM phosphate buffer (pH 6.7) at 37 °C. At this temperature, native gelatin may begin to dissolve. The modifications of the gelatin backbone through amination and thiolation may alter the swelling and cohesive properties, and at pH 6.7, thiol groups can form disulphide bonds, crosslinking the polymer and creating a more cohesive polymer (Bernkop-Schnürch *et al*.. 1999). The swelling properties of a polymer give an insight into the mucoadhesive properties, as over swelling of the polymer may lead to decreased adhesion and lack of cohesion may also inhibit mucoadhesion (Andrews *et al.*, 2009).

Native gelatin samples showed no cohesive properties or swelling abilities and were quick to dissolve, often within 15 – 20 s, in the 37 °C phosphate buffer. Aminated gelatin samples of differing bloom strengths were analysed and, as is shown in Figure 4.1, there was a linear trend in the swelling ability of the aminated samples. As the bloom strength, and therefore MW, of the samples increased so did the swelling abilities with the highest bloom strength/MW gelatins, 4568 and 4571, swelling to a greater extent than other low bloom strength/MW samples, gelatin 4567 and 4569. This suggests the potential a higher MW gelatin may have as a mucoadhesive polymer once thiolated, as its ability to swell will increase the surface area of the polymer allowing for more interactions between the polymer and the mucosal layer to occur (Andrews *et al*., 2009). However, it was observed in chapter 3 that thiolation levels were higher in gelatin 4569, the lowest MW gelatin.

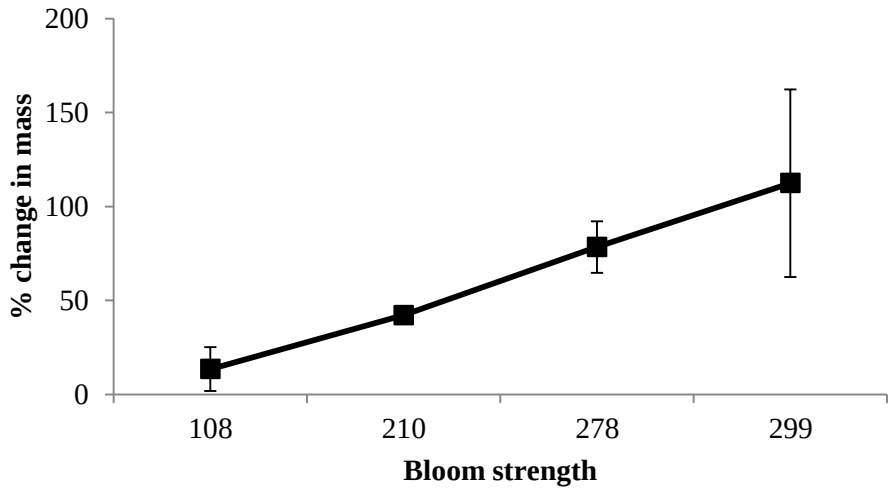

Figure 4.1 Swelling studies on aminated gelatin samples of differing bloom strengths (n=3)

Figure 4.2 shows the swelling abilities of gelatin 4569, which had been aminated at pH 5 and thiolated, resulting in a thiol content of 663.2 ± 32.3 µmol/g. The aminated control was prepared by treating the gelatin with ethylene diamine without the addition of EDC and its thiolated counterpart is described as the thiolated control.

The native, aminated and control samples had limited swelling abilities, all of which fell apart quickly within 3 min. This was in stark contrast to the thiolated sample which swelled to 300 times its original mass and, due to the potential formation of disulphide bonds at this pH, remained cohesive for a minimum of 3 h before it began to disintegrate in the phosphate buffer, pH 6.7 at 37 °C. The increase in cohesion and swelling ability upon thiolation of a polymer in comparison to unmodified and control samples was observed both with PAA in chapter 2, section 2.3.2 but also with other thiolated natural polymers; thiolated pectin with thiol content of 600 µmol/g (Sharma and Ahuja, 2011) and thiolated alginate with thiol content of 1561 µmol/g (Hauptstein *et al.*, 2015) both displayed a marked increase in swelling ability in comparison to the unmodified polymer samples. The thiol content of this thiolated sample after the two-step reaction process measured 663.2 µmol/g, which was a 10-fold increase in the thiol content in comparison to the thiolated control sample. As the swelling ability and cohesive properties of the polymers may influence mucoadhesion (Bernkop-Schnürch *et al.*, 1999; Andrews *et al.*, 2009), the improved cohesive properties of the thiolated 4569 sample shows great potential for the ability

of this thiolated material to adhere to a mucosal surface. Mucoadhesive studies will be discussed in section 4.3.2.

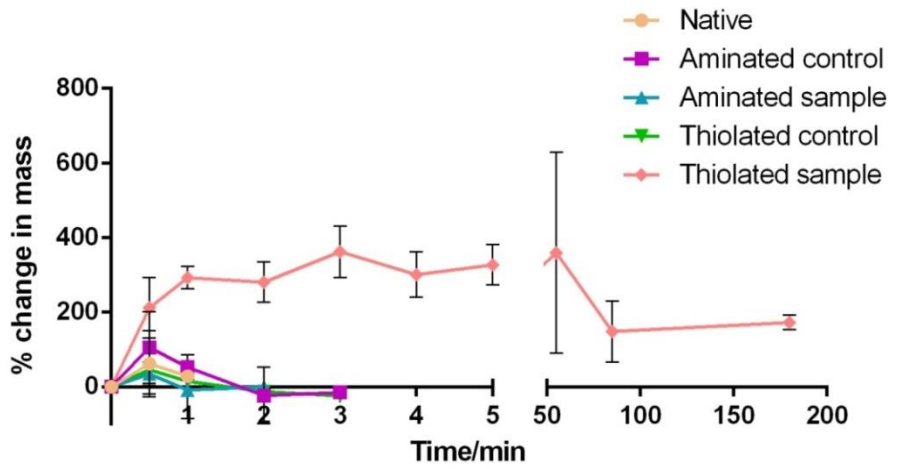

Figure 4.2 Swelling studies of gelatin 4569: aminated, control and thiolated samples. Thiol content of thiolated sample was 663.19 µmol/g (n=3)

The swelling abilities of the thiolated gelatin 4569 sample was compared to a thiolated PAA sample of similar thiol content (663.2 ± 32.3 µmol/g and 601.5 ± 45.3 µmol/g respectively), thus demonstrating the similarities and differences between the synthetic polymer, PAA, and natural polymer, gelatin (Figure 4.3). PAA was capable of swelling to a much higher capacity, with an increase in mass of up to 1700%, while gelatin swelled to its maximum capacity, approximately 300%, and stabilised within a number of minutes – this was in comparison to PAA which continued to swell over the 2 h analysis period. The structural properties of the two polymers are vastly different; native gelatin exists in a triple helix orientation due to electrostatic interactions between glycine, proline and hydroxyproline residues (Pratt and Cornely, 2011). Although modification through both amination and thiolation will have altered the structure of some of these residues, i.e. aspartic acid and glutamic acid, it may be possible that interactions between the glycine, proline and hydroxyproline residues prevented the gelatin sample from swelling to a greater extent. Similarly, as the carboxyl groups on the gelatin sample have been modified, the hydrophilic nature and swelling properties may have changed. This is different to PAA in which as little as 5 – 10% of the carboxyl groups along the PAA backbone

will have been modified through thiolation (Palmberger *et al.*, 2007); the hydrophilic nature of PAA will have been altered but to a lesser extent than that of gelatin.

Although the thiol content of the gelatin and the PAA samples were comparable, the MW of the samples may also have contributed to the different swelling abilities; PAA had a MW of 450 kDa whereas gelatin 4569 had a MW of only 20 – 25 kDa. This may have influenced the swelling ability of the samples, however, investigations into the swelling abilities of PAA of differing MW did not find a direct correlation between the swelling behaviour and its MW (V. M. Leitner *et al.*, 2003).

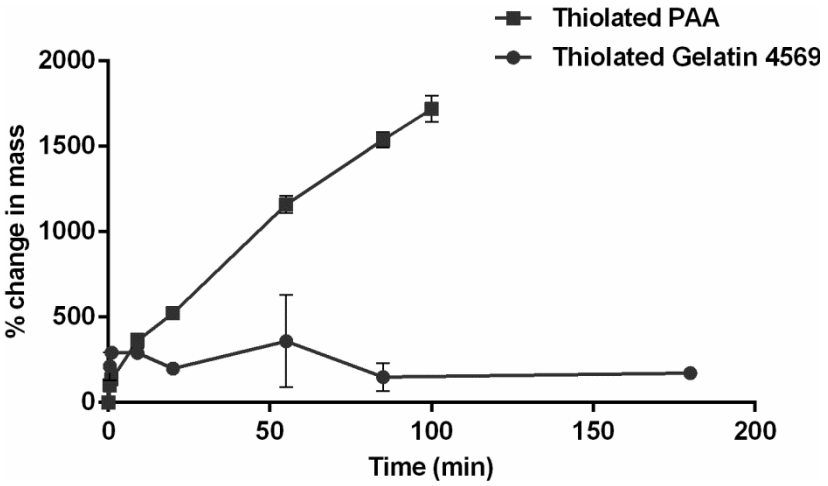

Figure 4.3 Swelling studies on thiolated gelatin 4569 and thiolated PAA of similar thiol contents (n=2)

The stabilisation of the swelling ability of the gelatin sample could be an advantage in terms of drug release, potentially allowing for a more controlled and slower release of drug in comparison to PAA. PAA could potentially release drug at a rate quicker than gelatin, as PAA continued to swell, increasing its surface area. As was observed in chapter 2, PAA released CPM over a period of 8 h but in swelling studies continued to swell and did not reach a maximum. The gelatin swelled to its maximum and stabilised. This could allow the incorporated drug to slowly diffuse from the matrix as the surface area of the matrix and swollen structure remained the same for a significant period of time. Drug release of CPM will be discussed in section 4.3.3.

Although the swelling abilities of the thiolated gelatin and PAA samples were considerable different, both samples had visual similarities, becoming clear, gel-like substances as they swelled. Both samples remained cohesive for a number of hours during the swelling studies, with further tests showing the ability of both samples to remain cohesive as a swollen polymer for a number of days before gradually dissolving in the 37 °C buffer. The cohesive properties and visual similarities between the natural and the synthetic polymers, in this case, again suggest the potential that this highly thiolated gelatin 4569 product has as a mucoadhesive polymer.

Swelling studies were then conducted on thiolated gelatin samples of differing MW. Figure 4.4 shows gelatin 4571 (MW = 50 – 100 kDa), in both its thiolated native form and thiolated aminated form. Although the thiol content of the thiolated sample (395.0 ± 106.1 µmol/g) was approximately 10-fold higher than the thiolated native sample (25.4 ± 9.3 µmol/g), this was not reflected in the swelling studies. The thiolated sample fell apart within 30 s while the thiolated native sample swelled to 140% of its initial mass before gradually disintegrating over 12 min. The swelling ability of both samples was poor and the tablet began disintegrating quickly. As this was the case, there was a loss of mass as the tablet disintegrated and therefore a negative change in mass, resulting in the negative curve shown in Figure 4.4.

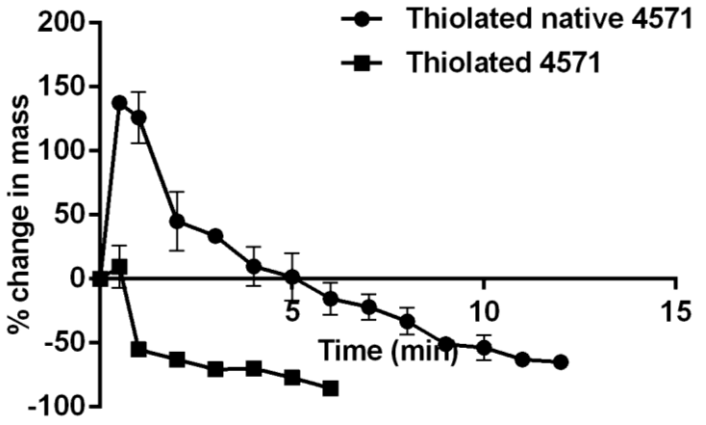

Figure 4.4 Swelling studies of thiolated native and thiolated aminated 4571 gelatin (n=2)

A similar occurrence was observed with thiolated 4568 samples and its control, as shown in Figure 4.5. Gelatin 4568 also has a MW of 50 – 100 kDA, similar to

gelatin 4571. Again, the thiolated sample had a thiol content of more than 10 times that of the thiolated native sample (579.6 ± 155.9 µmol/g in the thiolated sample and 35.5 ± 0.5 µmol/g in the thiolated native sample), yet the thiolated sample began to disintegrate quickly, with little to no swelling ability. The thiolated native sample swelled to approximately 140% of its original mass and disintegrated over 8 min. This again was a trend that was observed throughout testing. Regardless of the thiol content, the thiolated 4568 and 4571 samples were quick to fall apart, often faster than native or aminated samples; these thiolated samples did have a higher capacity to swell in comparison to the native and aminated samples which could influence mucoadhesion due to an increased surface area allowing for a potential increase in entanglements and interactions between the polymer and the mucosal layer (Andrews *et al.*, 2009). However, cohesion is required for mucoadhesion (Bernkop-Schnürch and Steininger, 2000) and both thiolated 4568 and thiolated 4571 displayed poor cohesive properties. If the polymer does not remain together, it may not adhere to the mucosal surface either.

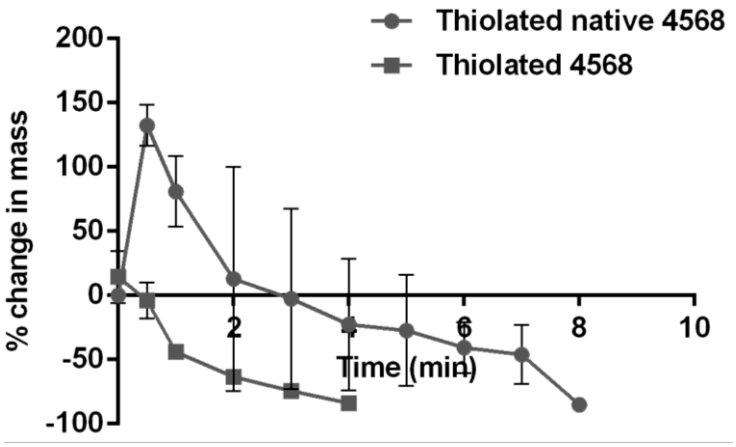

Figure 4.5 Swelling studies of thiolated native and thiolated aminated 4568 (n=3)

4.3.1.1 Ethylene diamine concentration

Gelatin 4567, a mid-range MW gelatin, was aminated at varying concentrations of ethylene diamine. The gelatin samples were initially aminated according to Seki *et al.* (2005) with the addition of 3.14 mL of ethylene diamine. To investigate the influence ethylene diamine had on the reaction, lower levels of ethylene diamine,

namely 10%, 25% and 50% of 3.14 mL, were added. These samples were then thiolated; the amine and thiol contents of each sample are displayed in Figure 4.6.

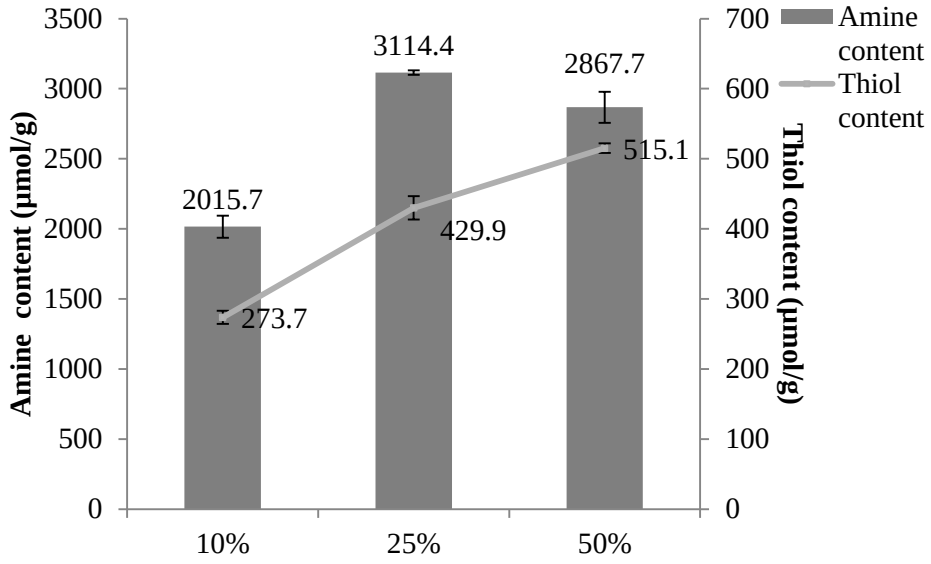

Figure 4.6 Amine and thiol content of ethylene diamine concentration experiment using gelatin 4567 (40- 50 kDa)

Swelling studies were conducted on these samples, results of which are shown in Figure 4.7. Again, the aminated samples did not swell to the same capacity as the thiolated samples, however, they did remain cohesive for 2 - 3 min longer than their thiolated counterparts. The thiolated samples were not cohesive, despite the fact that the thiol content was improved from the native gelatin and ranged from 273.7 µmol/g in the 10% sample to 515.1 µmol/g in the 50% sample (Figure 4.6). This suggests there was an optimum thiol content for a successfully cohesive tablet, as the initial gelatin 4569 tablet had a thiol content of 663.2 µmol/g and had excellent cohesive properties, whereas the 50% sample with a thiol content of 515.1 µmol/g was quick to disintegrate and did not swell significantly.

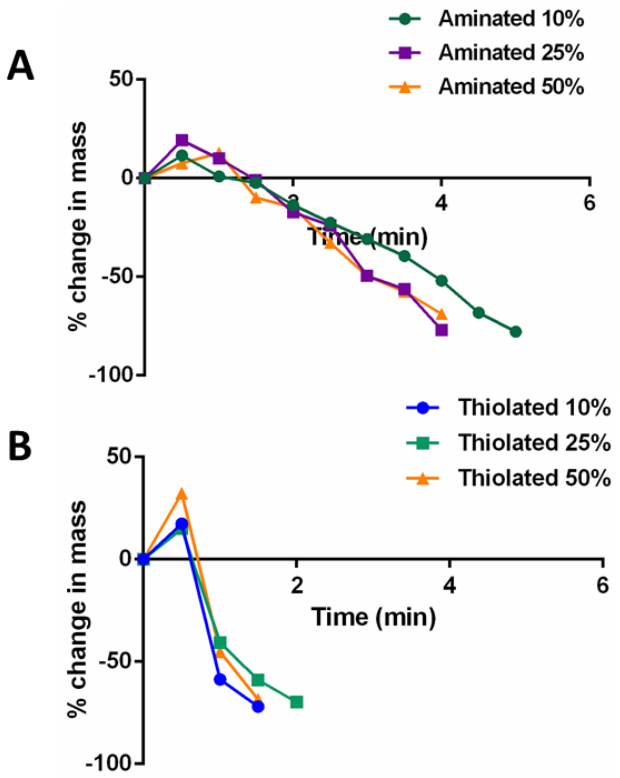

Figure 4.7 Swelling studies on gelatin 4567 samples aminated at varying ethylene diamine concentrations of (A) aminated samples and (B) thiolated samples (n=1)

Despite the fact the samples were dialysed exhaustively, the presence of any unbound thiol groups may affect the thiol content but also the swelling ability and ultimately the mucoadhesive properties. The apparent thiol content of the sample would increase as unbound thiol groups will also be measured by Ellman's reagent, but more importantly, unbound thiol will disrupt the formation of disulphide bonds in the polymer matrix decreasing the swelling ability of the sample (Verena M. Leitner *et al*., 2003). This will be discussed in more detail in section 4.3.2.

Molecular weight of the thiolated polymer is known to be of great importance as was discussed by Leitner *et al.* (2003) and observed in chapter 2, sections 2.3.2 and 2.3.3. Thiolated PAA samples of MW 450 kDa had greater cohesive and greater mucoadhesive properties than thiolated 250 kDa PAA. Optimal MW for mucoadhesive polymers varies (Andrews *et al.*, 2009), which will be discussed in more detail in section 4.3.2.2. Although the initial gelatin sample (Figure 4.2) had a marginally higher thiol content at 663.2 µmol/g in comparison to the 50% sample (Figure 4.7) which measured 515.1 µmol/g, the MW of the samples were different; gelatin 4569, a gelatin with a lower MW of 20 – 25 kDa displayed vastly improved

swelling and cohesion in comparison to the 50% sample gelatin 4567 which was a mid-range MW gelatin of 40 - 50 kDa. It may be that MW, as opposed to thiol content, is more important here in terms of swelling and cohesion and perhaps the MW of 20 - 25 kDa may be the optimum MW for mucoadhesion in gelatin. This may also explain the lack of cohesion in the 4568 and 4571 thiolated samples, both of which have high MWs of between 50 – 100 kDa, results of which are shown in Figure 4.4 and Figure 4.5.

Thiolated PAA with thiol contents of 450 µmol/g and lower have been reported in the literature as having improved swelling and mucoadhesive properties in comparison to unmodified PAA (Hornof *et al.*, 2003). PAA is known as a mucoadhesive polymer (Bernkop-Schnürch and Steininger, 2000; Smart *et al.*, 1984). In chapter 2, sections 2.3.2 and 2.3.3, mucoadhesive and cohesive properties were observed with unmodified PAA samples, which adhered to porcine tissue samples for an average of 70 min and swelled to approximately 100% of its original mass in swelling tests. This is in stark contrast to the natural polymer gelatin in its unmodified, native form. It was also observed in chapter 2 that increased thiol content improved both mucoadhesive and swelling properties, but, due to its inherent cohesive and mucoadhesive nature, increased thiol content may not be as important in terms of cohesion and mucoadhesion for PAA as for gelatin. Gelatin is not a naturally mucoadhesive material and it will begin to dissolve at body temperature, approximately 37 °C. Therefore, modification and increasing the thiol content of gelatin was vital in order to create a cohesive and mucoadhesive tablet.

4.3.1.2 pH studies

Samples of gelatin 4567 were aminated at varying pH values, namely pH 5, pH 5.5, pH 6 and pH 6.5, to investigate the influence pH had on the amination reaction. The thiol content of the gelatin 4567 samples aminated at varying pH values was much lower than other samples, including the ethylene diamine experiment samples and gelatin 4569 samples. Amine and thiol contents of each sample are listed in Table 4.2.

Table 4.2 Amine and thiol content of pH study samples using gelatin 4567

Sample	Amine content (µmol/g)	Thiol content (µmol/g)
pH 5	966. 7 ± 2.5	281.0 ± 13.8
pH 5.5	972.5 ± 5.0	300.5 ± 12.3
pH 6	991.3 ± 8.7	240.8 ± 6.2
pH 6.5	1029.0 ± 25.5	148.7 ± 4.0

The swelling abilities of the aminated and thiolated samples were poor, both of which displayed limited swelling and poor cohesive properties. The thiolated samples again disintegrated quicker than their aminated counterparts, as can be seen in Figure 4.8. Looking at the aminated samples, the swelling capacity of the samples aminated at pH 6 and 6.5 were much better than samples aminated at lower pH's. This again suggests aminating at higher pH's may be more advantageous.

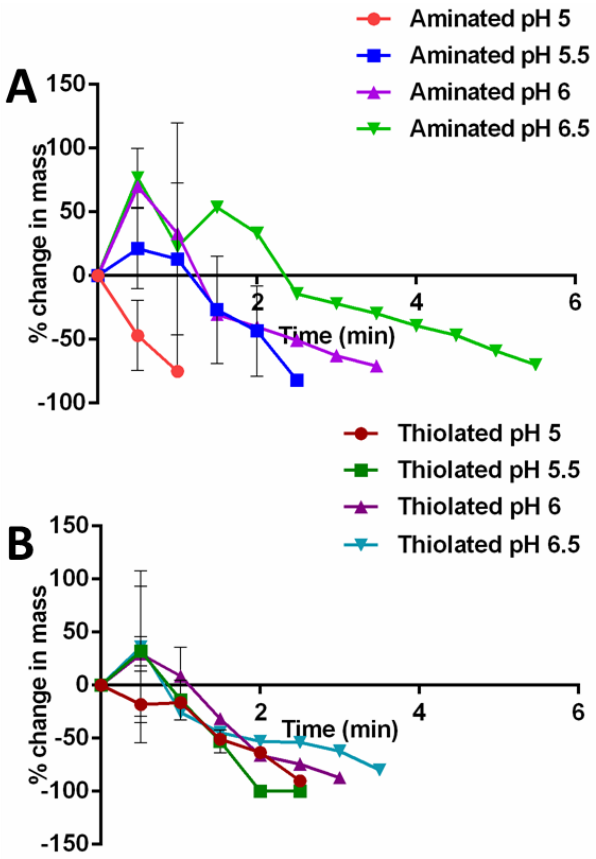

Figure 4.8 Swelling studies of gelatin 4567 samples aminated at varying pH values, of (A) aminated samples and (B) thiolated samples (n=2)

Looking at the amination reaction in more detail may give an explanation into why pH is changing the properties of the polymer. The reaction takes place with EDC which is most efficient at pH 4 - 6 but does work up to pH 7.5 (Hermanson, 2008) and, therefore, may not have too great an impact on the reaction in terms of pH changes. The protonation of ethylene diamine, however, may change with altered pH and it may be this that has an impact on the reaction. Hajós (2002) discussed the protonation states of ethylene diamine with changing pH, which is illustrated in Figure 4.9, and noted that between pH 6 - 8, ethylene diamine can exist as a mono-protonated or di-protonated compound or mixture of the two. Below pH 6, ethylene diamine exists as a di-protonated compound and this may be the reason for the decrease and variability in amination levels which has been observed. A di-protonated ethylene diamine compound would be less reactive toward the carboxyl groups, as it would be less nucleophilic.

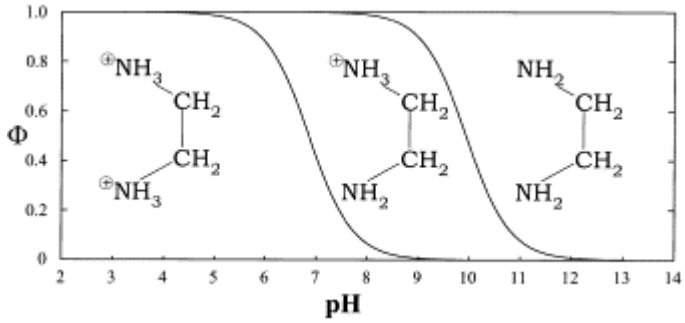

Figure 4.9 The protonation equilibria of ethylene diamine (Hajós, 2002)

The thiolated samples of both the ethylene diamine concentration study and the pH experiment showed poor cohesive and swelling properties. Comparing Figure 4.7 (B) and Figure 4.8 (B), the thiolated pH samples, although having very low thiol content in comparison to the ethylene diamine concentration samples, remained together marginally longer, despite the fact that the thiol contents of the ethylene diamine samples were up to four times higher. This suggests that thiol content is only a component of cohesion and that something else is required to form a stronger cohesive bond and, therefore, potentially a mucoadhesive polymeric tablet. Again, if any free thiol groups were present in the matrix of the ethylene diamine samples, it may explain why the thiol content was higher but also why their cohesive properties

were marginally lower, as the unbound thiol groups could form disulphide bonds with the polymer thus interrupting intramolecular crosslinking of the polymer.

The effect pH had on the amination of gelatin 4569 was also investigated, and the samples were thiolated. Shown in Figure 4.10 are the swelling results of thiolated gelatin 4569 which had been aminated at pH 6. The thiol content of the sample was approximately 257 µmol/g. Again, increased cohesion and swelling capacity were observed in the thiolated control sample in comparison to the thiolated sample. This is despite the fact that thiolation levels were 6-fold higher in the thiolated sample. The reason for this again may be due to the amino acid composition variability which is apparent in the protein gelatin. Thiolated areas may have been spread across the gelatin backbone, thus being too far from each other to bond and form the disulphide bridges that are required to keep the polymer cohesive when swelling.

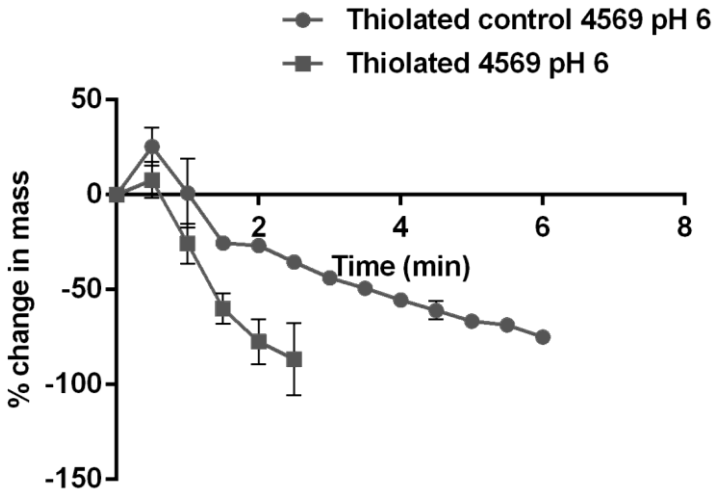

Figure 4.10 Swelling studies on thiolated control and thiolated samples of gelatin 4569 which had been aminated at pH 6 (n=2)

4.3.1.3 Amination with diethylene triamine

In an attempt to increase the amine content along the gelatin backbone further, and subsequently increase thiolation, gelatin 4569 was aminated with diethylene triamine. Swelling studies on gelatin 4569 aminated with diethylene triamine were conducted. As was discussed in chapter 3, amine content improved by a factor of 3 after amination with diethylene triamine in comparison to the aminated control. However, amination levels were not improved when compared to the sample

aminated with ethylene diamine. It was thought that steric hindrance due to the bulkier amine compound hindered the amination process resulting in lower than expected amine content with the triamine amination reaction. Thiolation levels of the diethylene triamine sample were also lower (430.1 ± 19.6 µmol/g) when compared to a similar gelatin sample aminated with ethylene diamine (564.2 ± 31.4 µmol/g). Shown in Figure 4.11 are both the aminated and thiolated gelatin samples, having been aminated with diethylene triamine. The thiolated sample did not swell significantly and began to disintegrate quickly. Aminating the gelatin sample with diethylene triamine did not improved thiolation, or swelling ability in comparison with amination ethylene diamine.

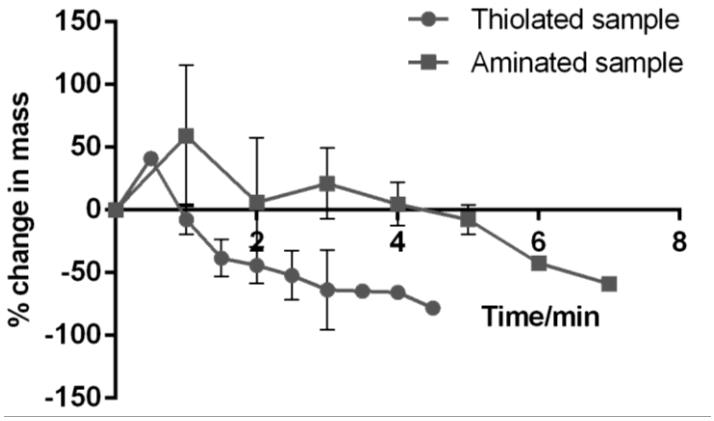

Figure 4.11 Swelling studies on gelatin 4569 samples aminated with diethylene triamine, showing aminated and thiolated samples (n=2)

4.3.2 Mucoadhesion testing

Mucoadhesive testing was conducted on native, aminated and thiolated gelatin samples of differing MW. The results of mucoadhesive testing mirrored the results obtained from the swelling studies; if the tablet was not cohesive in the swelling studies, it was not mucoadhesive either. The limited swelling ability of a sample may affect the mucoadhesive nature of the sample, as increased surface area of a swollen polymeric tablet allows for greater potential to interpenetrate and bind to the mucosal surface (Andrews *et al.*, 2009). Another factor to consider is the possibility of unbound thiol groups from unreacted Traut's reagent in the matrix of the thiolated polymer. If any unbound thiol were present in the polymer matrix after dialysis of the product, they could potentially disrupt swelling and also mucoadhesion. This is

due, in the case of mucoadhesion, to the formation of disulphide bonds between the mucosal layer and the unbound thiol groups, therefore, interrupting and decreasing the binding of the mucoadhesive polymer to the mucosal layer. Leitner *et al*. (2003) discussed the disruption that addition of L-cysteine had on the mucoadhesive properties of thiolated PAA samples. To solutions of thiolated PAA and unmodified control PAA, 1% (m/m) cysteine was added. The solutions were mixed, freeze dried and tableted. Mucoadhesive testing using the rotating cylinder method was then conducted. The presence of the free unbound thiol groups of the cysteine decreased the mucoadhesion time of thiolated PAA samples by approximately half when compared to samples without the presence of cysteine. In the unmodified control samples, mucoadhesion was not affected, suggesting that free unbound thiol groups of cysteine had the ability to disrupt the formation of disulphide bonds between the thiolated polymer and the mucosal surface. This again highlights the importance of dialysis and the complete removal of unbound thiol groups from the polymer matrix. Unlike the dialysis solution of the aminated samples, the thiolated dialysis process was not monitored by conductivity due to the changing pH of the solution; the solution was changed from 5 mM HCl to 1 mM HCl and back to 5 mM HCl to ensure complete removal of free thiols. Using the amination conductivity as a guideline, the dialysis of the thiolated samples was conducted for 7 days, to minimise the occurrence of free thiol groups within the matrix, similar to the dialysis times of thiolated PAA.

Mucoadhesive testing results were inconsistent with all gelatin samples, again highlighting the variability in natural polymers. Some of the tablets tested dislodged quickly, while others dissolved over a period of time. Mucoadhesive testing may also vary due to differences in the mucosal layers of tissue samples, with mucosal density and structure differing greatly between tissue samples as discussed in chapter 2, section 2.3.3. Similar to the swelling studies, the lower levels of thiolation resulted in decreased mucoadhesive properties, as is highlighted in Table 4.3.

Table 4.3 Thiolation levels of samples and their corresponding levels of swelling and mucoadhesion

	Levels of thiolation (µmol/g)	Highest swelling %	Mucoadhesion (min)
Thiolated 4569	663.2 ± 32.3	368	240
Thiolated 4568	579.6 ± 155.9	5.7	0.8
Thiolated 4571	395.0 ± 106.3	0	3

Native gelatin samples should in theory have minimal mucoadhesive properties, bar non-covalent bonding, such as hydrogen bonding and Van der Waal's forces, which are much weaker than covalent bonds such as the disulphide bond (Bernkop-Schnürch and Steininger, 2000; Wang *et al*., 2000; Verena M. Leitner *et al*., 2003). Unmodified gelatin begins to dissolve at 37 °C and in mucoadhesive testing, similar to the swelling studies, the native samples often began to dissolve during the 2 min incubation period of the tablet adhering to the tissue prior to being submersed into the 37 °C phosphate buffer. Figure 4.12 shows the results of mucoadhesive testing on native gelatin samples of 4569, 4567 and 4571. Mucoadhesion for all samples was minimal, with 4569 showing the greatest adhesion levels, adhering for 1.1 min.

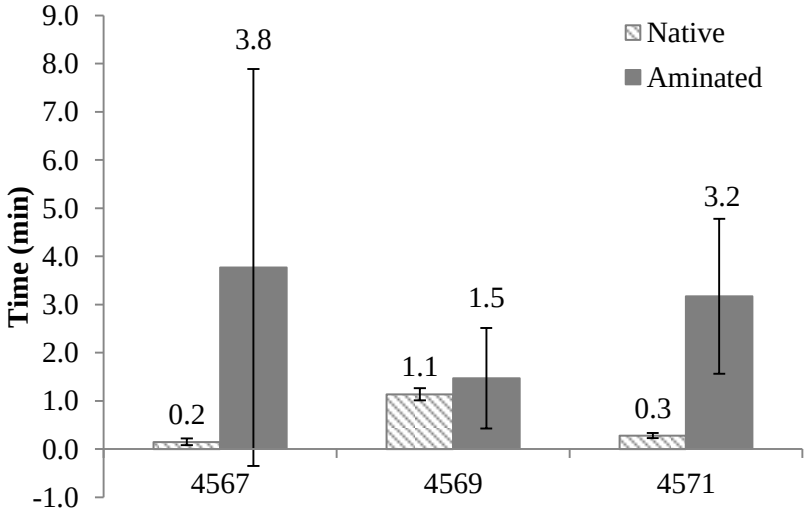

Figure 4.12 Mucoadhesive testing on native and aminated samples of gelatins 4567, 4546 and 4571 (n=3)

Also shown in Figure 4.12 are the mucoadhesive properties of aminated gelatin samples. The results again mirror the results of the swelling studies conducted on aminated gelatin samples (Figure 4.1) in which gelatin samples with higher bloom strengths swelled to a greater swelling capacity. There was variability in the mucoadhesive results however, as illustrated by the large error bars in the aminated samples (Figure 4.12). Aminated samples adhered for longer than native samples, therefore the mucoadhesive properties of the aminated material were improved, as was observed by Wang *et al*. (2001) and discussed in the introduction to this chapter; Wang observed an increase in mucoadhesion of approximately 30% with aminated gelatin microspheres in comparison to native gelatin samples. There was a greater than 30% increase in mucoadhesion observed in this study with samples 4567 and 4571. However, there was much variability with the aminated samples indicated by larger error bars.

The initial thiolated sample of gelatin 4569 which had been aminated at pH 5 and thiolated resulting in a thiol content of 663.2 ± 32.3 µmol/g showed great potential as a mucoadhesive polymer with adhesion times of greater than 4 h, Figure 4.13. However, due to the low product yields, there was minimal product available to test for mucoadhesion and this was the result of one test. Also shown in this graph was the mucoadhesive testing on a thiolated sample of gelatin 4569 with a thiol content of 267.5 ± 14.7 µmol/g, and a thiolated control sample of gelatin 4569 with a thiol content of 64.1 ± 5. 9 µmol/g (the control sample was aminated without the addition of EDC, therefore only the amine groups of native gelatin were thiolated with Traut's reagent). The increase in thiol content improved the mucoadhesive properties of the material and the degree of thiolation also had a significant effect. The sample with a thiol content of 267.5 µmol/g had increased mucoadhesion in comparison to the thiolated control sample, but had approximately 10-fold less mucoadhesion in comparison to the highly thiolated 663.2 µmol/g thiol content sample. This indicates the importance of a thiolated gelatin product with high thiol content in the formation of a mucoadhesive tablet for slow and controlled drug delivery, as increased thiol content will increase mucoadhesion. The improved mucoadhesive properties demonstrated by this thiolated gelatin 4569 sample indicated the potential this novel, biodegradable and natural material has for mucoadhesive drug delivery.

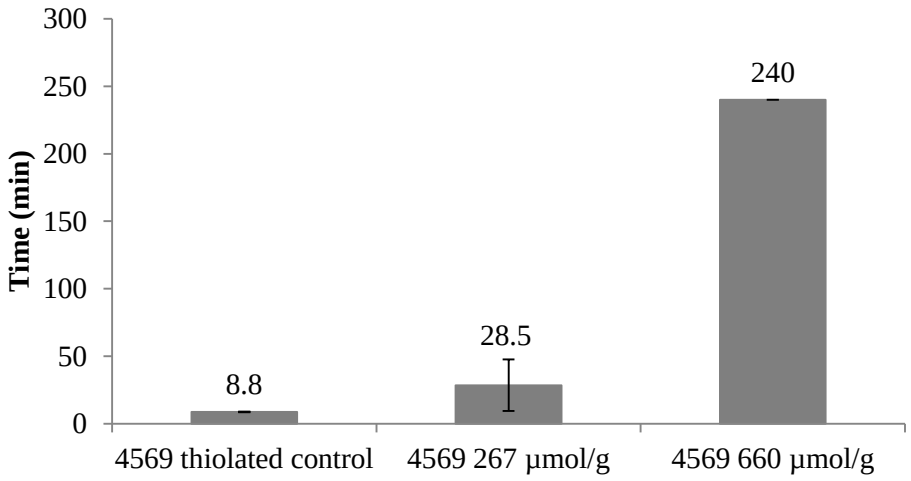

Figure 4.13 Mucoadhesive testing of gelatin 4569: thiolated control (n=3), with thiol content of 267 µmol/g (n=2) and with thiol content of 660 µmol/g (n=1). Samples were aminated at pH 5

Both thiolated samples of gelatin 4569 shown in Figure 4.13 were aminated and thiolated in the same manner; however, the thiol contents differed greatly, thus showing the variability of the results obtained through this two-step reaction system using gelatin 4569. The variability of the natural polymer may have significant effects on the creation of mucoadhesive drug delivery devices, as the polymer may contain pockets or areas which are highly thiolated, and similarly areas which have no thiol groups. This is due to the non-repeating amino acid structure of gelatin, unlike the backbone of continuous repeating units in synthetic polymers (e.g. PAA) or other natural polymers like polysaccharides. If areas of high thiolation and areas of low thiolation were to occur in the gelatin product, this would decrease the likelihood of intramolecular disulphide bond formation as the distance between thiol groups may be too large to bond, making the product material less cohesive. The lack of cohesion could in turn affect the mucoadhesive properties of the sample, as the tablet may disintegrate in solution prior to adhesion. A highly thiolated product, such as the gelatin 4569 sample (663.2 µmol/g) may not have this problem, it may have been thiolated along the length of the backbone, allowing sufficient crosslinking to occur in comparison to the less thiolated gelatin 4569 sample of 267.5 µmol/g.

Mucoadhesive testing was conducted on thiolated gelatin 4568 and 4571, the two highest MW gelatins, results are shown in Figure 4.14. The results of the mucoadhesive testing again mirrored the swelling tests of both samples (Figure 4.4

and Figure 4.5); in both cases the thiolated native samples had better swelling ability and a longer duration of cohesion. This was again highlighted in mucoadhesive studies (Figure 4.14) with the thiolated native samples of both 4568 and 4571 adhering to the mucosal tissue for up to 36 times longer in the case of gelatin 4568 and 3 times longer for gelatin 4571. The thiol contents between the thiolated native and thiolated aminated samples differed by approximately 10-fold, however, this did not have the desired effect in either the swelling studies or the mucoadhesive testing.

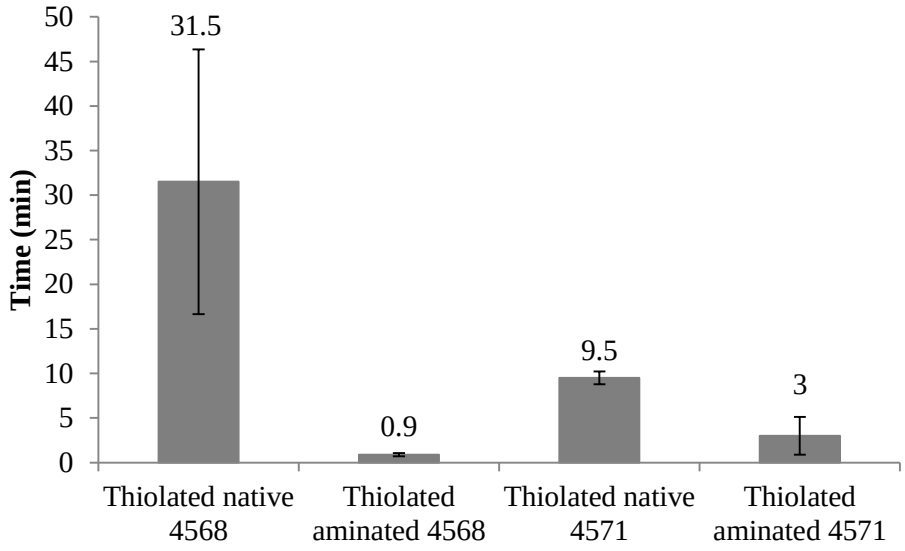

Figure 4.14 Mucoadhesive testing on thiolated native and thiolated aminated samples of gelatin 4568 and 4571 (n=2)

4.3.2.1 pH studies

Mucoadhesive testing was carried out on thiolated samples which had been aminated at different pH values, results are shown in Figure 4.15. Thiol content of all samples varied greatly, as indicated in Table 4.2. Although the pH 5.5 sample had higher levels of thiolation, it had the lowest level of mucoadhesion which was also the case in the swelling studies, Figure 4.8 (B). However, all samples had minimal mucoadhesive properties and the variability in mucoadhesion is again highlighted with large error bars.

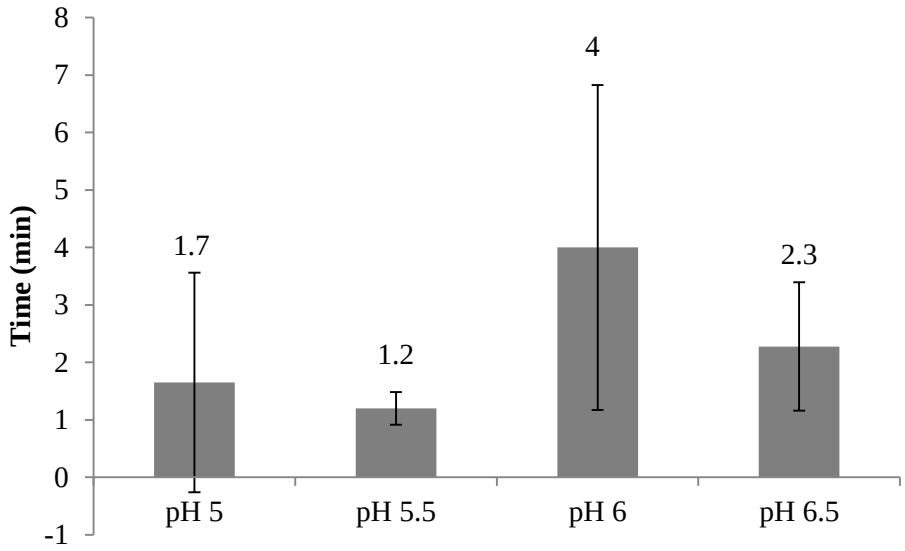

Figure 4.15 Mucoadhesive testing on thiolated gelatin 4567 samples which were aminated at varying pH values (n=3)

pH was seen to be of great importance in chapter 2 when thiolating PAA, resulting in different levels of thiol content, cohesion and mucoadhesion depending on the pH of the reaction. The pH of the PAA thiolation would have affected the concentration of EDC, due to hydrolysis and the possible reforming of the PAA polymer and it was this concentration difference which resulted in the range of thiol contents at specific pH levels observed in the PAA samples. As the EDC was added to the gelatin amination reaction after the pH was adjusted, the concentration of EDC would not have changed to the same degree. When thiolating gelatin at varying pH's, the thiol content and yield were also seen to be affected by pH differences, with the lower pH values of 5 and 5.5 resulting in higher levels of thiolation. Thiol levels of the four pH gelatin samples were low, and because of this, mucoadhesion was also low - particularly in comparison to PAA samples – however, pH again appeared to play a part in mucoadhesion. The thiolated samples which were aminated at higher pHs of 6 and 6.5, displayed slightly increased mucoadhesive properties in comparison to the thiolated samples aminated at pH 5 and 5.5 (Figure 4.15); however, the error was high on all samples.

Samples of thiolated gelatin 4569, aminated at pH 6, had a low thiol content measuring 267 µmol/g and displayed poor swelling ability in swelling tests (Figure 4.10). However, its mucoadhesive properties were excellent, with adhesion times of

up to 24 h as shown in Figure 4.16. Again, there was variation in adhesion between triplicate samples. However, all three samples adhered to the mucosal surface of the porcine tissue for a minimum of 8 h. This was a vast improvement in adhesion in comparison to the thiolated control sample of 4569 aminated at pH 6, which dislodged within 15 min, indicating that the two-step reaction had improved mucoadhesive times for gelatin by a large and significant amount. The lack of control of the level of thiolation, however, could lead to significant problems using the material as a mucoadhesive device.

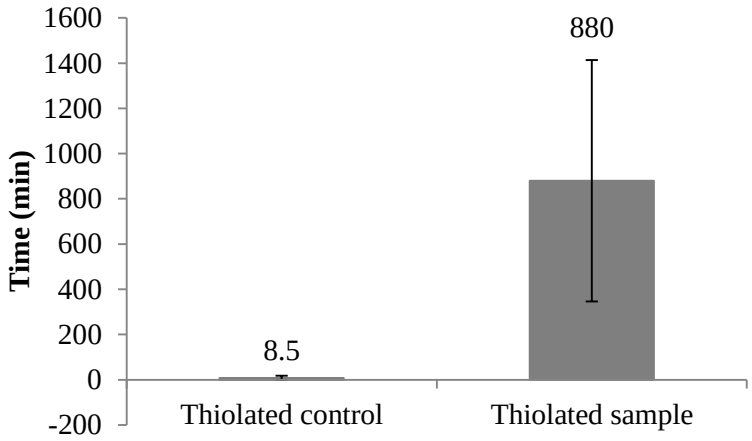

Figure 4.16 Mucoadhesive testing on thiolated controls (n=2) and thiolated samples (n=3) of gelatin 4569 which had been aminated at pH 6

The mucoadhesive abilities of the 4569 pH 6 thiolated sample again reemphasises the importance of the MW of the polymer. The low thiol content of this thiolated gelatin 4569 sample exhibited excellent mucoadhesive properties, whereas thiolated gelatin 4567 samples, which had comparable thiol content of but higher MW than gelatin 4569, had poor mucoadhesive properties. This was also observed in chapter 2 with PAA of two different MW, 250 kDa and 450 kDa. In this case, the higher MW PAA sample exhibited better mucoadhesive and cohesive properties. The length of the chain and flexibility of that chain influences the mucoadhesive and cohesive properties of the polymer (Huang *et al*., 2000); a minimum MW of 100 kDa has been stated as required for mucoadhesion (Andrews *et al.*, 2009) and therefore, theory would suggest that the higher MW gelatins of 4568 and 4571 would have better cohesive and mucoadhesive properties in comparison to lower MW samples due to

the increased chain length and increased potential for interpenetration into the mucosal layer. However, literature has also shown that excessively long polymer chains can inhibit or decrease mucoadhesion (Andrews *et al.*, 2009; Huang *et al.*, 2000) and that there is an optimal MW for mucoadhesion for each polymer (V. M. Leitner *et al.*, 2003; Andrews *et al.*, 2009). Andrews *et al.* (2009) describes each polymer as being "unique preventing the definition of an optimum molecular weight". With PAA, a mid-range MW of 450 kDa was concluded to be optimal for mucoadhesion; however, this may not be the case with the natural polymer gelatin, with a lower MW of 20 – 25 kDa, and, therefore, shorter chain length, being potentially more suitable for mucoadhesion. This may explain why the mucoadhesive properties of gelatin 4569 are superior to other gelatin samples of higher MW, regardless of the thiol content. The chain length of gelatins 4567, 4568 and 4571 may be too long for sufficient interpenetration and diffusion into the mucosal surface, therefore decreasing their mucoadhesive properties. This was observed by Leitner *et al.* (2003) when comparing a high MW thiolated polycarbophil to thiolated PAA samples of varying MW, as discussed in chapter 2. The polycarbophil had reduced mucoadhesive properties in comparison to the 450 kDa PAA.

Although mucoadhesive, the thiolated gelatin 4569 pH 6 sample tablets did not visually swell and they remained solid throughout the experiment, dislodging as opposed to dissolving or disintegrating. This was in contrast to both thiolated PAA and the gelatin pH 5 4569 sample (660 µmol/g, shown in Figure 4.13), both of which had excellent swelling ability and visually swelled, becoming a clear gel-like material during both swelling and mucoadhesive testing. This gel-like material, which is illustrated in Figure 4.17, held liquid readily and did not seep liquid when touched.

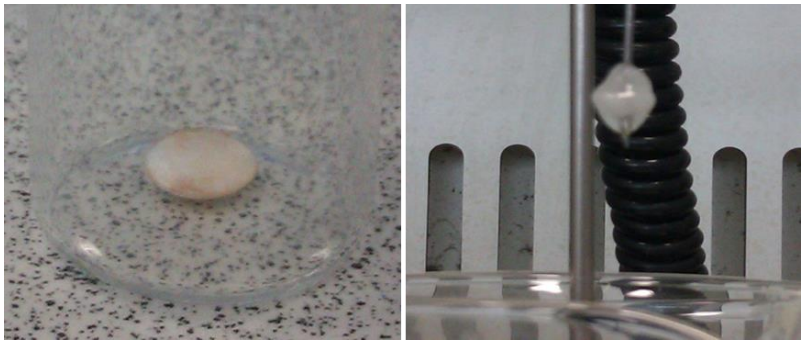

Figure 4.17 Images of gelatin sample as a dry tablet, approximately 1 cm in diameter (left) and swollen during swelling tests after approximately 10 min in buffer (right).

4.3.2.2 Amination with diethylene triamine

The sample of gelatin 4569 which was aminated at pH 5 with diethylene triamine was also tested for mucoadhesive properties. However, again due to low levels of product, only one test could be conducted. The sample had a thiol content of 430 µmol/g but did not have good swelling properties and the mucoadhesive properties mirrored this, with the tablet disintegrating in 20 min. This was an improvement in comparison to the native and control gelatin samples, with more than a 2-fold increase in the adhesion time; however, mucoadhesive properties were still low.

4.3.3 Drug release studies

CPM was incorporated in the matrices of thiolated control samples (i.e. gelatin samples aminated at pH 6 without EDC addition and then thiolated) and thiolated samples of gelatin 4569 pH 6 and drug release studies were conducted (n=3). With the incorporation of CPM, the control remained cohesive for 24 h, however drug was released quickly over 10 - 20 min. The thiolated sample broke apart and began to disintegrate in the vial within a number of minutes, therefore, completely releasing the drug. Although the thiolated tablet displayed excellent mucoadhesive properties, the cohesive properties of this thiolated tablet were extremely poor in swelling tests. The release of CPM was fast from both samples, and the increased thiol content of the thiolated sample in comparison to the thiolated control sample had little effect on controlling the release of drug. It is possible that interactions, such as hydrogen bonding, occurred between the thiolated control sample and the drug, CPM, which created a more cohesive matrix. Interactions between a polymer and an incorporated drug generally will decrease the release of the drug (Zhu *et al.*, 2002b), in this case it

may have increased the cohesion of the thiolated control sample. As the thiolation of the sample resulted in a thiol content of 42.4 µmol/g, there may have been hydrogen bonding between the carboxyl groups of the thiolated control sample and the drug which occurred to a lesser extent than in the thiolated sample, due to the increase in thiol content.

The drug release profile of the above gelatin sample was poor in comparison to that of PAA in chapter 2. The cohesive nature of PAA allowed for a better drug release profile. The gelatin samples tested here, on the other hand, had poor cohesive properties and released the drug quickly from both the control and thiolated samples. The lack of swelling and cohesion these samples possessed had a major impact on the drug release, as was to be expected. The polysaccharide, chitosan, has been investigated extensively in the literature in terms of its mucoadhesive properties, which is observed through hydrogen bonding and electrostatic interactions in its unmodified form (Khutoryanskiy, 2011) and disulphide bonding in its thiolated form (Bernkop-Schnürch *et al*., 2003; Bernkop-Schnürch *et al*., 2004; Kast *et al*., 2002). As was discussed in chapter 2, thiolated chitosan samples of differing thiol content were analysed for swelling ability, mucoadhesion and drug release studies of clotrimazole, and were compared to unmodified chitosan (Kast *et al*., 2002). Both thiolated chitosan samples displayed a faster release of drug in comparison to the unmodified chitosan sample. The drug release correlated to the swelling studies performed, and the higher thiol content sample displayed a faster release of thiol. Although thiolation of the polymer did not offer slower release of drug, it was concluded that, due to the improved disintegration rates and mucoadhesive properties, thiolated chitosan had great potential for the delivery of clotrimazole. In this study, the thiolated 4569 pH 6 sample also displayed improved mucoadhesive properties. The fast release of drug at the targeted mucoadhesive site may be beneficial if high concentrations of localised drug were required.

4.3.4 Polymer characterisation

4.3.4.1 Scanning electron microscopy (SEM)

SEM imaging was conducted on freeze dried samples of aminated gelatin, thiolated gelatin and thiolated gelatin-CPM conjugates, as shown in Figure 4.18. The texture and morphology of the aminated sample changed greatly once the sample was thiolated. After each modification step of the polymer, a new material was produced which had a different morphological structure. Similar to the incorporation of CPM in PAA samples, the structure again changed slightly between the thiolated gelatin sample and the thiolated gelatin-CPM conjugate; this was observed visually as the gelatin-CPM conjugates had a different texture and appearance in comparison to the non-drug incorporated samples once freeze dried, as was also the case with the PAA-CPM conjugates. The ratio of drug:polymer in the drug incorporated samples is 1:4, making it quite a large proportion of the tablet. The drug may freeze forming a more crystalline-like structure differing from that of the polymer, therefore, changing the appearance of the drug incorporated tablets in comparison to the polymer alone tablets. A similar occurrence happened when CPM was incorporated into Eudragit RS PO, in which the crystalline structure of CPM was observed in the polymer/drug mixture when analysed using X-ray diffractometry (Zhu *et al.*, 2002a).

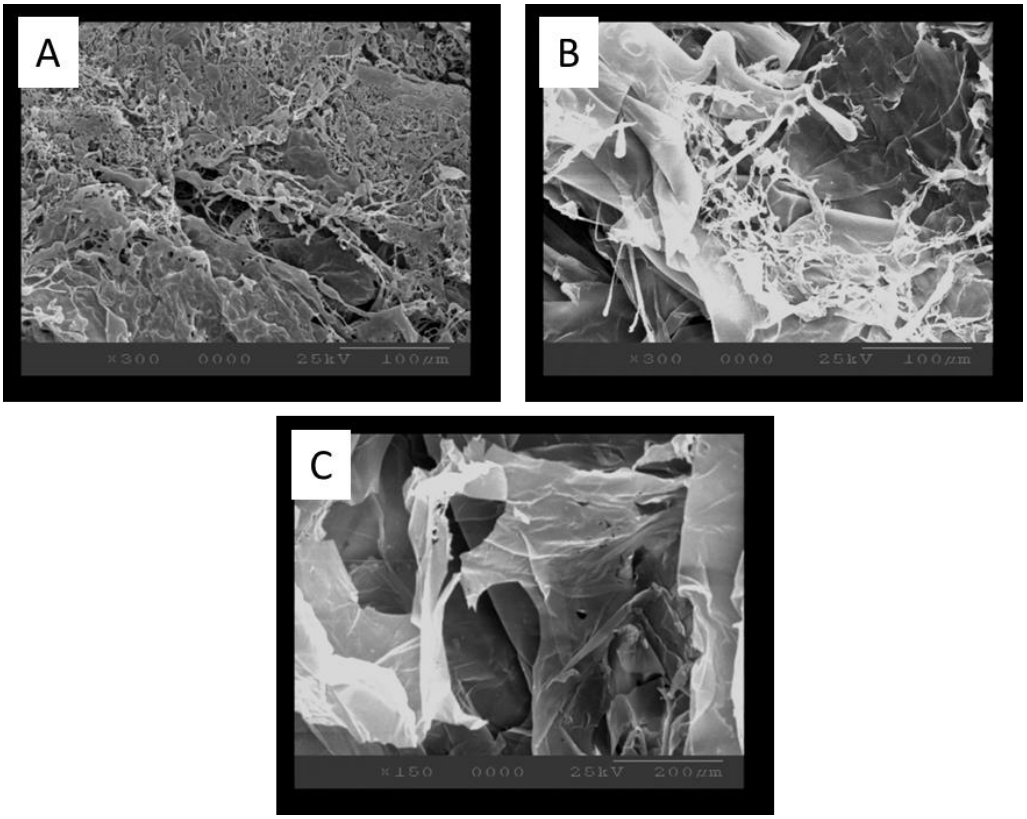

Figure 4.18 SEM imaging of (A) aminated gelatin, (B) thiolated gelatin and (C) thiolated gelatin-CPM conjugate

4.3.4.2 Thermogravimetric analysis (TGA)

Shown in Figure 4.19 is the TGA thermogram of gelatin 4571 in its native (freeze dried), aminated and thiolated forms. All samples showed water loss below 100 °C; in the native sample there was a loss of 12.4% while the aminated sample showed a loss of 5.6% and the thiolated samples showed 4.5% loss. Gelatin contains up to 15% water in its native form (PBgelatins, 2009); although the sample was freeze dried, loosly bound water may still be within the matrix and this weight loss may, again, be due to the loss of this loosly bound water. The TGA curve for native gelatin was comparable to that found in the literature (Barreto *et al*., 2003). All samples, native, aminated and thiolated, had comparable degradation patterns, with the native sample displaying a larger loss of mass up to 160 °C in comparison to the aminated and thiolated samples due to the water content of the sample.

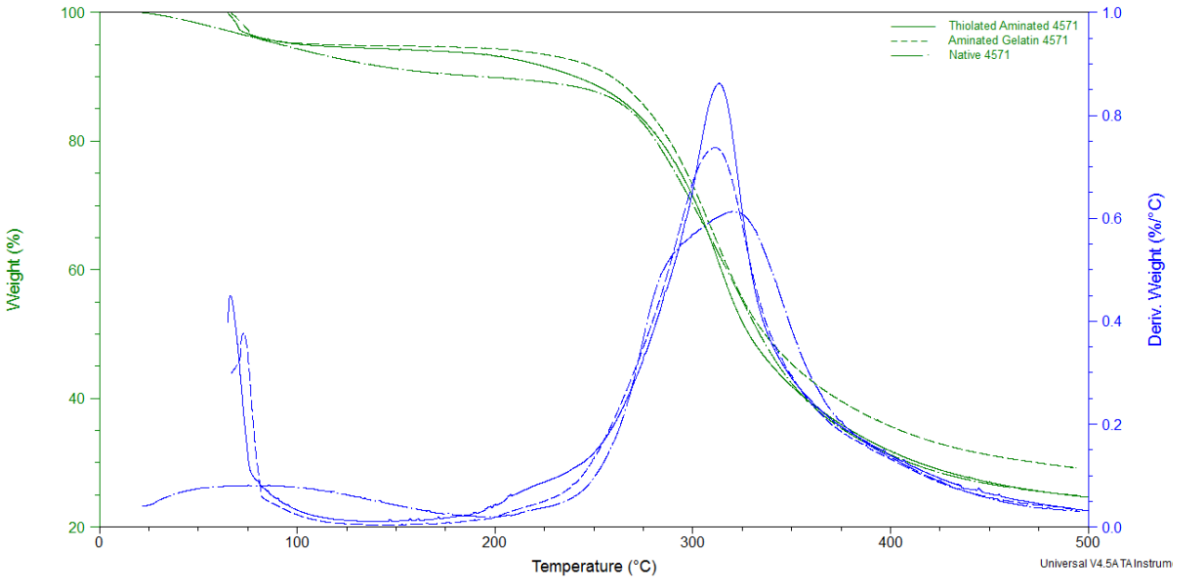

Figure 4.19 TGA analysis of freeze dried native gelatin, aminated and thiolated gelatin 4571 samples

4.3.4.3 Differential scanning calorimetry (DSC)

Shown in Figure 4.20 is the DSC profile of native gelatin 4569, and its aminated and thiolated counterparts; the complete heat-cool-heat-cool cycle is shown in Figure 4.20 (A) and the T_g is highlighted in the second heating cycle in Figure 4.20 (B). The importance of running the sample in a heat-cool-heat-cool cycle is apparent in Figure 4.20 (A), as a large broad peak at approximately 90 °C, indicating water release from the samples, was observed in the 1st heating cycle. Each sample also displayed an endothermic peak within the first heating cycle, labelled in Figure 4.20 (A) at 214 °C in the native 4569 samples, 200 °C in the aminated sample and 192 °C in the thiolated sample. These observed endothermic peaks occurred close to the glass transition (T_g) of the materials, and are classified as enthalpic relaxation peaks (Hohne *et al*., 2003). These relaxation peaks occurred only in the first heating cycle masking the T_g of the materials, but once the samples had been cooled and heated again, the relaxation peaks were no longer visual allowing the T_g of the samples to be measured. The T_g of the samples can be seen more clearly in the second heating cycle and are shown in Figure 4.20 (B) and clarified in Table 4.4. The T_g of native gelatin in its dry state is stated as being 217 °C (Apostolov *et al*., 1999) but the T_g does decrease with the presence of water. In this study, the T_g was measured as 204 °C and the presence of water, as indicated by TGA results, may explain this observed decrease. With each reaction step, the polymer backbone was modified

further, changing the properties of the material and the T_g decreased with each modification; as is shown in Table 4.4, the T_g of the aminated 4569 sample decreased to 199 °C while the T_g of the thiolated samples measured 191 °C. This indicated that the thiolation of the polymer acted as a plasticiser and thus reduced the T_g. In addition to this, Traut's reagent is bulkier than ethylene diamine. This bulkiness, and indeed the functionality of the thiolated sample, may allow the polymer to be less rigid and, therefore, be able to move and melt more easily, again allowing for a lower T_g.

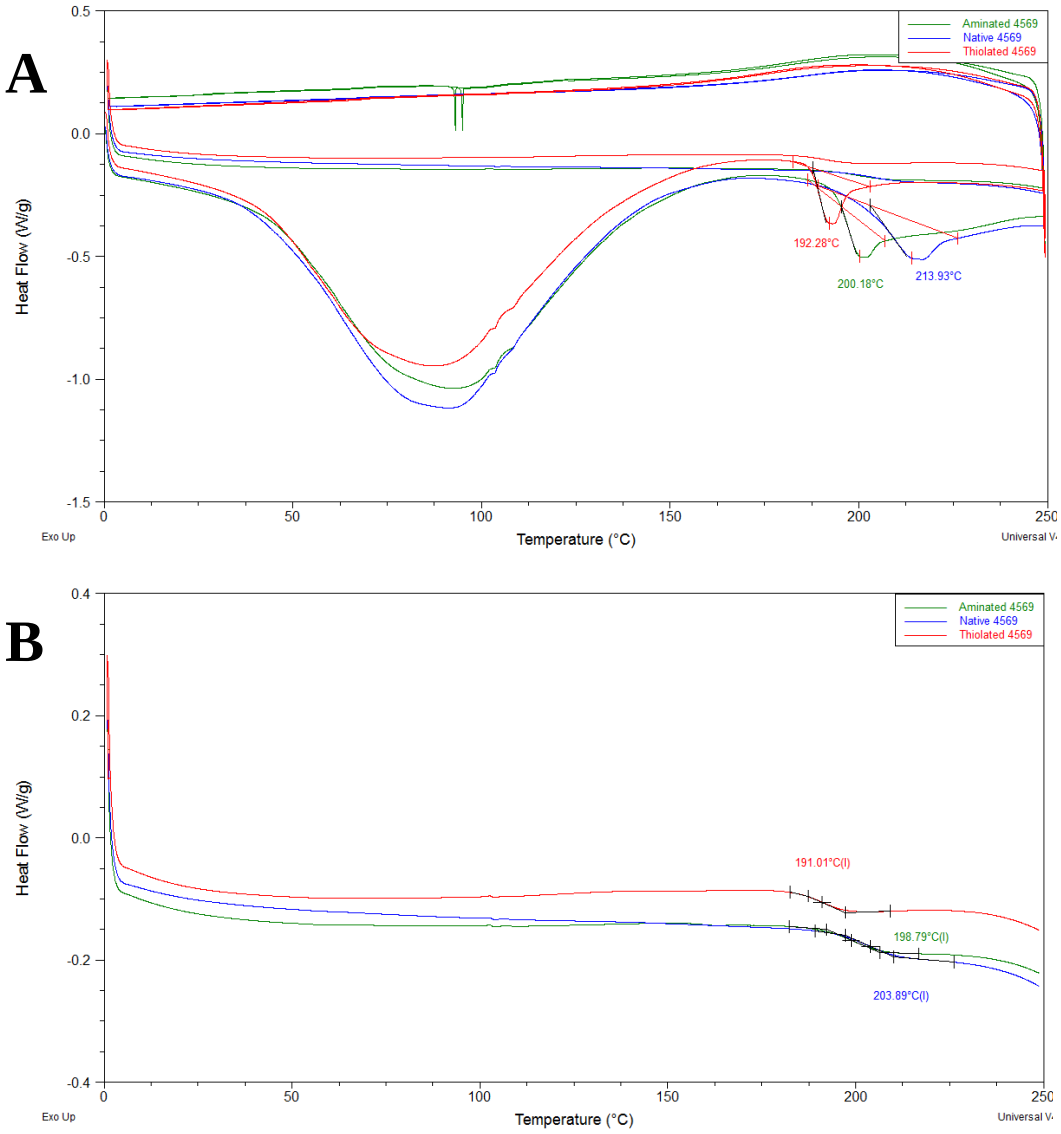

Figure 4.20 DSC profile of native, aminated and thiolated samples of gelatin 4569 in (A) the complete heat-cool-heat-cool cycle and (B) 2nd heat-cool cycle

Table 4.4 T_g measurements of freeze dried native, aminated and thiolated gelatin 4569 samples

Sample	Measured Tg temperature (°C)
Native gelatin 4569	204
Aminated 4569	199
Thiolated aminated 4569	191

The enthalpic relaxation peak in the thiolated sample in Figure 4.20 (A) was much sharper than the aminated or native gelatin samples, which were broad and more sloping. Gelatin has been described as a partially crystalline material, containing

regions of amorphous and crystalline structure but, once melted and cooled, a fully amorphous material was created (Badii *et al*., 2005). Badii *et al.* (2006) suggested the T_g was due to the amorphous portion of gelatin and the endothermic peak observed after the T_g was the melt of the partially crystalline portion of the material. The endothermic peak observed by Badii *et al.* was also only apparent in the first heating cycle, as was the case in this study, and so concluded the initial heating cycle was performed on a partially crystalline material while the second cycle was on a fully amorphous material. The endothermic peak became more pronounced due to increased periods of aging, and these relaxation peaks can occur due to the storage of a material at a temperature below its T_g. This may be a reason why the endothermic peak was more prominent in the aminated sample than the thiolated sample in Figure 4.20 (A). Due to the length of time the thiolation reaction takes, i.e. reaction time, dialysis, freezing and freeze-drying, there was a significant time delay between the synthesis of both products and, therefore, the thermal history of the samples could be different even though the samples were stored at 4 °C after freeze drying. As the backbone of gelatin was modified further with the conversion of amine groups to thiol groups, this may explain why the endothermic peaks were shifted slightly between the aminated and thiolated samples.

4.3.4.3.1 Ethylene diamine concentration

The gelatin 4567 samples which were aminated with differing concentrations of ethylene diamine (10%, 25% and 50%) and were then thiolated were also analysed with DSC; the T_g measurements of both the aminated and thiolated samples are displayed in Table 4.5.

Again, the T_g of the thiolated samples were lower than that of the aminated samples across all samples. The 10% and 25% aminated samples had comparable T_g measurements at 199 °C, as did the 10% and 25% thiolated samples measuring 186 °C. As the concentration of ethylene diamine increased to 50% in the reaction, the measured T_g fell to 197 °C in the aminated samples and to 185 °C in the thiolated samples.

All the ethylene diamine concentrations samples were aminated at pH 5 and all the pH studies samples were aminated using 100% ethylene diamine; therefore, utilising

the pH 5 sample from the pH studies experiment gives a T_g measurement of 100% ethylene diamine concentration, which is also displayed in Table 4.5. When 50% ethylene diamine was added to the reaction, the T_g of the aminated sample measured 197 °C, which was a decrease in temperature when compared to both the 25% and 10% samples. A similar occurrence happened when 100% ethylene diamine was added to the reaction; the T_g of the aminated 100% sample decreased further in comparison to the aminated 50% sample with the T_g shifting from 197 °C in the 50% sample to 186 °C in 100% sample.

Table 4.5 T_g measurements of aminated and thiolated samples of gelatin 4567 aminated with varying concentrations of ethylene diamine

Samples	T_g of aminated samples (°C)	T_g of thiolated samples (°C)
10%	199	187
25%	199	187
50%	197	185
100%	186	186

The increase in ethylene diamine used within the reaction affected the T_g of the samples, decreasing it, which again displays a plasticising effect similar to the effect thiolation has had. The amine content of the ethylene diamine experiment samples were above the theoretical values, all of which measured 2000 – 4000 µmol/g in amine content. The T_g of the samples varied with increasing ethylene diamine, therefore showing that the amount of ethylene diamine used within the experiment has altered and modified the thermal characteristics of the gelatin material. As discussed in chapter 3, section 3.3.1.2, dialysis solutions of the aminated samples were monitored to ensure complete removal of unreacted ethylene diamine; however, if any unreacted ethylene diamine was trapped within the matrix, this may explain the decrease in T_g as concentration of ethylene diamine increased.

4.3.4.3.2 pH studies

Samples of gelatin 4567 which were aminated with ethylene diamine at varying pHs were analysed by DSC. Shown in Figure 4.21 is the 2nd heating cycle, highlighting the T_g of each sample in their (A) aminated and (B) thiolated forms. For clarity, Table 4.6 also shows the T_g measurements of the aminated and the thiolated samples.

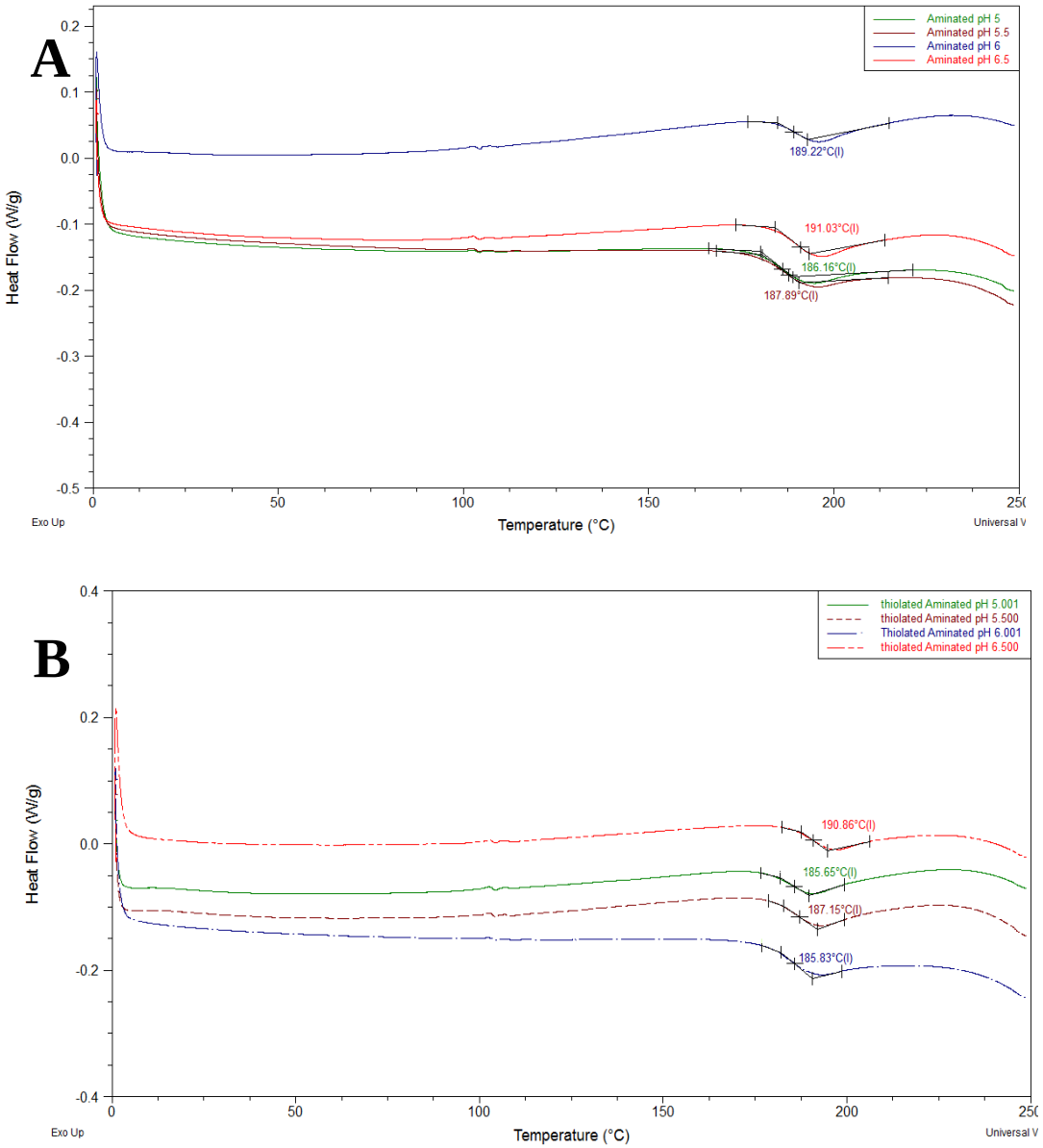

Figure 4.21 DSC curve showing 2nd heating cycle and T_g of gelatin 4567 (A) aminated at varying pHs and (B) thiolated samples

Table 4.6 T_g measurements of aminated and thiolated samples of gelatin 4567 aminated at varying pHs

Samples	T_g of aminated samples (°C)	T_g of thiolated samples (°C)
pH 5	186	188
pH 5.5	188	187
pH 6	189	186
pH 6.5	191	191

In the pH study samples, the amine contents of the aminated samples were comparable (Table 4.2). The T_g of these aminated samples has, however, varied; as the pH of the reaction increased in the aminated samples, so did the T_g, shifting from 187 °C in the pH 5 sample to 190 °C in the pH 6.5 sample. As was mentioned previously, the addition of thiol groups on the polymer backbone acted as a plasticiser, and so, the T_g of each thiolated sample was marginally lower than its aminated counterpart. However, the trend was similar in the thiolated samples where the T_g increased from 188 °C in the pH 5 sample to 191 °C in the pH 6.5 sample. Looking at the pH 5 and pH 5.5 samples, there was less of a decrease in T_g between the aminated and thiolated samples of the same pH. The T_g of these samples almost remained constant once the aminated sample has been thiolated. Regardless of the reaction conditions during the amination reaction (samples were aminated at varying pH's), the conditions of the thiolation reaction were maintained and consistent. It may be this reason why the thiolated samples T_g are more constant, as all were thiolated in the same fashion and at the same time, unlike the aminated samples which were aminated at varying pH's.

In mucoadhesive testing, samples of gelatin 4569 were tested; one aminated at pH 5 with a resulting thiol content of 267 µmol/g and the second aminated at pH 6 with a resulting thiol content of 257 µmol/g. Both had similar thiol contents but performed very differently, with the pH 6 samples adhering to the tissue for an average of 880 min while the pH 5 sample adhered for 28.5 min. The properties of the two materials were very different, even though amination had occurred with only one pH unit in the difference. DSC was performed on both the aminated and the thiolated samples of gelatin 4569 pH 5 and 4569 pH 6 in order to determine the differences between them. Shown in Figure 4.22 are the DSC curves of (A) aminated gelatin 4569 pH 5

and pH 6 samples and (B) thiolated gelatin 4569 pH 5 and pH 6 samples. Again, for clarity, the T_g measurements are also shown in Table 4.7.

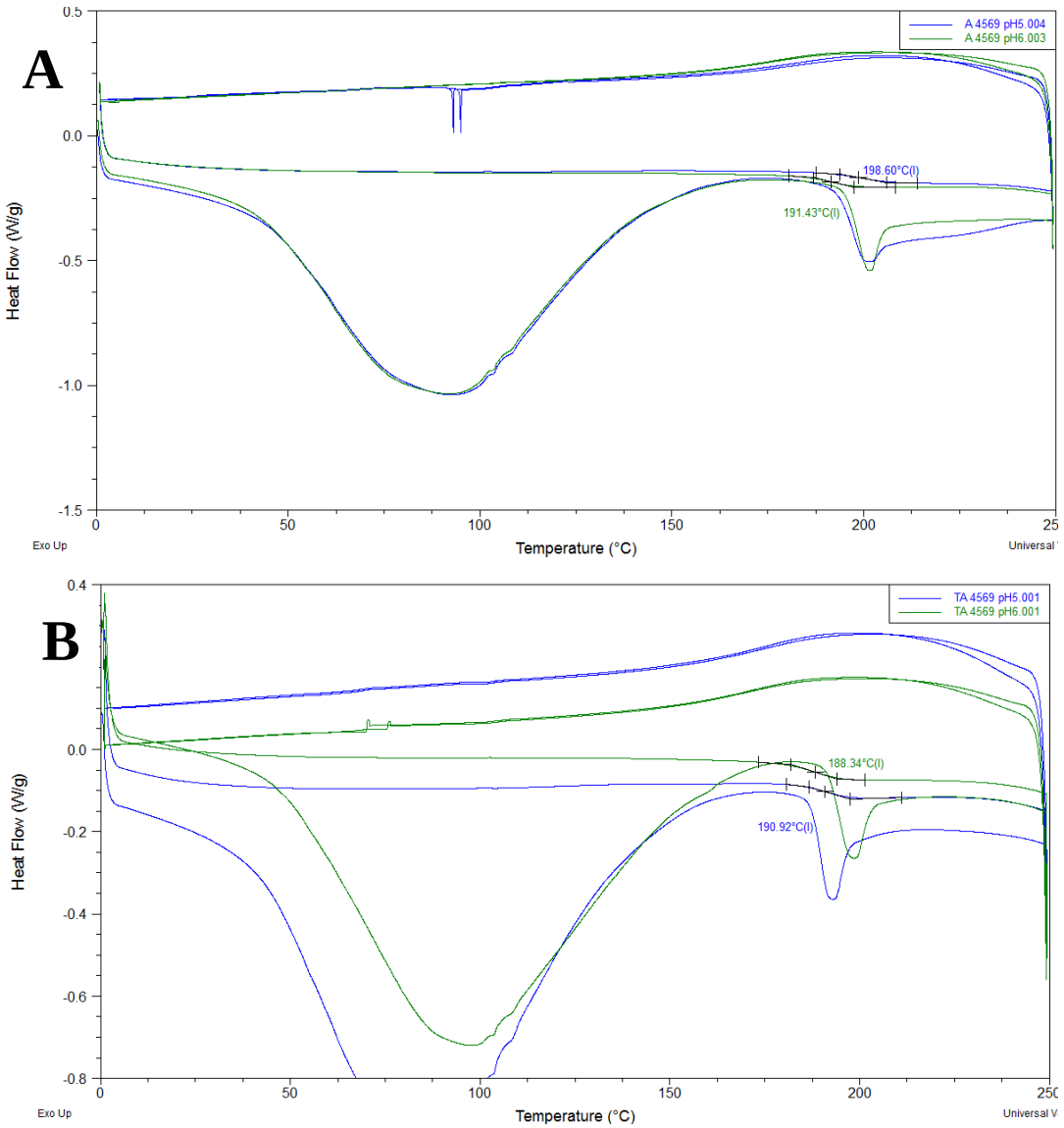

Figure 4.22 DSC curves of gelatin 4569 aminated at pH 5 and pH 6: (A) aminated samples and (B) thiolated samples

Table 4.7 T_g of 4569 samples aminated at pH 5 and pH 6; aminated and thiolated samples

Sample	Aminated sample T_g	Thiolated sample T_g
4569 pH 5	199	191
4569 pH 6	191	188

There was a notable decrease in T_g between the two aminated samples, falling from 199 °C in the pH 5 sample to 191 °C in the pH 6 sample. This is opposite to the results observed in the pH study using gelatin 4567, where increasing pH increased the T_g (Table 4.6). As was observed previously, the thiolation of the two 4569 pH samples decreased the T_g further again in both cases but in the pH 5 sample, there was a large shift in T_g of over 7.5 °C between the aminated and the thiolated sample. The shift was not as pronounced in the pH 6 sample, with a temperature change of 3.1 °C between the aminated and thiolated sample. In the pH studies of gelatin 4567 (Table 4.6), there was also a decrease in T_g but the decrease was less marked compared to the gelatin 4569 samples (Table 4.7). The difference of one pH unit could have an effect on the protonation of ethylene diamine and also the efficiency of EDC which will in turn affect the properties of the final product. The presence of water and the MW of the polymer can both influence the measurement of the T_g but so can the presence of electrostatic interactions or crosslinking between polymer chains (Brown, 2001; Steendam *et al.*, 2001; Rahman *et al.*, 2008). As was observed in the mucoadhesive studies of these two gelatin 4569 samples, the pH 6 displayed excellent mucoadhesion in comparison to the pH 5 sample; it may be that intramolecular interactions within the pH 6 sample have altered the T_g in comparison to the pH 5 sample, changing the alignment or orientation of the polymers chains within the pH 6 sample in comparison to the pH 5 sample due to the change in pH (Staroszczyk *et al.*, 2012).

4.3.4.4 Isoelectric point (IEP) titrations

IEP titations were conducted on all native gelatin, aminated and thiolated samples. The IEP is the point at which gelatin holds no net charge. IEP can influence the characteristics of gelatin and, as the backbone of gelatin was modified with each step of reaction process, it is reasonable to think the IEP value of the aminated and thiolated samples may also have altered. All gelatin samples were titrated against both 0.1 M NaOH and 0.1 M HCl, resulting in a typical graph as shown in Figure 4.23 which displays native gelatin 4569. The pKa 1 and pKa 2 values were deduced from the titration graphs and the IEP value was calculated. In Table 4.8 are the pKa and IEP values of native, aminated and thiolated gelatin 4569 samples. The IEP value of each sample did not change significantly upon each modification step and

this was typical of all MW gelatin samples, including both the ethylene diamine concentration experiment and the pH study samples. Although the overall IEP value didn't change considerably once the samples were aminated or thiolated, the pKa values did alter upon both amination and thiolation (Table 4.8). The change in pKa may have an influence on the structural orientation or potential coiling of the gelatin molecule, due to changes in electrostatic interactions between chains. This may be a reason as to why the T_g measurements of the gelatin 4569 pH 5 and pH 6 samples were different (Table 4.7).

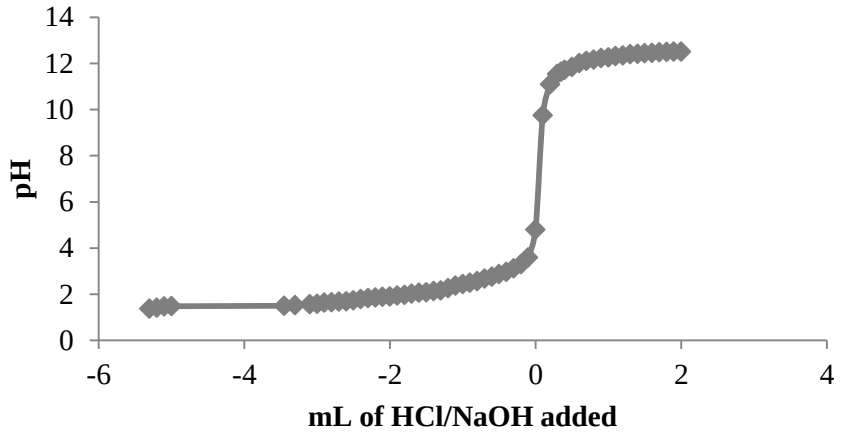

Figure 4.23 IEP titration of native gelatin 4569

Table 4.8 IEP values of gelatin 4569

Sample	pKa1	pKa2	IEP
Native	2.1	12.4	7.2
Aminated	2.2	12.3	7.3
Thiolated	2.7	11.8	7.2

4.4 Conclusions

Highly thiolated gelatin products were produced and were tested for swelling abilities, cohesion and mucoadhesion. Initial swelling tests gave good indications as to the mucoadhesive properties of the products, as without cohesion, mucoadhesion may not occur. The swelling abilities of many of the thiolated gelatin samples which

had undergone the two-step amination/thiolation reaction were poor, which was reflected in the rotating cylinder test for mucoadhesion. The MW of the gelatin used in the reaction played on integral part in the creation of the mucoadhesive tablet, as was illustrated with regards to PAA (V. M. Leitner *et al*., 2003). In the cases of gelatin 4567 (MW of 40 - 50 kDa), 4568, and 4571 (both having MW of 50 – 100 kDa), the thiol content of the aminated thiolated samples were approximately 10-fold greater than the thiolated native samples, yet the thiolated native samples had better swelling abilities, cohesion and mucoadhesion. The chain length of the polymer will increase with increasing MW. Mucoadhesion requires interpenetration of the polymer chain in order to obtain a strong bond; however, too long a chain will also inhibit the formation of the bond. This may be due to the lower flexibility of the chain with increasing chain length, which may impede the interpenetration required and inhibit mucoadhesion (Huang *et al*., 2000). This may be the reason for the poor mucoadhesive properties of the above mentioned gelatins. Different polymers will have an optimum chain length for mucoadhesion (Andrews *et al.*, 2009) and from the swelling studies and mucoadhesive testing conducted on all thiolated gelatin samples, gelatin 4569 is thought to be the most optimal.

Gelatin 4569, the lowest MW gelatin sample used with a MW of 20 - 25 kDa, had the greatest potential for mucoadhesion. A thiolated sample aminated at pH 5 resulted in an amine content of approximately 1200 µmol/g and once thiolated, had a thiol content of 660 µmol/g. The sample had excellent swelling abilities and mucoadhesive properties. In an attempt to increase the product yield, the amination reaction was conducted at pH 6. The thiolated sample resulted in a thiol content of 257 µmol/g. The swelling abilities of this pH 6 sample were poor, with the tablet disintegrating quickly. However, in mucoadhesive testing, the tablet adhered for greater than 24 h, displaying vastly improved mucoadhesion in comparison to the thiolated control samples. The tablet, however, did not swell during the mucoadhesive testing and remained a solid tablet throughout the experiment. The lack of cohesion and lack of swelling abilities of this material affected the drug release profile of chlorpheniramine maleate. The thiolated samples and thiolated controls had poor drug release patterns and the increase in thiol content did not improve the rate of drug release. However, as this thiolated gelatin 4569 pH 6

sample displayed improved mucoadhesion, a drug delivery device can be formed which allows for the fast release of drug at the specific mucoadhesive site.

The polymer backbone was modified with each step of the reaction, forming a new material. This was observed in both SEM and DSC analysis. In DSC analysis, the T_g decreased with each step of the reaction process across all samples. In the analysis of the pH study samples using gelatin 4567, there was no clear difference in DSC analysis and all samples behaved similarly in swelling and mucoadhesive testing. The gelatin 4567 samples which had been aminated with differing concentrations of ethylene diamine did display a change in T_g; In the aminated samples, increasing concentrations of ethylene diamine decreased the T_g. Again, the thiolated samples had a marginally lower T_g than the aminated counterparts. The gelatin 4569 samples aminated at pH 5 and pH 6 displayed substantially difference mucoadhesive abilities although the thiol content was similar. DSC showed a marked difference in T_g between the pH 5 and the pH 6 aminated and thiolated samples. This difference was also greatly different to that of the 4567 pH study samples, and the T_g of the pH 5 4569 sample was markedly higher than that of the pH 6 4569 sample.

No paper has previously been published investigating the mucoadhesive properties of thiolated gelatin. This novel two-step reaction process of amination followed by thiolation conducted on this natural polymer has resulted in an increase in thiolation of up to 10-fold in comparison to control samples. Of the four gelatin samples which were investigated in this study, of MW 20 – 25 kDa, 40 – 50 kDa and 50 – 100 kDa, gelatins 4569, 4567, and 4568 and 4571 respectively, it was concluded that gelatin 4569 was the most promising for utilisation of gelatin as a mucoadhesive system. It showed improved cohesive and mucoadhesive properties in comparison to the samples of higher MW. Increased thiol content is an important factor in cohesion and mucoadhesion and samples of gelatin 4569 did, in general, have higher thiol content than the other samples as discussed in chapter 3. However, samples of differing MW and comparable thiol content were investigated and gelatin 4569 did have improved cohesive and mucoadhesive properties. Therefore, it was concluded in this study using four gelatin samples of varying MW that a MW of 20 – 25 kDa was the most optimal as a mucoadhesive drug delivery system and that an investigation of optimal MW is critical in the design of a novel mucoadhesive polymeric system.

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Chapter 5 *Synthesis of thiolated polyallylamine and analysis of mucoadhesive properties*

5.1 Introduction

Synthetic polymers, such as polyacrylic acid, have a definite, repeating structural unit in their backbone. They would, therefore, display less variability in MW in contrast to natural polymers, such as the protein gelatin which was observed in chapter 3 to have marked variability in MW, with each sample having a broad range of MW as was demonstrated by SDS-PAGE. The variability of gelatin (chapter 4) led to inconsistent levels of thiolation and, consequently, inconsistent levels of cohesion and mucoadhesion. As such, it was thought that returning to a synthetic polymer may result in more repeatable results, which would allow for better control of thiol content and ultimately mucoadhesive levels. Therefore, an investigation into the thiolation of another synthetic polymer, polyallylamine will be conducted and the thiolated product will be examined for its cohesive and mucoadhesive properties. Polyallylamine has a simple structure with repeating primary amine moieties attached to the backbone. It is available in a solution in its free base form, PAAm, (Figure 5.1 (A)) or in a salt form as polyallylamine hydrochloride, PAH (Figure 5.1 (B)) or polyallylamine carbonate.

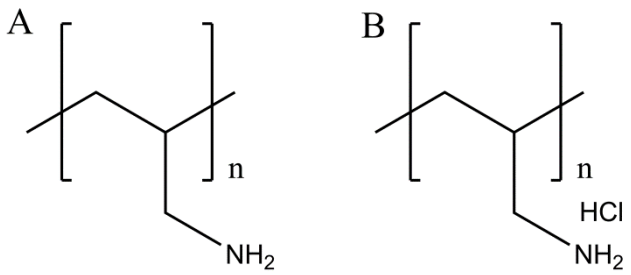

Figure 5.1 Structure of (A) polyallylamine (PAAm) and (B) polyallylamine hydrochloride (PAH)

Similar to thiolated gelatin, limited work has been published on the thiolation of polyallyalmine or its potential use as a mucoadhesive drug delivery system. As the polymer backbone comprises entirely of primary amines, the potential levels of thiolation could be extremely high. Modification of just 5% of these primary amines using a 15 kDa polymer would result in approximately 900 µmol/g thiol content once thiolated. Thiolation of polyallylamine has been conducted using two different methods: thiolation with N-acetyl cysteine and EDC (Vigl *et al.*, 2009) or thiolation

with Traut's reagent (Bacalocostantis *et al.*, 2012). Both methods have also been used in the thiolation of the polysaccharide, chitosan, in which reaction with N-acetyl cysteine resulted in a thiolated chitosan product with a thiol content of 325.5 µmol/g (Schmitz *et al.*, 2008) and thiolation with Traut's reagent resulted in a thiolated chitosan product with up to 408.9 µmol/g thiol content (Bernkop-Schnürch *et al.*, 2003).

Using a method similar to the thiolation of PAA in chapter 2, Vigl *et al.* (2009) thiolated polyallylamine with N-acetyl cysteine, the reaction of which is shown in Figure 5.2. In chapter 2, PAA was reacted with L-cysteine and EDC; it was the carboxylates on PAA which were activated with EDC, which in turn allowed for the amine group on L-cysteine to react and bind to the polymer. In the thiolation of polyallylamine, it is the carboxylates on the cysteine derivative which are activated with EDC and, therefore, N-acetyl cysteine must be used to prevent unwanted side reactions of intermolecular crosslinking between cysteine molecules due to reactions with EDC. Vigl *et al.* dissolved N-acetyl cysteine in water and added EDC. The pH of this solution was adjusted to 4.5 and activation of the carboxylate groups by EDC occurred for 45 min, after which, polyallylamine hydrochloride (PAH) was added. The reaction was left stirring for 3 h prior to dialysis and lyophilisation. PAH crosslinked with dimethylsuccinate was also thiolated and thiol levels were compared to non-crosslinked thiolated samples. The resulting thiol content of the thiolated PAH samples were low, measuring 77.6 µmol/g in the non-crosslinked samples to 162.5 µmol/g in the crosslinked sample. The thiolated PAH products were investigated for their efflux pump inhibitory properties and neither the cohesive nor mucoadhesive properties of the thiolated product were explored.

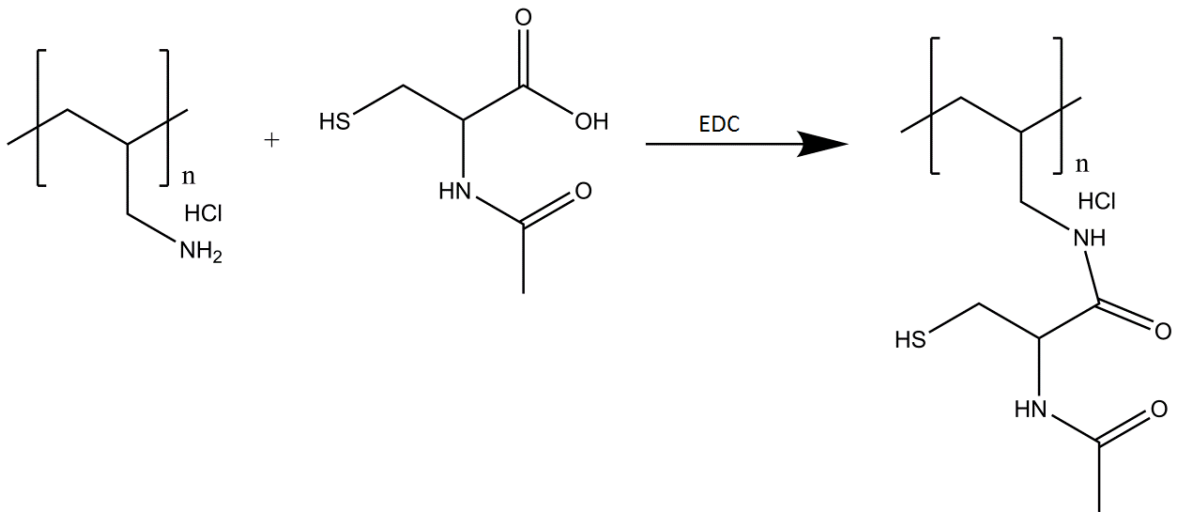

Figure 5.2 Thiolation reaction of PAH and N-acetyl cysteine and the crosslinker, EDC (Vigl *et al.*, 2009)

Using the free base form of polyallylamine, PAAm, Bacalocostantis *et al.* (2012) thiolated PAAm with Traut's reagent (Figure 5.3). Bacalocostantis *et al.* adjusted the amounts of Traut's reagent added, creating a number of products with differing levels of theoretical thiolation, 5%, 13% and 20%; the actual thiol content of the three thiolated PAAm samples was verified with both ^{1}H NMR and the Ellman's reagent assay and resulted in 4.59 ± 2.43%, 13.07 ± 1.61% and 19.27 ± 0.07% thiolation of the PAAm backbone. Again, this paper did not explore the mucoadhesive properties of thiolated PAAm but investigated the use of thiolated PAAm as a delivery vector for plasmid DNA. DNA-polymer binding, complex size, buffering capacity at endosomal pH and pH-sensitive gene release were all analysed using unmodified PAAm and the three levels of thiolated PAAm. All three thiolated samples showed complexation to the plasmid DNA which was not displayed by the unmodified PAAm. It was shown that 13% thiolation had the greatest potential as a gene delivery vector. The 5% thiolated sample was observed to have the highest levels of crosslinking through disulphide bond formation which affected release rate of the enclosed DNA. The 20% sample had the highest levels of buffer capacity, however, it was also inefficient at the release of DNA and also provided poor protection of the enclosed DNA.

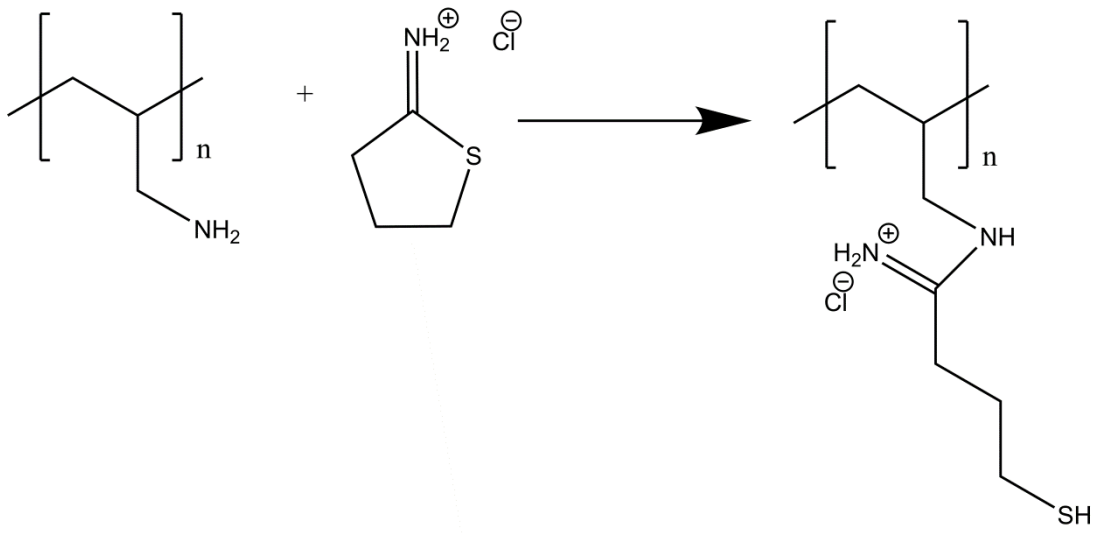

Figure 5.3 Thiolation of PAAm with Traut's reagent (Bacalocostantis *et al.*, 2012)

Both methods of thiolation, reacting with Traut's reagent or with N-acetyl cysteine/EDC, were used by Ibie *et al.* (2015) and the mucoadhesive properties of the resulting thiolated polyallylamine products were tested. Ibie *et al.* used PAH in both thiolation reactions. The pH of the PAH used was, however, adjusted with NaOH and dialysed to yield the free base form of polyallylamine, PAAm, which was then thiolated with both Traut's reagent and N-acetyl cysteine. Similar to results observed by Vigl *et al.* (2009), the thiol content of the resulting samples thiolated with N-acetyl cysteine were low, measuring 60 µmol/g which was in vast contrast to the greatly improved thiolation levels observed with Traut's reagent, which resulted in a thiol content of 490 µmol/g. Disulphide bond formation of the samples was also examined. The samples displayed high levels of disulphide bond formation, with samples thiolated with N-acetyl cysteine resulting in disulphide bond levels of 280 µmol/g and reaction with Traut's reagent measuring 590 µmol/g formed as disulphide bonds. Mucoadhesion testing was conducted using a porcine mucin solution; solutions of both the thiolated polymers and unmodified polyallylamine were mixed with the mucin solution and tested by UV/Vis spectrometry to determine the levels of adsorption of mucin to the polymer. Thiolation of the polymer displayed improved mucin interactions in comparison to unmodified samples. The percentage of mucin adsorption increased from approximately 40% in the unmodified PAAm sample to 65 – 70% in the thiolated PAAm samples. However,

no swelling tests or mucoadhesive testing on porcine mucosal tissue were conducted with either of the thiolated samples.

5.1.1 Aims and objective

The aim of this study is to create a thiolated polyallylamine (PAAm) product using Traut's reagent as the method of thiolation. By varying the concentration of Traut's reagent added to the reaction, thiolated products with a range of thiol contents will be synthesised and compared to unmodified samples for their swelling ability and their cohesive and mucoadhesive properties, with a view to assessing their suitability for use as a mucoadhesive polymer.

5.2 Materials and methods

5.2.1 Materials

15% PAAm solution of 15 kDa MW was purchased from Polyscience, USA. D_2O was purchased from Sigma Aldrich, Ireland. Iodine was obtained from Riedel-de Haen.

5.2.2 Thiolation of polyallylamine (PAAm)

5.2.2.1 Thiolation using Traut's reagent

Thiolation of PAAm was conducted according to the thiolation of gelatin method in chapter 3, section 3.2.6. Using a 15% PAAm solution of 15 kDa MW, 500 mg (corresponding to 75 mg of polymer) was dissolved in 50 mL of deionised water. The pH of the solution was adjusted from pH 9.5 to approximately pH 7.5 with 1M HCl. To this, 0.375 mg, 0.1875 mg and 0.075 mg of Traut's reagent per mg of polymer were added to achieve 5%, 2.5% and 1% theoretical thiolation of the polyallylamine backbone, respectively. Once Traut's reagent was added, the pH of the solution dropped marginally, from pH 7.5 to 7.36 and the solution was left stirring at room temperature for 20 min, before it was readjusted to pH 5 with 1 M HCl. The solution was then left stirring, again at room temperature, for 3 h before

being dialysed exhaustively in the dark in a 3.5 kDa cellulose membrane against 5 mM HCl and 1 mM HCl. After dialysis, the samples were frozen and freeze dried.

5.2.3 Thiol content determination

5.2.3.1 Ellman's reagent

Thiol content determination was conducted using Ellman's reagent solution, as outlined in chapter 2, section 2.2.3. A 1 mg/mL solution of unmodified PAAm in D.I. water was prepared as a reference standard.

5.2.3.2 2,4,6-trinitrobenzene sulfonic acid (TNBS) method of amine quantification

Amine quantification was conducted as outlined in chapter 3, section 3.2.5.

5.2.3.3 Proton nuclear magnetic resonance (^{1}H NMR)

Thiolation of the unmodified polymer was verified with proton NMR using a Joel ECX-400 operating at 400 MHz. Samples of unmodified and thiolated PAAm were dissolved in D_2O at a concentration of 8 mg/mL.

5.2.3.4 Iodometric titration

Iodometric titration was also used to determine the thiol content of the samples. This was conducted in accordance to Vigl *et al.* (2009). 1 mg/mL thiolated polymer solutions were made in deionised water and the pH of the solutions was adjusted to between 1 – 2. This was then titrated against a 1 mM iodine solution, also made up in water. 300 µL of a 1% starch indicator was added to the polymer solutions to aid end point detection. The end point was noted as a colour change from colourless to a permanent light blue colour. Thiol content was determined from a set of standards ranging from 0 - 60 µg cysteine/mL (0 - 1000 µmol thiol/g polymer) which were also titrated against a 1 mM iodine solution.

5.2.3.5 Determination of disulphide bond formation

Thiolated PAAm samples were reduced using $NaBH_4$ to analyse the formation of disulphide bonds. Samples at a concentration of 1 mg/mL were made in 50 mM Tris buffer, pH 7.4. To each sample, 4% $NaBH_4$ was added and the samples were incubated for 1 h at 37°C with gentle stirring. The pH was then adjusted with 5 M HCl to pH 1 – 2 and the solutions were incubated for 10 min to quench $NaBH_4$. The iodometric titration was then conducted on the samples as detailed in section 5.2.3.4.

5.2.4 Tablet formation

Unmodified PAAm solution was freeze dried for tabletting, however, the samples were difficult to compress. Unmodified PAAm were dissolved in 20 mL D.I. water at 37 °C with the addition of 1%, 5%, 10% or 15% (w/w) gelatin 4569 (MW = 20 – 25 kDa) to act as an excipient and aid binding. These samples were then freeze dried and compressed into tablet form with 150 bar of pressure before swelling studies analysis of the tablets was carried. Thiolated samples were compressed with and without the addition of 5% gelatin and compared to the unmodified PAAm-gelatin conjugates.

5.2.5 Swelling studies

Swelling studies were conducted on unmodified PAAm with the addition of gelatin and on thiolated PAAm samples and thiolated PAAm samples with 5% gelatin addition according to the method outlined in chapter 2, section 2.2.4.

5.2.6 Mucoadhesion testing

Mucoadhesive testing was conducted on thiolated samples using porcine gastrointestinal tissue according to the method outlined in chapter 2, section 2.2.5.

5.3 Results and discussion

5.3.1 Thiolation of PAAm

Thiolation of PAAm using Traut's reagent was conducted using the thiolation of gelatin method as described in chapter 3, section 3.2.6. Similar to this study, using Traut's reagent, Bacalocostantis *et al.* (2012) aimed to create thiolated PAAm samples with 5%, 13% and 20% total thiolation of the polymer backbone. 5% total thiolation could yield a polymer which had approximately 900 µmol/g thiol content, and, as this was the case, it was decided in this study to also aim for 5% total thiolation, with the addition of 0.375 mg Traut's reagent per 1 mg polymer, as used by Bacalocostantis *et al.* (2012). A thiol content of 900 µmol/g would result in a polymer with high thiol content from the perspective of a mucoadhesive polymer; as observed with thiolated PAA (chapter 2), samples with higher degrees of thiolation than approximately 900 µmol/g showed lower levels of mucoadhesion and higher rates of swelling in comparison to samples of lower thiol content, therefore, potentially making them unsuitable for mucoadhesive drug delivery. Similarly, too high a thiol content could result in a polymer which crosslinks to a large degree, which will in turn affect the mucoadhesive nature of the polymer due to lower levels of free thiol groups being available for interaction with the thiol residues of the mucosal layer (Marschütz and Bernkop-Schnürch, 2002). Because of this, two more thiolated samples were also synthesised by the addition of half and one fifth of the initial 0.375 mg Traut's, 0.1875 and 0.075 mg Traut's per mg polymer, respectively. Therefore, the overall theoretical thiolation from these three reactions could produce 5%, 2.5% and 1% levels of thiolation of the PAAm backbone, potentially yielding 900, 450 and 180 µmol/g thiolation. In this chapter, the thiolated samples are described as 5% TPAAm, 2.5% TPAAm and 1% TPAAm, respectively. Thiolation levels of the 5% TPAAm, 2.5% TPAAm and 1% TPAAm samples are displayed in Table 5.1.

Table 5.1 Thiol content of thiolated PAAm samples

Sample	Thiol content (µmol/g)
1% TPAAm	133.6 ± 43.3
2.5% TPAAm	329.5 ± 13.4
5% TPAAm	487.4 ± 18.3

Bacalocostantis *et al.* thiolated polyallylamine in a PBS buffer at pH 7.4; as it was thought this would encourage the formation of disulphide bonds within the polymer matrix, the thiolation reaction in this study was conducted in water, similar to the gelatin thiolation reaction in chapter 3. As also mentioned in chapter 3, the efficiency of Traut's reagent is greatest between pH 7 – 10. Traut's reagent can hydrolyse in solution, ring opening to form methyl 4-mercaptobutyrimidate and the half-life of the compound decreases as the pH increases (Hermanson, 2008; Goddard and Hotchkiss, 2008). As this was the case, the pH of the PAAm solution was adjusted with 1 M HCl from approximately pH 9.5 to between pH 7 – 8 prior to the addition of Traut's reagent. Once Traut's reagent was added, similar to the thiolation of gelatin in chapter 3, the pH of the reaction was again adjusted with 1 M HCl from pH 7 to pH 5 after 20 min. The efficiency of Traut's reagent is rapid (Jue *et al.*, 1978), and the change in pH should lessen the formation of intramolecular disulphide bonds, as thiol groups are more reactive at higher pH. The total reaction time was 3 h and the samples were then dialysed against 5 mM HCl and 1 mM HCl, prior to freeze drying.

It was observed during the reaction and dialysis process that the dialysis membrane used was of great importance. The thiolated samples were initially dialysed using a 3.5 kDa membrane. When a 12 kDa membrane was used, the samples were visually different post freeze-drying, and their cohesive and mucoadhesive properties were diminished. As the PAAm used in this study had a MW of 15 kDa, it was possible that the thiolated polymer was dialysing through the membrane. Therefore, a 3.5 kDa membrane was used throughout.

5.3.2 Thiol content determination

5.3.2.1 Ellman's reagent and 2,4,6-trinitrobenzene sulfonic acid (TNBS) method of amine quantification

Ellman's reagent was used to determine the thiol content of both thiolated PAA and thiolated gelatin, as described in chapters 2 and 3. It was also utilised for thiol determination of the thiolated PAAm samples; however, due to precipitation during the Ellman's assay, a true value of thiol content of the thiolated PAAm samples could not be determined. The samples were both diluted and filtered in an attempt to

quantify the thiol content, both of which were unsuccessful. The TNBS method for amine quantification was also employed to determine the thiol levels in an indirect fashion; Traut's reagent reacts with primary amine groups yielding free thiol groups, therefore, the amine content should decrease with increasing levels of thiolation. However, similar to the Ellman's assay, the polymer samples precipitated and an accurate amine level could also not be determined. The samples were also diluted in an attempt to measure the samples, but absorbance levels were then too low for an accurate reading. Because of this, other methods of thiol quantification were utilised, and the samples were initially analysed by Proton nuclear magnetic resonance (^{1}H NMR) to determine if modification had occurred. An iodometric titration was then employed to determine the thiol content of the three thiolated samples (Vigl *et al.*, 2009; Clausen and Bernkop-Schnürch, 2000; Bernkop-Schnürch and Steininger, 2000; Ibie *et al.*, 2015).

5.3.2.2 Proton nuclear magnetic resonance (^{1}H NMR)

Due to the issues encountered with both Ellman's and TNBS assays, the thiolated PAAm samples were firstly analysed by ^{1}H NMR and compared to unmodified PAAm samples. Shown in Figure 5.4 are the NMR spectra of (A) unmodified PAAm and (B) 1% TPAAm. At approximately 4.6 ppm, in both the unmodified and all the thiolated samples there was a solvent peak, corresponding to D_2O (Harwood and Moody, 1994). The NMR spectrum of unmodified PAAm was similar to that observed by Cai *et al.* (2008). When comparing the unmodified PAAm sample to the thiolated samples, there was a marked difference in the NMR spectra, with the addition of a number of extra peaks within the thiolated sample. This was to be expected as the backbone of the polymer was altered greatly by the reaction of Traut's reagent. As mentioned in chapter 3 and highlighted in Figure 5.3, Traut's reagent reacts in a ring opening reaction, binding to the primary amine group of polyallylamine, resulting in a free thiol group at the end of the chain; both the unmodified and thiolated structures of PAAm are shown in the insets in Figure 5.4. Therefore, there has been a discernible addition to the polymer backbone upon thiolation, which is evident from the NMR spectra. Looking at the spectra between 0 – 4 ppm, there was a number of additional peaks, including the smaller peaks along the baseline, in the thiolated sample in comparison to the unmodified sample, which

correspond to the ring opening addition of the Traut's reagent. Looking at the structure of the thiolated product, both the thiol group and the imine group will have an electron withdrawing effect on the resulting chemical shift, resulting in the peaks observed at between 3 – 4 ppm. As some of the peaks within the spectra are broad and may be masking other signals, the proposed peak assignment for both unmodified and thiolated PAAm is shown in Figure 5.4. The integration values between and ratios of the protons marked a, b, c and d did not change upon modification through thiolation, however, the large chemical shift in these protons in the thiolated sample, in particular the proton labelled d, was due to the addition of the Traut's reagent chain and its electronegative effect. Similarly, the primary amine groups of the unmodified PAAm will have decreased in intensity due to thiolation and it may be that the small peak of the NH group in thiolated spectrum in Figure 5.4 (B) is in a similar position as the NH_2 group in the unmodified spectrum. The proposed position of the thiol group was at 3.6 ppm and the electronegativity value of that thiol group has caused a chemical shift in the protons beside it (marked i). There are ten protons identified within the thiolated structure and only nine clear peaks in the structure. The imine group, marked f in Figure 5.4 (B), has not been identified in the thiolated sample spectrum. It may be that this peak is being shadowed by the larger and broader peaks within the sample; for example, there is a shoulder on the peak marked b in the thiolated sample which may be the proton of the imine group.

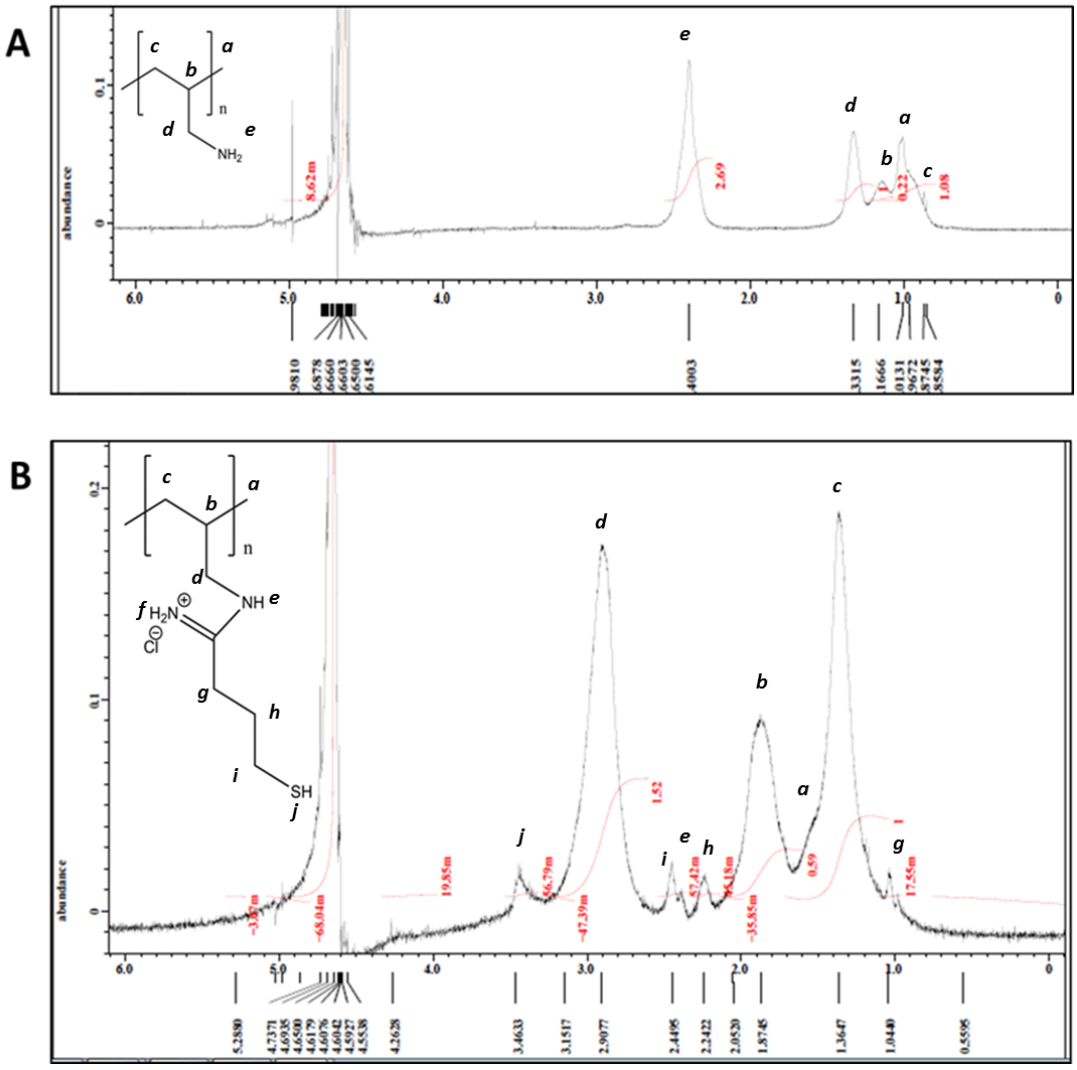

Figure 5.4 NMR spectra of (A) unmodified PAAm and (B) 1% TPAAm

5.3.2.3 Iodometric titration

The quantification of thiol content between the three thiolated samples could not be achieved by NMR, and therefore, an iodometric titration was conducted to determine the thiol content of the samples.

The iodometric titration uses iodine in excess which is oxidised to iodide by the thiol groups of the thiolated polymer, changing from the brown-orange colour associated with iodine to a clear solution. Using starch as an indicator, the iodide ions bind to the starch changing to a light blue at the end-point. Low pH and low temperatures were found to be of benefit for an iodometric titration (Virtue and Lewis, 1934), stabilising the iodine in solution. Because of this, the pH of the aqueous thiolated sample solutions was adjusted to pH 1 – 2 before the titration was conducted.

As shown in Figure 5.5, the thiol content of the samples increased with increasing levels of Traut's reagent added to the reaction process, resulting in thiol contents of 133.6 ± 43.3 µmol/g in the 1% TPAAm sample, 329.5 ± 13.4 µmol/g in the 2.5% TPAAm sample and 487.4 ± 18.3 µmol/g in the 5% TPAAm sample. It was observed, however, that the levels of thiolation achieved in the 5% TPAAm samples were lower than that of the theoretical levels of thiolation, with 487.43 µmol/g achieved and a 900 µmol/g theoretical value.

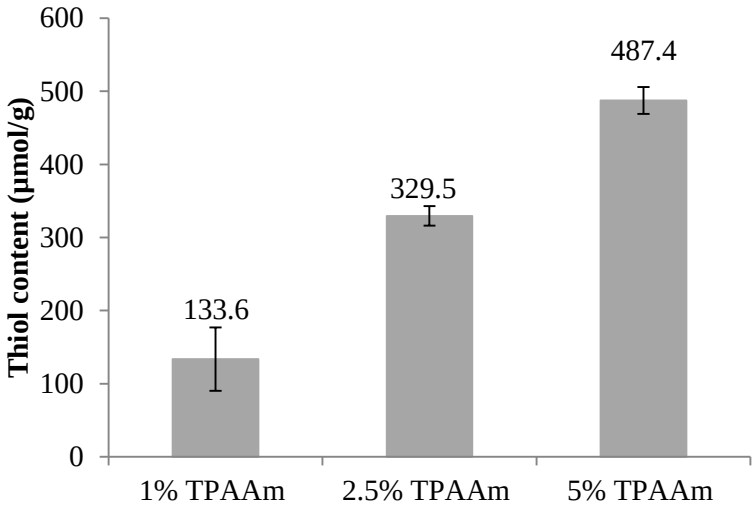

Figure 5.5 Thiol content of PAAm samples, displaying thiol content of the TPAAm samples (n=3)

The thiolation reaction was initially conducted at between pH 7 - 8 and, after 20 min, the pH was then adjusted to pH 5. During the reaction of 5% TPAAm sample, as the highest mass of Traut's reagent was added to this reaction, it was more difficult to maintain the pH at between 7 – 9, the pH at which Traut's reacts most efficiently and often the pH fell to between 6 – 6.5 within the 20 min period. The Traut's reagent reaction proceeds rapidly at pH 7 – 9 (Hermanson, 2008) and the drop in pH over the first 20 min may have had an effect on both the efficiency of the Traut's reagent reaction and the resulting thiolation levels as the reaction may have slowed. The reaction pH of both the 1% TPAAm and 2.5% TPAAm samples remained above pH 7 throughout the 20 min period and both samples of 1% TPAAm and 2.5% TPAAm are comparable to the theoretical values of 180 µmol/g and 450 µmol/g, yielding

products with thiol contents of 133.6 ± 43.3 µmol/g and 329.5 ± 13.4 µmol/g respectively.

A similar thiol content to the content of 5% TPAAm was achieved by Ibie *et al* (2015) when thiolating PAAm with Traut's reagent. However, to 500 mg of PAAm, Ibie *et al*. added 400 mg of Traut's reagent. Therefore, Traut's reagent was added at a ratio of 0.8 mg per mg of polymer in comparison to 0.375 mg per mg polymer as was used in the 5% TPAAm samples in this study.

5.3.2.4 Determination of disulphide bond formation

All the thiolated samples were reduced using $NaBH_4$ and were then analysed using the iodometric titration to investigate the levels of disulphide bonds formed during both the reaction and dialysis processes. The disulphide bond contents are highlighted in Table 5.2.

Table 5.2 Thiol content and disulphide bond content of thiolated PAAm samples

Sample	Thiol content (µmol/g)	Disulphide bond content (µmol/g)	Total thiol content (µmol/g)
1% TPAAm	133.6 ± 43.3	111.1 ± 78.6	244.7
2.5% TPAAm	329.5 ± 13.4	81.9 ± 57.9	411.4
5% TPAAm	487.4 ± 18.3	219.3 ± 5.9	706.7

The total thiol content of the three thiolated samples is also highlighted in Table 5.2. The theoretical thiol content of the 1% TPAAm sample measured 180 µmol/g; looking at the total thiol content of the 1% TPAAm sample in Table 5.2 shows that it is marginally higher than the theoretical thiol value which was not the case for either the 2.5% or 5% TPAAm samples. However, the error associated with both the thiol content and the disulphide bond content of the 1% TPAAm was markedly higher than that of the other two samples which may explain the higher total thiol content. Similar to the gelatin thiolation reaction, the pH of the TPAAm reaction solution was altered after 20 min, from pH 7 to pH 5, in order to minimise the formation of disulphide bonds. Additionally to this, all the thiolated samples, PAA, gelatin and PAAm, were dialysed in acidic conditions to again minimise disulphide bond

formation during the dialysis process. The formation of disulphide bonds within both the 1% TPAAm and 2.5% TPAAm samples was measured at 111.1 and 81.9 µmol/g, respectively. Disulphide bond formation within the 5% TPAAm sample was higher than the 1% TPAAm and 2.5% TPAAm samples. The thiol content of the 5% TPAAm sample was lower than the theoretical value and the higher levels of disulphide bonds observed may have contributed to this lower thiol content. It was noted by Ibie *et al*. (2015) that high levels of disulphide bonds formed in PAAm when thiolated with Traut's reagent; thiol content of the samples was measured at 490 µmol/g and disulphide bond content was measured at 590 µmol/g in the Traut's thiolated samples. The reaction of the thiolated samples, as conducted by Ibie *et al.*, was carried out under nitrogen to minimise thiol oxidation and the samples were dialysed against a HCl solution; however, in comparison to this study, the pH of the reaction was not altered and was conducted in the dark for 14 h at pH 6.5. At this pH, disulphide bond formation would be higher. In comparison, in this study, the change in pH from 7 to 5 during the reaction has minimised disulphide bond formation, resulting in lower levels of disulphide formation within the samples in comparison to Ibie *et al.*, as is shown in Table 5.2. Also, as noted above, the ratio of Traut's reagent added to the thiolation reaction by Ibie *et al.* was more than twice the ratio added in this study. As a higher amount of thiolating agent was added, it would follow that disulphide bond formation would also be higher, particularly when the reaction was conducted at the higher pH.

5.3.3 Swelling studies

Swelling studies were conducted on unmodified and thiolated PAAm samples. The unmodified PAAm solution was freeze dried to enable the compression of a tablet, and also to be consistent with the analysis, for comparison to other thiolated polymers. However, the freeze dried sample did not compress into a uniform tablet and quickly became a tacky material once compressed, possibly due to the hygroscopic nature of the polymer (Hoskins *et al.*, 2012). PAH was also utilised as a control, however, due to the crystalline nature of PAH, it also did not compress. Because of this, gelatin was added to unmodified PAAm to aid the binding of the samples, allowing unmodified PAAm to be used as a control in swelling studies, mucoadhesive testing and drug release studies. As was observed in chapter 4, native

gelatin had limited swelling ability but it is a substance that is commonly used in the pharmaceutical industry as an excipient (Jones *et al.*, 2011). Initially, 1%, 5%, 10% and 15% (w/w) gelatin was added to the unmodified tablet and swelling studies were conducted on the samples. As shown in Figure 5.6, as the ratio of gelatin increased, the initial rate of swelling also increased. Martínez-Ruvalcaba *et al*. (2009) also observed an increase in swelling ability due to the increased ratio of gelatin addition to polyacrylamide hydrogels. Similar to this study, 5%, 10%, 15% and, additionally, 20% gelatin (w/w) was added to polyacrylamide and swelling studies in water were conducted. As the addition of gelatin increased, so did the swelling ability of the sample and Martínez-Ruvalcaba *et al*. surmised that the addition of the hydrophilic gelatin into the hydrogel increased the swelling ability and therefore, as gelatin concentration increased so did the hydrophilicity and swelling capacity. In this study, although the initial rate of swelling increased with gelatin addition, the ratio of added gelatin did not affect the overall degradation times of the unmodified samples, with all PAAm-gelatin samples disintegrating within 2 min (Figure 5.6). It was concluded that all levels of gelatin aided compression; the addition of 1% gelatin, although aiding compression, did result in a sticky material similar to the unmodified PAAm sample without gelatin addition and because of this, it was not used. The addition of 5%, 10% and 15% had similar swelling profiles, but with 5% addition having the lowest influence on the swelling ability and, therefore, the addition of 5% gelatin to unmodified PAAm was used henceforth as a control.

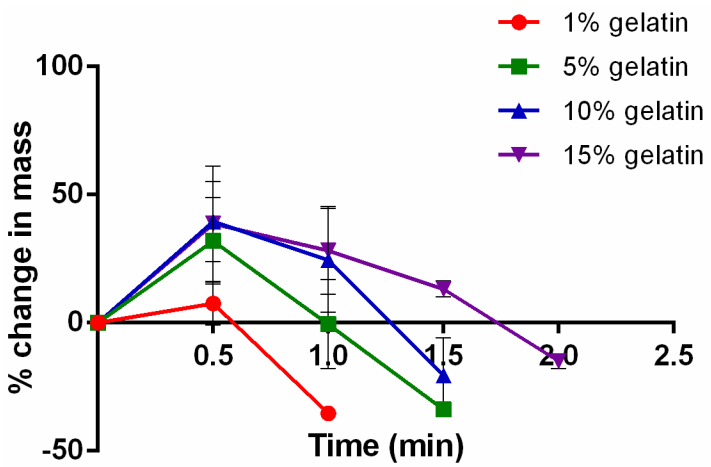

Figure 5.6 Swelling studies of unmodified PAAm with the addition of varying levels of gelatin

Swelling studies were conducted on thiolated samples of PAAm with differing ratios of Traut's reagent added, as shown in Figure 5.7. All thiolated samples, 1% TPAAm, 2.5% TPAAm and 5% TPAAm, had high levels of swelling initially, with an increase in mass of up to 1500% within the first minute. The swelling ability of the samples increased with increasing amount of Traut's added, therefore, 5% TPAAm swelled to a greater extent than the 1% TPAAm sample. The swelling results again seem to contradict theory, similar to the results of thiolated PAA in chapter 2. The 5% TPAAm sample had a thiol content of approximately 490 µmol/g, as shown in Figure 5.5, which was higher than both the 1% TPAAm and 2.5% TPAAm samples; therefore, there should be a higher potential for crosslinking due to disulphide bond formation and thus, in theory, the swelling ability of the 5% TPAAm sample should be lower than that of both the 1% TPAAm and 2.5% TPAAm samples. Bacalocostantis *et al*. (2012) thiolated 5%, 13% and 20% of the total PAAm backbone with Traut's reagent and noted that the sample with the lowest level of thiolation had in fact the highest level of crosslinking. Looking at the disulphide and thiol contents of the three thiolated PAAm samples shows that the overall ratio of disulphide content to total thiol content, i.e. free thiol content and disulphide content added together, is highest in the 1% TPAAm sample. Therefore, similar to Bacalocostantis *et al.* the sample with the lowest thiol content had the highest level of crosslinking. As this was the case, this may also explain why the swelling rate was lower in the 1% TPAAm in comparison to the 5% TPAAm sample, as higher degrees of crosslinking had already occurred within the 1% TPAAm sample creating a more cohesive system.

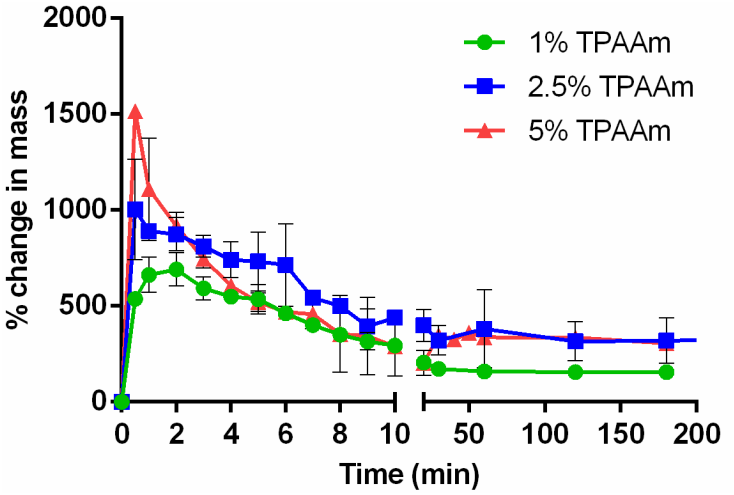

Figure 5.7 Swelling studies of thiolated PAAm samples with varying thiol content (n=3)

The swelling profiles of the thiolated PAAm samples were all similar. Although the initial swelling of all the samples was rapid, over the subsequent 10 min all samples began to decrease in mass, indicating a loss of buffer uptake or disintegration. However, all samples then plateaued at approximately 150 - 300% of their original masses, and remained in this swollen state for a number of hours. It is possible that the initial swelling rate of the samples was so large and so rapid, that it became the dominating factor within the matrix and inhibited the crosslinking ability of the samples. All samples may then have reached swelling maxima and, as the samples crosslinked, the samples may have relaxed into a crosslinked yet swollen state at which they remained for a number of hours. The thiolation of the samples was quite limited, as little as 1% of the amine groups were theoretically thiolated during the reaction process within the 1% TPAAm sample. Because of this, the crosslinking ability of the samples may also have been limited and the distance between thiol groups may have been too great, particularly when the initial swelling rate was so fast. However, increased thiolation displayed increased levels of swelling. As discussed in section 5.1, Ibie *et al.* (2015) thiolated polyallylamine using two methods of thiolation, with N-acetyl cysteine and with Traut's reagent. Using UV/Vis spectrometry, adsorption rates of the thiolated polymer solutions with a porcine mucin solution were tested and indicated mucoadhesive properties. Interestingly, the mucoadhesive properties of the N-acetyl cysteine thiolated product were superior to the system thiolated with Traut's reagent, in spite of the marked increase in thiol content in the Traut's reagent sample, thiol contents were measured

at 60 µmol/g and 490 µmol/g respectively. Ibie *et al.* discussed the role steric hindrance may have played within the two thiolated products; the shielding of the groups within the more thiolated sample, i.e. the sample thiolated with Traut's reagent, was thought to limit the mucoadhesive properties of the samples. Similarly, higher levels of substitution were thought to cause interchain repulsions which resulted in changes in conformation. This, in turn, led to decreased chain flexibility, also reducing mucoadhesion. It was these reasons which were thought to decrease the mucoadhesive properties of samples thiolated with Traut's reagent in comparison to those thiolated with N-acetyl cysteine. Swelling tests were not conducted by Ibie *et al.* on unmodified or thiolated samples. However, as swelling ability is related to mucoadhesion, the potential effect steric hindrance and interchain repulsions discussed by Ibie *et al.* had on the thiolated samples may explain the higher degree of swelling observed in the thiolated PAAm samples with higher thiol content. A similar theory was proposed by Bacalocostantis *et al.* (2012); as mentioned above, the highest levels of disulphide bond formation were measured in samples with lowest thiol content. Bacalocostantis *et al.* surmised that the lack of disulphide bond formation in the 20% thiolated sample was due to steric hindrance and repulsive interactions between polymer chains caused by the addition of Traut's regent.

The thiolated PAAm samples, once swollen, behaved and looked visually different than that of the thiolated PAA samples in chapter 2. During swelling and mucoadhesive testing, the thiolated PAA samples visually became a clear gel-like substance which held its shape and its moisture content when a light force was applied to it, i.e. when dabbed with a tissue. This is in contrast to the thiolated PAAm samples, which seeped moisture from their swollen form when the same light force was applied. Therefore, thiolated PAAm may have a much weaker gel structure than both thiolated PAA and thiolated gelatin samples. This may also explain the loss of mass in the swollen thiolated PAAm samples; the swelling ability of thiolated PAAm, as shown in Figure 5.7, was very rapid in the initial 30 s of the test, but the samples were not as capable in retaining the liquid or maintaining the forces within the swollen gel structure. The thiolated PAA samples (chapter 2) displayed a lower rate of swelling in comparison to the thiolated PAAm samples and were also capable of maintaining the swollen structure up to an increase of over 2000% of their original mass over a longer period of time. The thiolated PAAm samples were more likely to

break during the swelling tests, unlike the thiolated PAA samples, which again suggested a weaker gel structure within the thiolated PAAm samples. As the initial swelling of the thiolated PAAm samples was quite rapid, this may affect both the mucoadhesive properties and the drug release profile of these samples. Drug release may be poor, with potentially a rapid release of drug within the first number of minutes due to the increased swelling ability. This will be discussed further with the release of the model drug, CPM, in chapter 6, section 6.3.1.

Swelling studies were also conducted on thiolated samples which contained 5% gelatin as an excipient and, similar to the unmodified samples, the addition of 5% gelatin altered the swelling properties of the thiolated samples. By comparing the thiolated samples with gelatin addition in Figure 5.8 (A) to those without gelatin addition in Figure 5.7, the initial swelling of the samples has changed; this is also highlighted with the 1% TPAAm sample, with and without gelatin addition, over the first 10 min of swelling in Figure 5.8 (B). The initial swelling ability of the 5% TPAAm sample decreased from approximately 1500% to approximately 1100%; this may be beneficial for the 5% TPAAm sample from a drug release prospective as the large swelling within the first minute was lessened. However, similar to the unmodified PAAm/gelatin samples, the addition of the hydrophilic gelatin into the 1% TPAAm sample has increased the initial swelling rate, increasing it from a percentage swelling rate of 500% to over 1000% when gelatin was added to the matrix, which is highlighted in Figure 5.8 (B). This may again influence the drug release profiles of the 1% TPAAm sample, potentially increasing the initial release of drug. The swelling rate of the 2.5% TPAAm sample was comparable with and without gelatin addition. The overall degradation pattern of all 3 samples remained similar to that of the samples without gelatin addition.

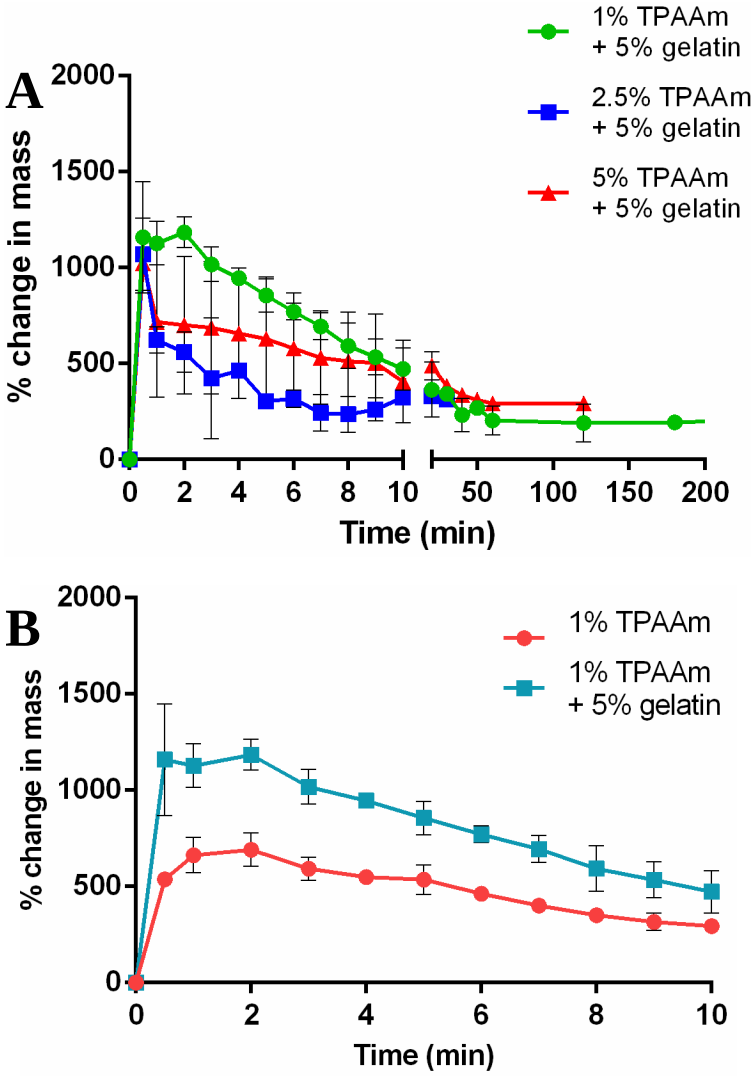

Figure 5.8 Swelling studies of (A) thiolated PAAm samples with the addition of 5% gelatin (n=3) and (B) 1% TPAAm with and without gelatin addition

5.3.4 Mucoadhesive testing

Mucoadhesive testing using the rotating cylinder method was performed on unmodified/5% gelatin control samples, the three thiolated PAAm samples, and on TPAAm/5% gelatin samples.

The mucoadhesive testing of the unmodified PAAm/5% gelatin control samples reflected the swelling studies, and the samples displayed poor mucoadhesive properties. The samples were initially pressed against the intestinal tissue and allowed to adhere for a 2 min period prior to the tissue and tablets being submersed into the phosphate buffer. The samples were tested in triplicate and, during this 2 min incubation period, all three tablets visually began to dissolve; this had also

occurred with the native, unmodified gelatin tablets (chapter 4). The control PAAm/5% gelatin tablets were submerged in the pH 6.8 buffer (37 °C), rotating at 50 rpm, and the tablets quickly dislodged and partially dissolved, adhering for an average of 1.5 ± 0.2 min.

The results of the mucoadhesive testing of the three thiolated samples, 1% TPAAm, 2.5% TPAAm and 5% TPAAm, were vastly different to that of the unmodified/5% gelatin control samples and there was a marked improvement in mucoadhesion upon thiolation, as is shown in Figure 5.9. Despite the difference in thiol content, the mucoadhesive properties of all the thiolated samples in this study, 1% TPAAm, 2.5% TPAAm and 5% TPAAm, were similar and all samples showed excellent mucoadhesion on porcine intestinal tissue. All three thiolated samples adhered for a minimum of 22 h with some tablets adhering for over 48 h. The 1% TPAAm tablets dissolved in the buffer while both the 2.5% TPAAm and 5% TPAAm samples dislodged; this may be due to the difference in the thiol content of the 1% TPAAm sample in comparison to the 2.5% TPAAm and 5% TPAAm samples. This increase in mucoadhesive properties of thiolated PAAm was also observed by Ibie *et al.* (2015), as indicated by the adsorption of a mucin solution onto the thiolated PAAm samples, measured by UV/Vis spectroscopy.

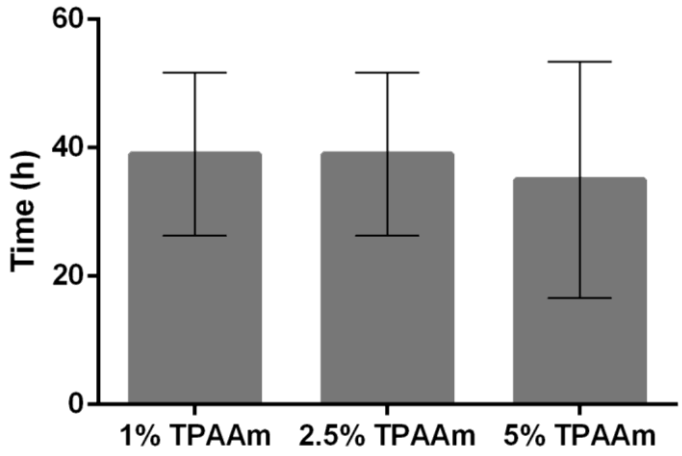

Figure 5.9 Mucoadhesive testing of thiolated PAAm samples (n=3)

The samples swelled rapidly once submerged into the 37 °C phosphate buffer, which was similar to the swelling studies, however, the swelling rate appeared not to be to

the same extent. As the thiolated samples swelled, the surface area of the samples also increased rapidly, much more so than the thiolated PAA samples had in chapter 2, and this is illustrated in Figure 5.10. The increase in surface area is seen to be advantageous as there is a greater ability for interpenetration of the polymer chains into the mucosal layer, allowing for a stronger mucoadhesive bond (Andrews *et al.*, 2009). Therefore, the increased surface area may have contributed to the improved mucoadhesive properties of the thiolated PAAm samples. Similarly, the functionality of the polymer may also have encouraged secondary bonding to the mucosal layer, with hydrogen bonding occurring between the polymer and the mucus (Andrews *et al.*, 2009). As was mentioned in section 5.3.3, during swelling tests, the texture of the thiolated PAAm samples was very different to that of the thiolated PAA samples. This was also the case in mucoadhesive testing; the thiolated PAAm samples remained a white tablet throughout testing and did not become a clear gel-like material, in contrast to the thiolated PAA samples in chapter 2. Again, similar to the swelling studies, the swelling ratio of the thiolated PAAm samples appeared to lessen as the test progressed. This was in complete contrast to the thiolated PAA samples (chapter 2), which visually continued to swell during the mucoadhesive testing, becoming a clear gel-like material after 4 h. The visual difference between the thiolated PAA samples and thiolated PAAm samples is displayed in Figure 5.10; the image shows the difference in swelling between thiolated PAA (left) and thiolated PAAm samples (right) after a period of 10 min during mucoadhesive testing. The samples displayed in Figure 5.10 were analysed at the same time, thus highlighting the difference between the two thiolated polymers; the thiolated PAA samples on the left in Figure 5.10, retained the ~1 cm diameter but had begun to change to a clear gel, which can be observed on the edges of the samples. This is in contrast to the thiolated PAAm sample, Figure 5.10 (right), which had increased in diameter, by approximately double, and remained a white tablet throughout testing.

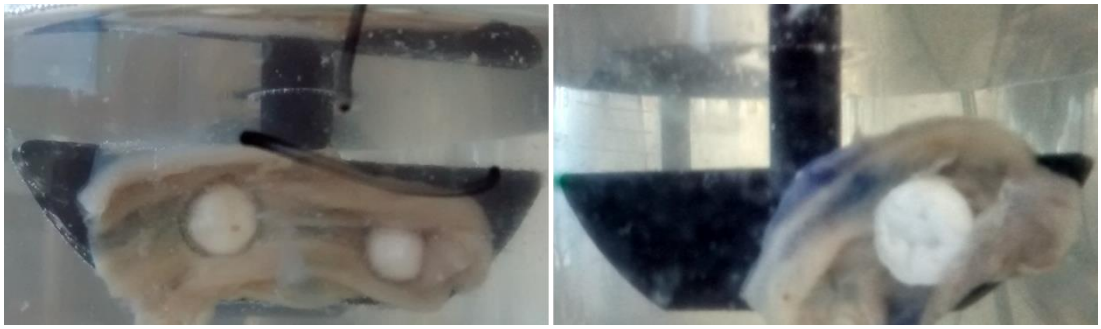

Figure 5.10 Mucoadhesive testing of thiolated PAA samples, pH 6 and pH 5.5 (left) and 5% TPAAm sample (right) after 10 min

Thiolated PAAm samples with the addition of 5% gelatin were also tested for mucoadhesive properties. The addition of gelatin decreased the mucoadhesive properties of the samples, as is shown in Figure 5.11. In the swelling studies, in Figure 5.8, the initial swelling rate of the thiolated samples was lessened by the addition of gelatin; this too occurred in the mucoadhesive testing. Throughout the mucoadhesive testing of the TPAAm/5% gelatin samples, the samples did not swell to the same extent as the TPAAm samples without gelatin addition. Therefore, as the swelling ability of the samples was diminished, the increase in surface area, which was observed in the samples without gelatin addition, did not occur and this may have affected the interpenetration of the polymer chains into the mucosal layer, resulting in the observed decrease in mucoadhesive properties of the TPAAm/5% gelatin samples. The TPAAm/5% gelatin tablets also did not hold together as well, implying that the addition of gelatin into the TPAAm samples affected the cohesive nature of the tablets.

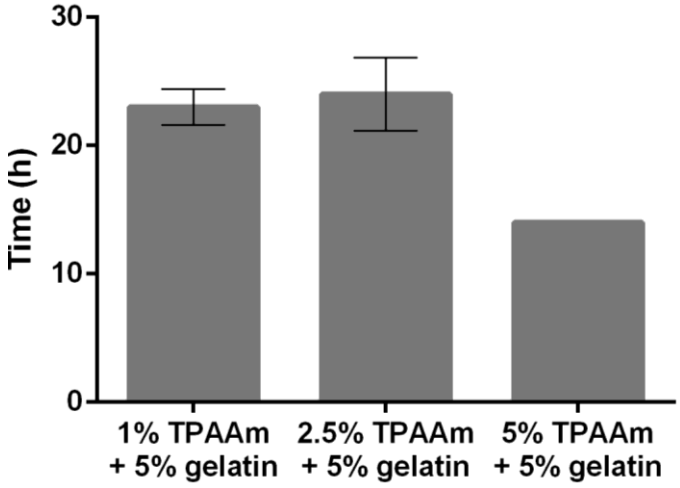

Figure 5.11 Mucoadhesive testing of TPAAm samples with the addition of 5% gelatin (n=2)

The intestinal tissue displayed a notable influence on the mucoadhesive properties of the samples. As was discussed in detail in chapter 2, section 2.3.3, the thickness and quantity of mucus on the tissue will vary between animals and between the areas the tissue was taken from; great variability was noted along the GI tract of pigs with both thickness and mucus levels changing from the mouth to the colon (Varum *et al.*, 2010). The intestinal tissue used in this study was taken from a number of different pigs, aged 7 - 8 weeks. Because of this, a degree of variability due to the change in tissue samples used was evident; the tissue thickness itself varied noticeably between samples, and often the quantity of mucus on the sample was also noticeably different which did influence the mucoadhesive properties of the samples. The three TPAAm samples on one occasion adhered for less than 30 min, gradually dislodging over that time. The samples dislodged as a whole, yet swollen tablet. The thiolated tablets did not visually appear to have dissolved, suggesting that they had crosslinked and become cohesive when submerged in the pH 6.8 phosphate buffer. This was in vast contrast to the unmodified samples which had visually halved in size due to dissolution in the same buffer. The same thiolated samples which did not adhere to the porcine intestinal tissue were tested on a different tissue sample which had visually higher levels of mucus, and they adhered for more than 30 h, thus demonstrating the effect the intestinal mucosal tissue can have on mucoadhesive properties.

5.4 Conclusion

In this study, the synthetic polymer, polyallylamine in its free base form (PAAm), was thiolated using Traut's reagent. As the polymer contains free amine groups along the backbone, the polymer was reacted with varying ratios of Traut's reagent to yield a range of thiolated polymers, with the aim to thiolate 1%, 2.5% and 5% of the total amine content of the polymer; The thiolation of 5% of the 15 kDa PAAm backbone in theory could create a highly thiolated product with up to 900 µmol/g thiol content. The thiol content of the thiolated PAAm samples was determined by iodometric titration, due to precipitation formation in both the Ellman's and TNBS assays. The addition of increasing levels of Traut's reagent yielded products with increasing levels of thiolation and resulted in thiol contents of 133.6 ± 43.3 µmol/g, 329.5 ± 13.4 µmol/g and 487.4 ± 18.3 µmol/g for the 1% TPAAm, 2.5% TPAAm and 5% TPAAm samples, respectively. It was observed that 5% TPAAm did not achieve full theoretical thiolation of 900 µmol/g. Traut's reagent is a fast and efficient reagent, but it is highly pH dependent. Similar to the thiolation reaction of gelatin in chapter 3, the pH of the reaction was changed after 20 min from pH 7 to pH 5; this was to minimise the formation of disulphide bonds which could alter the swelling and mucoadhesive properties of the final thiolated product. It was noted upon the change in pH from 7 to 5 that the pH of the 5% TPAAm reaction solution was often lower than pH 7 and, therefore, the efficiency of the Traut's reagent reaction may have decreased. The drop in reaction pH during the initial 20 min may have been due to the addition of the higher amounts of thiolating agent to the 5% TPAAm solution, as this was not observed in either the 1% TPAAm or 2.5% TPAAm samples and the thiol content of these samples were comparable to the theoretical values.

Disulphide bond formation was also analysed by reducing the thiolated samples with $NaBH_4$ before determining the thiol content by iodometric titration and comparing the results to non-reduced samples. Disulphide bonds content ranged from 111 µmol/g in the 1% TPAAm to 219 µmol/g in the 5% TPAAm. This was in contrast to Ibie *et al.* (2015) where 560 µmol/g of disulphide bonds had formed during reaction and dialysis. The Traut's reagent reaction used in this study also differed slightly to Ibie *et al.* as the pH of the reaction was altered from pH 7 to pH 5 in order to

minimise thiol oxidation. This may be the reason why disulphide bond formation was much lower in this study in comparison to Ibie *et al.*

Unmodified PAAm was freeze dried but tablet formation was difficult. Because of this, 5% gelatin was added to aid binding and the unmodified PAAm/5% gelatin sample was used as a control in both swelling and mucoadhesive studies. In swelling studies, the unmodified PAAm/5% gelatin samples quickly dissolved and had limited swelling ability. In comparison to this, the thiolated samples had improved cohesive and swelling abilities, remaining cohesive for a number of hours. The swelling profile of the thiolated PAAm samples differed from that of both thiolated PAA and thiolated gelatin samples; the thiolated samples initially displayed a high swelling rate, with a percentage increase in mass of up to 1500% in the first 30 s. Over the following number of minutes, the thiolated samples then began to lose mass, before stabilising at a mass of approximately 300% in a visually swollen state. It was thought that the initial swelling rate dominated over the ability of the polymer samples to crosslink, allowing the samples to continue to take on liquid. The samples then reached a maximum level of swelling and gradually formed a swollen, and potentially crosslinked, system which was cohesive and did not dissolve.

The thiol content of the resulting products influenced the swelling ability of the thiolated samples. As was observed in chapter 2 with thiolated PAA, increasing levels of thiolation did not increase the cohesive nature of the polymer, and higher levels of swelling were observed in samples with higher thiol content. This was also observed in this chapter with PAAm. The 5% TPAAm sample with a thiol content of 487 µmol/g had higher degrees of swelling in comparison to both the 1% TPAAm and 2.5% TPAAm samples, which had lower levels of thiolation. When discussing the mucoadhesive properties of thiolated PAAm, Ibie *et al.* (2015) suggested increased levels of thiolation resulted in both interchain repulsions within the polymer and steric hindrance, both of which decreased mucoadhesive properties. Swelling studies were not conducted by Ibie *et al.*, however, steric hindrance and interchain repulsions may have also affected the cohesive and swelling ability of the thiolated polymers in this study. Furthermore, the overall ratio of disulphide bond formation was highest in the 1% TPAAm sample. The 1% TPAAm had the lowest thiol content and this increased degree of crosslinking within the polymer matrix of the 1% TPAAm sample may have resulted in the observed lower initial rate of

swelling. Swelling studies were conducted on TPAAm samples which also contained 5% gelatin within the matrix. The addition of gelatin did not change the overall swelling profile of the TPAAm samples, but the initial rapid rate of swelling was altered, decreasing the swelling rate in the 5% TPAAm sample, while increasing it in the 1% TPAAm.

As was the case in the swelling tests, in mucoadhesive testing, the unmodified/5% gelatin control samples also quickly dissolved within 2 min. Unlike polyacrylic acid in chapter 2, polyallylamine is not described as a mucoadhesive polymer. Without the modification through thiolation, the mucoadhesive and cohesive properties of polyallylamine would be limited, with only the potential of electrostatic interactions occurring both intra- and intermolecularly. This was evident throughout this study, and the cohesive and mucoadhesive properties of the unmodified PAAm/5% gelatin control samples were poor. In comparison to the unmodified PAAm/5% gelatin controls, the thiolated PAAm samples displayed vastly improved mucoadhesive properties, with mucoadhesion onto porcine small intestine of up to 48 h for all thiolated samples. The thiol content of the three samples did not appear to affect the mucoadhesive abilities of the samples, and mucoadhesive properties were comparable between the 1% TPAAm, 2.5% TPAAm and 5% TPAAm samples. The surface area, due to the swelling ability of the thiolated samples, increased greatly during mucoadhesive testing and this may have contributed to the improved mucoadhesive properties of the samples. Upon the addition of 5% gelatin into the TPAAm samples, mucoadhesive properties decreased; this may have been due to the decrease in swelling ability, and subsequent decrease in surface area.

By thiolating the synthetic polymer, polyallylamine, with Traut's reagent, a highly mucoadhesive polymeric system has been created. Varying the degree of thiolation was reproducibly achieved by altering the amount of Traut's reagent added to the reaction. The thiolation levels did affect the swelling ability of the samples, but had minimal influence on the mucoadhesive properties of the samples. Thiol content may have an impact on drug release profiles of the samples due to swelling rate differences; this, along with further characterisation of the thiolated samples, will be examined in chapter 6.

5.5 References

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Chapter 6 Characterisation of thiolated polyallylamine

6.1 Introduction

Using similar techniques as used in chapters 2 and 4 to characterise thiolated polyacrylic acid (PAA) and thiolated gelatin, the characterisation of thiolated polyallylamine (PAAm) will be discussed in this chapter. This will include drug release studies of the model drug, chlorpheniramine maleate (CPM), thermal analysis, rheology and, additionally, biological analysis of the thiolated PAAm samples. As was mentioned in chapter 5, limited work on the thiolation and mucoadhesive properties of polyallylamine has been conducted, and as this is the case, characterisation of the thiolated polyallylamine products has also been minimal. Papers have discussed the thiolation of polyallylamine whereby the polymer is thiolated by either the addition of Traut's reagent or reaction with EDC and N-acetyl cysteine. Much work focused on using thiolated polyallylamine as a DNA delivery vector (Bacalocostantis *et al.*, 2012) or an efflux pump inhibitory (Vigl *et al.*, 2009). Ibie *et al.* (C. O. Ibie *et al.*, 2015; C. Ibie *et al.*, 2015) did investigate thiolated PAAm as a mucoadhesive polymer, thiolating the polymer with both Traut's reagent and N-acetyl cysteine, as was discussed in chapter 5. The thiolated polymers were characterised using differential scanning calorimetry (DSC), however, no rheological analysis of the samples was conducted. Similar to the analysis of PAA in chapter 2, the viscoelastic properties of the thiolated PAAm (TPAAm) samples and the interactions of the TPAAm samples with a mucin solution can be assessed, giving further insight into the mucoadhesive properties of PAAm. This will also allow for a direct comparison to the thiolated PAA samples and, therefore, both the strength of the gel structures of the two different synthetic polymers can be compared, as well as any differences in mucoadhesive bonding between the two polymers.

As was discussed in chapter 1, section 1.8.6, polyallylamine is known to be highly toxic to both cells and microbes due to its cationic backbone. Modification of the polymer, by a variety of methods, has decreased the toxic effects associated with the polymer allowing it to be used in the delivery of, for example, DNA (Bacalocostantis *et al.*, 2012; Oskuee *et al.*, 2015). The thiolation of PAAm with Traut's reagent has been shown to decrease the cytotoxicity of PAAm in comparison to unmodified samples (C. Ibie *et al.*, 2015). In this study, further analysis will be conducted to investigate the effect thiolation has on the antimicrobial properties of the polymer.

The potential to decrease the antimicrobial activity of PAAm may be advantageous when targeting the gastrointestinal (GI) tract, due to the high levels of bacterial growth within the intestine which is required for the digestion of foods (Pratt and Cornely, 2011).

6.1.1 Aims and objectives

In the chapter, the previously thiolated polyallylamine will be characterised. The model drug, chlorpheniramine maleate, will be incorporated in both the control and thiolated samples and tested for drug release. The rheological properties of the three thiolated products, 1% TPAAm, 2.5% TPAAm and 5% TPAAm, will be examined and compared to that of the unmodified PAAm. The unmodified and thiolated polymers will also be mixed with a mucin solution to investigate their mucoadhesive interactions by rheology. SEM and thermal analysis will be conducted on all samples. The thiolated polymer products will also be tested for antimicrobial ability and will be compared to unmodified samples.

6.2 Materials and methods

6.2.1 Materials

Mueller Hinton Agar was purchased from Difco, France. Brain Heart Infusion Broth (BHI) was purchased from Oxoid, England. Novobiocin was obtained from Fluka, Ireland.

6.2.2 Drug release studies

Drug release studies were conducted on thiolated PAAm and unmodified PAAm/5% gelatin samples. Studies were conducted as described in chapter 2, section 2.2.6, with a minor modification to the buffer solution concentration. Previously, a 10 mM phosphate buffer was made at pH 3; this was replaced by to a 1 mM phosphate buffer at pH 3. A new set of CPM standards as described in chapter 2, section 2.2.6 were then analysed using the new mobile phase.

6.2.3 Polymer characterisation

6.2.3.1 Rheological properties

Rheological studies were performed on a TA instruments AR 2000 ex rheometer, using a 40 mm parallel plate geometry. An 8% solution of Type II porcine mucin was created as outlined in chapter 2, section 2.2.7.1. Thiolated PAAm samples were made in D.I. water, at a concentration of 1.5% (m/v). The mucin solution was mixed in equal parts with 3% (m/v) aqueous polymer solutions. Samples were loaded onto the rheometer and allowed to equilibrate for 3 min prior to analysis. All tests were conducted at 37 °C.

Dynamic oscillatory tests were conducted on polymer samples alone, at a concentration of 1.5% (m/v), and with the addition of mucin solution. Amplitude sweeps were conducted over a stress range of 0.1 – 100% strain at constant frequency of 1 Hz in which the storage modulus (G´) and the loss modulus (G´´) were determined. Frequency sweeps were conducted on polymer samples alone and with the addition of mucin, at a constant strain of 1% and a frequency range of 0.1 – 20 Hz. Time sweeps were also conducted at a strain of 1% and frequency of 1 Hz over a period of 30 min. Polymer samples were analysed at a concentration of 1.5% (w/v) made in 50 mM phosphate buffer, pH 6.8 and also at a concentration of 1.5% (w/v) made in D.I water. Polymer samples, at a concentration of 3% (w/v) in D.I. water with the addition of equal volumes of 8% mucin were also analysed.

6.2.3.2 Scanning electron microscopy (SEM)

SEM imaging was conducted on unmodified and thiolated samples according to the method outlined in chapter 2, section 2.2.7.2. Samples were analysed at various magnifications with a voltage of 22 kV.

6.2.3.3 Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) analysis

Samples were analysed according to the method outlined in chapter 2, sections 2.2.7.3 and 2.2.7.4. All samples were analysed by TGA prior to DSC analysis; this gave the degradation temperature of the sample and the differential weight loss in

relation to temperature. Samples for DSC were hermetically sealed and pin-holed to release water vapour. All samples were run on a heat-cool-heat-cool cycle, so that any water present would be released during the initial heating cycle. The samples were equilibrated at -30 °C, heated to 190 °C at a ramp rate of 10 °C/min, and then cooled to 0 °C, creating one cycle, using N_2 at a flow rate of 50 mL/min as a carrier gas. Samples were also analysed by modulated DSC (MDSC), using the method outlined in Table 6.1.

Table 6.1 MDSC settings

Modulated amplitude	±0.75 °C
Modulation	60 s
Ramp rate	1 °C/min
Start temperature	-10 °C
Final temperature	200 °C

6.2.4 Antimicrobial testing: well diffusion test

Unmodified and thiolated PAAm solutions were aseptically made up in sterile water at concentrations of 0.5 mg/mL, 1 mg/mL and 2 mg/mL. These samples were heated to approximately 100 °C to ensure they were sterile prior to testing. As a precaution, samples of unmodified PAAm and 2.5% TPAAm were also both made up aseptically in sterile water but without heating, as heating may cause conformational changes within the polymer, thus changing its antimicrobial characteristics.

Agar plates which were spiked with bacteria were also made up. 7.6 g Mueller Hinton Agar was dissolved in 200 mL of water and was autoclaved. 7.4 g Brain Heart Infusion Broth (BHI) was made up in 200 mL water and was also autoclaved. Both representative Gram-positive and Gram-negative bacteria were used: *Staphylococcus aureus* and *Escherichia coli*, respectively. To 5 mL of BHI solution, 50 µL of bacteria was added and this solution was incubated at 37 °C overnight to culture the bacteria. To make the agar plates, 500 µL bacteria were added to 200 mL of agar; therefore, a set of agar plates spiked with *E. coli* was produced and a set of plates spiked with *S. aureus* was produced. A set of control agar plates were also

made, without the addition of bacteria, to ensure there was no contamination of the agar solution.

As a positive control, the antibiotic Novobiocin was used. 50 mg of novobiocin was dissolved in 10 mL water. This was then diluted 1 in 4 and filtered using a sterile 45 µm nylon filter. As a negative control, sterile water was used. Once the agar had set, a number of wells were created in the agar using a sterile glass capillary tube, to which 50 µL of either the polymer solutions, or the positive and negative control samples were added. Each plate contained a positive and negative control, an unmodified sample and the 1% TPAAm, 2.5% TPAAm and 5% TPAAm thiolated samples. Each polymer concentration was analysed in triplicate. The plates were incubated overnight at 37 °C. The plates were examined for zones of inhibition of bacterial growth around the wells the following day.

6.3 Results and discussion

6.3.1 Drug release studies

A small change to the concentration of the phosphate buffer used in the HPLC anlaysis method of PAAm in comparison to both PAA and gelatin was made. The 10 mM phosphate buffer, as used in chapters 2 and 4 with both PAA and gelatin, was changed to 1 mM phosphate buffer for use with PAAm. As before, the pH of this buffer solution was adjusted to pH 3 with ortho-phosphoric acid and the same gradient method was used. It was noted that the decrease in concentration in the buffer solution sharpened the peak shape of the chlorpheniramine peak due to a decrease in retention time with the 1 mM buffer. A new set of CPM standards were analysed using the new phosphate buffer solution. A typical chromatogram of the PAAm-CPM drug release using the new buffer is shown in Figure 6.1; the maleate peak eluted at 3.6 min while the chlorpheniramine peak eluted at 5.6 min.

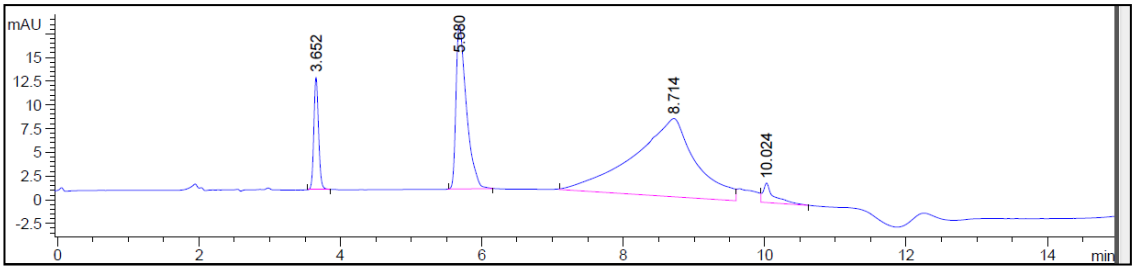

Figure 6.1 Typical chromotgram the release of CPM from thiolated PAAm, displaying the maleate peak eluting at 3.6 min and the chlorpheniramine peak eluting at 5.6 min.

Drug release studies of CPM were conducted on unmodified PAAm/5% gelatin control samples and the three thiolated PAAm samples, 1% TPAAm, 2.5% TPAAm and 5% TPAAm; the thiolated samples did not include the addition of gelatin. Once the unmodified PAAm/5% gelatin control samples were added to the 37 °C phosphate buffer for the drug release studies, they began to dissolve immediately. Within 30 s, the buffer had changed from a clear solution to a cloudy white solution as the tablets quickly disintegrated; therefore, the unmodified sample was not included in the drug release graph in Figure 6.2. This was in vast contrast to the thiolated samples. The thiolated samples, similar to the swelling studies, swelled quickly and to a large degree once they were added to the buffer solution. This fast ability to swell may have had a negative impact on the release of drug from the tablet, with a burst release occurring quickly. Figure 6.2 shows the release profiles of the three thiolated PAAm samples; similar to the drug release profiles of PAA, the values in Figure 6.2 are based on the ratio of actual release to 100% theoretical release. All three thiolated samples reached drug release of approximately 50 – 60% within 2 h. The overall profiles of the three thiolated samples were similar. Looking at the first 60 min of release in Figure 6.2 shows that both the 1% TPAAm and 2.5% TPAAm samples had comparable drug release, both of which had an equivalent slope for the first 30 min measuring approximately 1.7. The 5% TPAAm sample showed a slower rate of drug release over the same period of time in comparison to the other two samples. This is surprising as the swelling rate of the 5% TPAAm sample in the swelling studies, in chapter 5, section 5.3.3, was higher in comparison to either the 1% TPAAm or 2.5% TPAAm samples, and thus it would be expected that the rate of drug release would be faster in the 5% TPAAm sample. However, the 5% TPAAm sample did have the highest thiol content, measuring 487.4 µmol/g and,

therefore, the potential to form intramolecular disulphide bonds, which would crosslink the polymer and alter drug release (Andrews *et al.*, 2009), would also be greater than the 1% TPAAm or 2.5% TPAAm samples, both of which had lower thiol content at 133.6 µmol/g and 329.5 µmol/g, respectively. Higher levels of thiolation has been shown to slow the release of acyclovir from thiolated PANAM dendrimers (Yandrapu *et al.*, 2013). PANAM dendrimers were thiolated with cysteamine at different molar ratios resulting in thiol contents of 10.56 µM/mg and 68.21 µM/mg. Acyclovir was loaded into both thiolated dendrimers samples and, although results were not significant, acyclovir release was slower from the thiolated sample with higher thiol content, similar to this study.

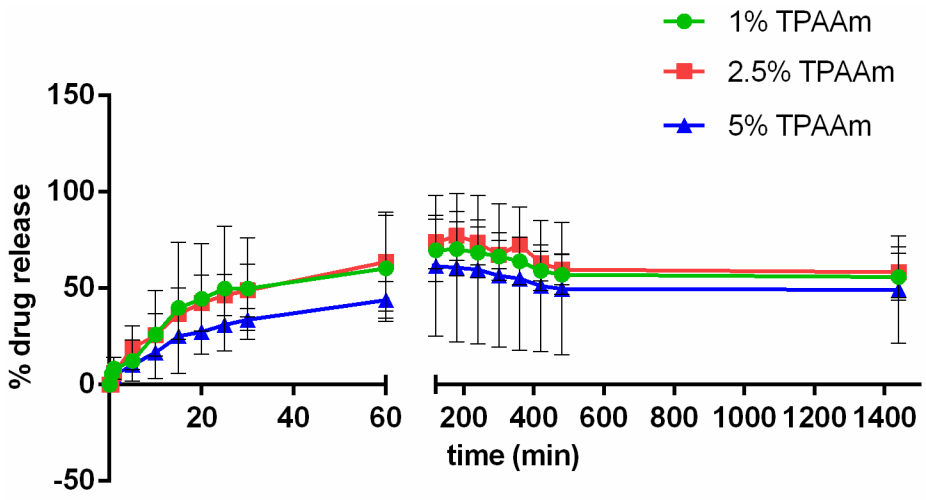

Figure 6.2 Drug release of CPM from thiolated PAAm samples (n=3)

Comparing the release of CPM from the thiolated PAAm samples to that of the thiolated PAA samples (chapter 2, section 2.3.4) shows that the release of drug from the TPAAm samples was quicker than that of the thiolated PAA samples. The release of drug from the PAA samples was over 8 h, in comparison to the 2 h release from the thiolated PAAm samples. In 30 min, the thiolated PAA samples had released approximately 6% of drug whereas all three TPAAm samples had released between 30 – 50% of drug, which is substantially higher. The thiolated PAA samples in question did have higher thiol content at 600 and 1000 µmol/g and they also had a much slower rate of swelling in comparison the thiolated PAAm samples which may have allowed for the slower release of drug over time.

6.3.2 Polymer characterisation

6.3.2.1 Rheological studies

Unmodified and thiolated PAAm samples, with and without the addition of an 8% mucin solution, were analysed by rheology. Rheological studies of thiolated PAAm has not been published in the literature and, therefore, the results were compared to unmodified and thiolated PAA samples from chapter 2, section 2.3.5.1.

6.3.2.1.1 Dynamic amplitude tests

PAAm samples were initially analysed at a constant frequency of 1 Hz over a percentage strain of 0.1 – 100%. The storage modulus (G') and the loss modulus (G") were determined for all thiolated PAAm and unmodified PAAm samples; neither the unmodified PAAm nor the thiolated PAAm samples contained 5% gelatin. As shown in Figure 6.3 (A), the G' values for all the thiolated PAAm were markedly higher than the unmodified PAAm sample; this was also observed in chapter 2 with the thiolated PAA samples. The increase in G' in the thiolated samples in comparison to the unmodified samples may be due to the more cohesive properties possessed by the thiolated samples and demonstrates the ability of the thiolated samples to swell. In swelling studies in chapter 5, section 5.3.3, the unmodified samples disintegrated quickly, whereas the thiolated samples swelled up to 1500% their original mass and retained their structure. When testing the samples with rheology, the thiolated PAAm samples quickly turned to a gel, whereas the unmodified sample remained in liquid form.

All three thiolated samples, 1% TPAAm, 2.5% TPAAm and 5% TPAAm, displayed similar responses to increasing strain; Figure 6.3 (B) shows the G' and G" values of 5% TPAAm, which was typical of all thiolated samples. All G' values were higher than G" values in the initial stages of the test, as is shown in Figure 6.3 (B), indicating a predominantly solid-like response in the thiolated samples. At a certain strain, both G' and G" began to drop, and there was a crossover in the two values after which G" became higher than G', indicating structural breakdown of the sample; this had also occurred with the thiolated PAA samples in chapter 2.

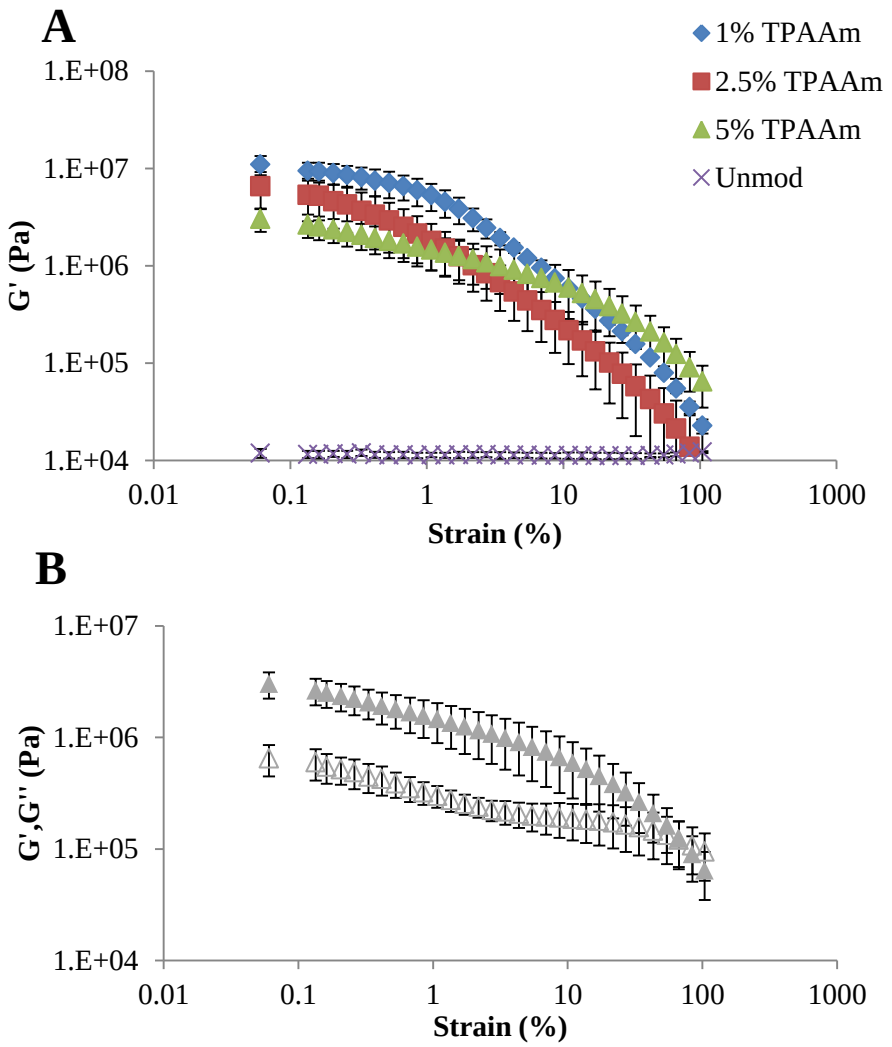

Figure 6.3 Strain sweep of thiolated PAAm samples displaying (A) G' values of thiolated PAAm in comparison to unmodified PAAm and (B) G' (closed) and G'' (open) values of 5% TPAAm (n=2)

Interestingly, when comparing the G' values of the thiolated PAAm samples to those of the thiolated PAA samples from chapter 2, section 2.3.5.1.1, there was a marked increase in G' values in the PAAm samples. Table 6.2 shows the G' values of the thiolated PAAm samples at a strain of 0.1% and the corresponding values of the thiolated PAA samples. This increase is particularly important when comparing the pH 5 PAA sample with the 5% TPAAm which had comparable thiol content (400.6 ± 61.3 and 487.4 ± 18.5 µmol/g, respectively). Again, the swelling ability of the TPAAm samples differed greatly in comparison to the thiolated PAA samples, swelling to a much higher extent in the initial 5 min, which may explain the marked difference in observed G' values. This noticeable difference in G' values can also be

seen in Figure 6.4, when comparing the 5% TPAAm sample with a pH 5.5 PAA sample.

Table 6.2 G' values of thiolated PAAm and thiolated PAA samples at a strain of 0.1%

PAAm samples	G' (Pa)	PAA samples	G' (Pa)
1% TPAAm	9463500	**pH 5**	63.7
2.5% TPAAm	5336667	**pH 5.5**	29.0
5% TPAAm	2643000	**pH 6**	70.0
		pH 6.5	141.6

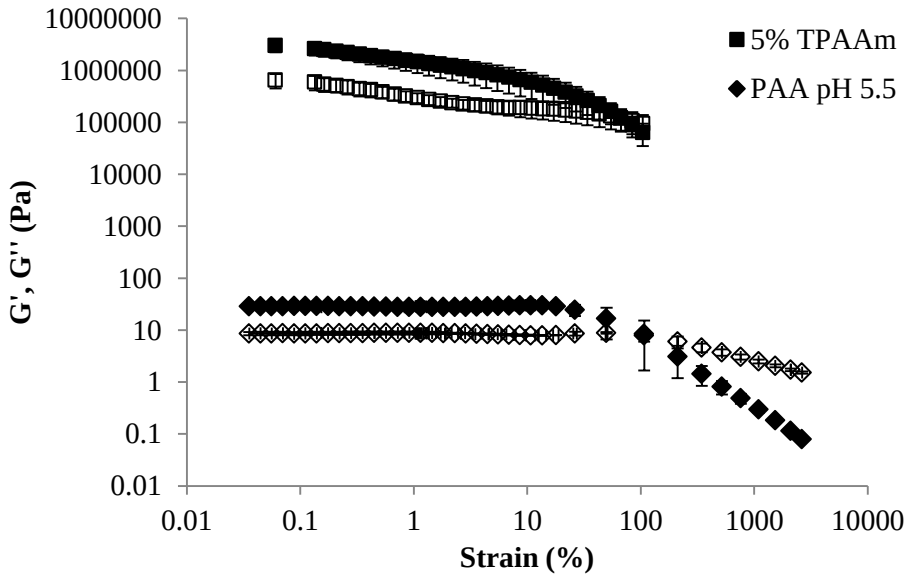

Figure 6.4 Strain sweep of 5% TPAAm and thiolated PAA pH 5.5, displaying both G' (closed symbols) and G'' (open symbols) values (n=2)

Although the TPAAm samples had higher G' values, the strain at which the G'/G" crossover occurred, i.e. the structural breakdown point of the samples, was much lower in comparison to the thiolated PAA samples, as is displayed in Table 6.3. This indicates that the gel structure of the PAAm samples was more sensitive to deformation in comparison to the thiolated PAA samples. This was also observed during the swelling studies, as discussed in chapter 5, section 5.3.3; when a light force was added to the swollen PAAm tablet it seeped moisture. This did not occur to the thiolated PAA samples, which retained their swollen structure when the same light force was applied. Similarly, when comparing the data in Figure 6.4, the

complete breakdown (the crossover point of G' and G") of the 5% TPAAm sample occurred at 66.88% strain; however, the onset of structural breakdown occurred at a much lower strain in comparison to the PAA pH 5 samples, and breakdown was more prolonged. The G' values of the PAA pH 5.5 sample are linear up until a certain strain at which G' then drops; this is in contrast to the 5% TPAAm sample, in which the G' values began to drop at a much earlier strain. This again suggests a weaker gel structure within the TPAAm samples in comparison to the thiolated PAA samples. The thiol content of the PAA pH 5.5 is higher than the 5% TPAAm; no crossover point was observed in the PAA pH 5 sample, as can be seen in Table 6.3, but the G' response was linear for this sample, similar to the pH 5.5 sample displayed in Figure 6.4. The stronger gel structure within the thiolated PAA may be due to the greater degree of intramolecular bonding and entanglement within the PAA matrix. The PAA polymer used was 450 kDa in comparison to PAAm which had a MW of 15 kDa and this may also have had an impact on the strength of the formed gel.

Table 6.3 Strain at which G'/G'' crossover occurred in thiolated PAAm and thiolated PAA samples

PAAm samples	Crossover point (% strain)	PAA samples	Crossover point (% strain)
1% TPAAm	4.3	**pH 5**	NA
2.5% TPAAm	2.2	**pH 5.5**	107.3
5% TPAAm	66.9	**pH 6**	62.3
		pH 6.5	45.5

As discussed in chapter 2, rheological studies of polymer/mucin interactions can give further information about the mucoadhesive properties of the polymer, and can give an insight into the strength of the bonds formed between the polymer and mucin. Upon the addition of an 8% mucin solution to the thiolated PAAm samples, both the values of G' and G" increased in comparison to the samples without mucin, as demonstrated with the 1% TPAAm sample in Figure 6.5. The increase in G' and G" values indicated a higher degree of interactions occurring, due to entanglements and chemical interactions between the mucin solution and the polymers, which is indicative of mucoadhesion (Hägerström *et al.*, 2000).

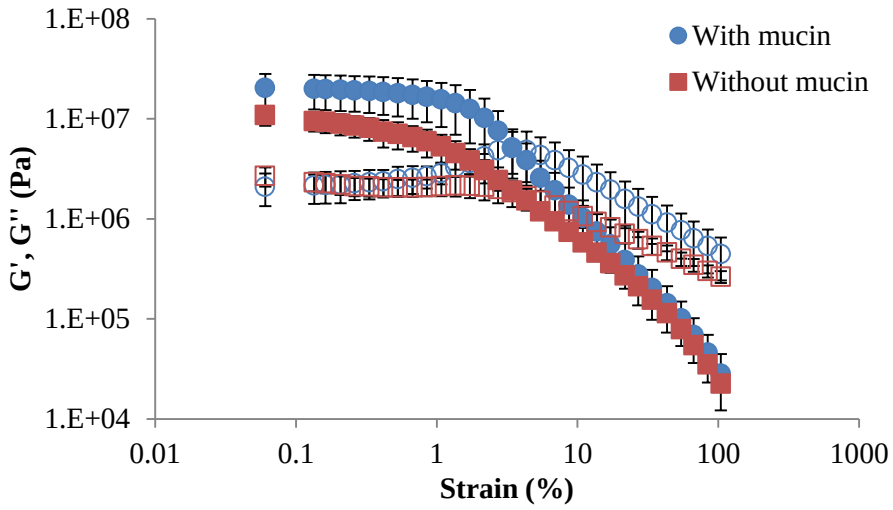

Figure 6.5 G' (closed symbols) and G'' (open symbols) values of 1% TPAAm with and without the addition of an 8% mucin solution (n=2)

Again, when comparing the results of the TPAAm samples to the PAA samples, there is a noticeable difference in G' values. Once mucin was mixed with the polymer solution, there was an increase in G' values observed with both polymers. However, the increase was much more pronounced in the thiolated PAA samples in comparison to the TPAAm samples, as is shown in Figure 6.6. The G' values of both polymers with the addition of mucin are comparable, suggesting a similar gel structure and bonding within both polymer/mucin systems.

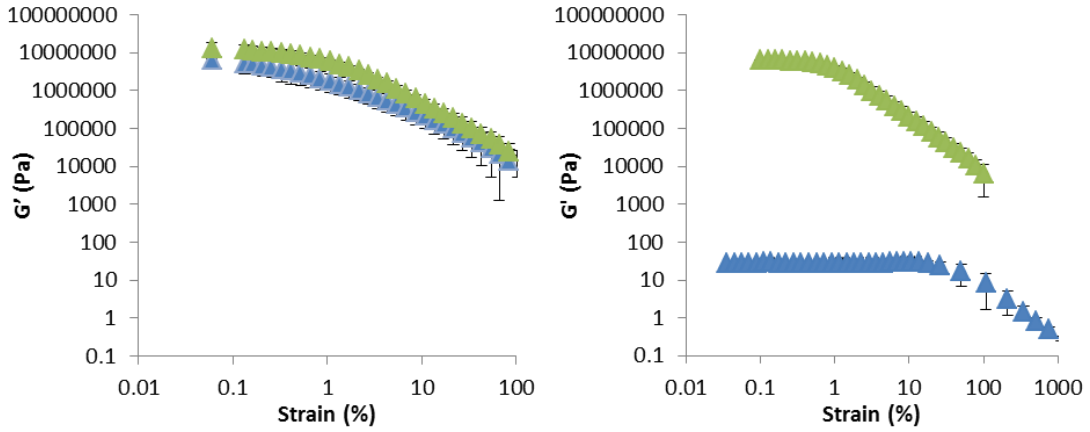

Figure 6.6 G' values of polymer samples with (green) and without (blue) mucin addition. 2.5% TPAAm is on the left and PAA pH 5.5 is on the right (n=2)

For a direct comparison of the strength of the polymer/mucin bonds between the different polymer samples, the relative G' values were calculated using Equation 6.1 (Riley *et al.*, 2001):

Equation 6.1

$$\textbf{Relative } G' = \frac{G'\textbf{mix} - (G'\textbf{poly} + G'\textbf{mucin})}{G'\textbf{poly} + G'\textbf{mucin}}$$

where G' mix = polymer/mucin mixtures, G' poly = polymer and G' mucin = mucin

Shown in Table 6.4 are the relative G' values of the three thiolated PAAm samples and the PAA pH 5 sample, as a comparison of strength of interactions between the two polymers when mixed with mucin. Both the 1% TPAAm and 2.5% TPAAm samples had comparable values, whereas the 5% TPAAm sample was higher; this may be due to the increased thiol content, and potential increase in intermolecular disulphide bond formation, in the 5% TPAAm sample compared to the 1% and 2.5% TPAAm samples. When comparing the 5% TPAAm sample to the PAA pH 5 sample, both of which had approximately 450 µmol/g thiol content, there was a marked increase in the relative G' value of the PAA sample. This suggests there is a 3-fold increase in interaction levels between the PAA pH 5 sample and mucin in comparison to the 5% TPAAm sample and mucin.

Table 6.4 Relative G' values of thiolated PAAm samples and PAA pH 5 sample at a strain of 0.1%

Sample	Relative G'
1% TPAAm	1.1
2.5% TPAAm	1.2
5% TPAAm	3.6
PAA pH 5	9.0

6.3.2.1.2 Frequency sweeps

Frequency sweeps were conducted on the unmodified and thiolated PAAm samples within the viscoelastic region. The thiolated samples displayed an initial linear

response across the frequency range of 0.1 – 10 Hz. Similar to the strain sweep, the values of G' were higher than the G" values, as is shown in Figure 6.7. The addition of an 8% mucin solution increased the values of both G' and G", as also occurred in the strain sweeps, indicating an increase in interactions between the polymer and the mucin solution; this was typical of all three thiolated samples. Similar to the PAA samples in chapter 2, the G' values at a frequency of 0.1 Hz were compared, as shown in Table 6.5; the unmodified PAA and thiolated PAA pH 5.5 samples are also included in Table 6.5 as a comparison. The G' values of the unmodified PAAm sample did increase with mucin addition but to a lesser extent than the unmodified PAA sample, again highlighting the lower inherent mucoadhesive properties that unmodified PAAm has in comparison to unmodified PAA. This mirrors the mucoadhesive testing of the unmodified PAAm and PAA samples. The PAAm samples quickly dissolved within minutes whereas the unmodified PAA samples remained cohesive and mucoadhesive for approximately 1 h. The G' values of the thiolated PAAm samples were substantially higher than the thiolated PAA samples and, therefore, upon mucin addition, the increase in G' values of the PAAm samples was not as pronounced as in the PAA samples. This is highlighted further with the ratio of G' with mucin to G' without mucin, also shown in Table 6.5; a 1.7-fold increase in G' was observed in the 5% TPAAm in comparison to a 254620-fold increase in the thiolated pH 5.5 PAA sample.

The PAA samples in chapter 2 showed a linear response throughout the frequency sweep, with and without mucin addition; this was indicative of a crosslinked matrix system (Riley *et al.*, 2001). The PAAm samples appear to be behaving as an intermediate gel, with characteristics of both a crosslinked and entangled network (Mortazavi *et al.*, 1993); G' in both the polymer samples with and without mucin remained linear on the whole, while G" decreases at lower frequencies. This may be due to entanglement of the polymer chains into the mucin occurring prior to the crosslinking effect of disulphide bond formation.

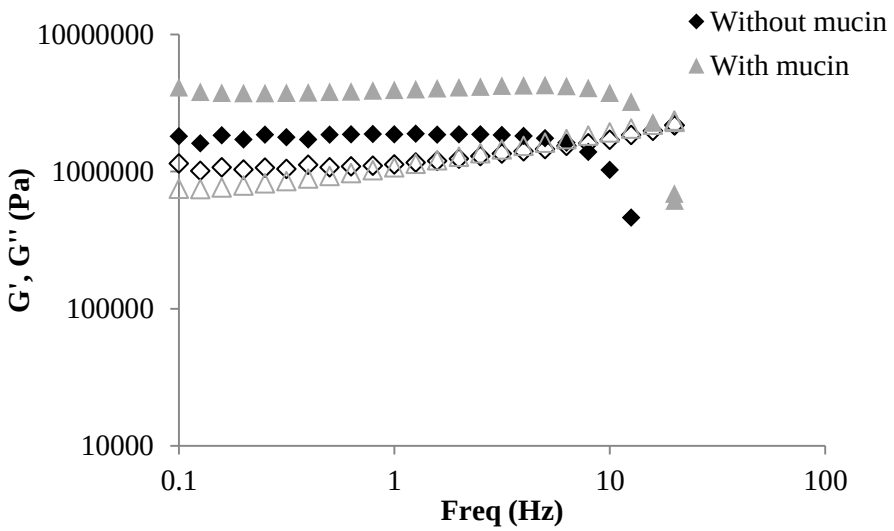

Figure 6.7 Frequency sweep of 2.5% TPAAm with and without mucin displaying G' (closed symbols) and G'' (open symbols) values (n=2)

Table 6.5 G' values of unmodified PAAm and thiolated PAAm samples at 0.1 Hz, with and without mucin addition

Samples	G' (Pa) at 0.1 Hz		
	without mucin	with mucin	Ratio
Unmodified PAAm	0.6	3.1	4.8
1% TPAAm	4937000	8219500	1.7
2.5% TPAAm	1807500	4105500	2.3
5% TPAAm	2606500	4352500	1.7
Unmodified PAA	0.8	1223.8	1480.3
PAA pH 5.5	22.6	5745500	254620

Tan δ is described as the Loss Tangent and is the ratio of G" to G'. Upon the addition of mucin, there was a difference in the Tan δ values of the thiolated PAAm samples in comparison to the unmodified samples, which again is suggestive of the different intermolecular bonds and interactions which occurred between the unmodified and modified samples. When the 5% TPAAm sample was compared to the PAA pH 5 sample, as shown in Figure 6.8, the Tan δ values were comparable. This highlights that the mucoadhesive interactions and bonding which have occurred between the more novel thiolated PAAm sample and mucin are similar to those which occurred between the known mucoadhesive polymer, PAA and mucin.

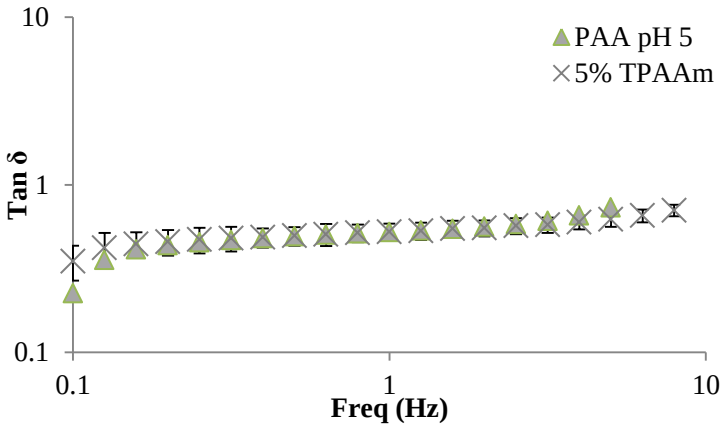

Figure 6.8 Frequency sweep of PAA pH 5 with mucin and 5% TPAAm with mucin, displaying Tan δ values

6.3.2.1.3 Time sweeps

Time sweeps were conducted on all thiolated and unmodified PAAm samples at a constant strain of 1% and a constant frequency of 1 Hz. Polymer samples were dissolved in D.I. water, and in 50 mM phosphate buffer (pH 6.8) and were also mixed with a mucin solution (8%). All samples were analysed over a period of 30 min. When comparing the results of the water samples to the buffer samples, G' and G" were both higher in the water samples, displayed in Figure 6.9 over a period of 3 min. The thiolated PAAm samples were quick to dissolve and form a gel in D.I. water. When dissolved in 50 mM phosphate buffer (pH 6.8), the thiolated samples did not dissolve homogenously and there were obvious polymer particles within the solution; this may have been due to the formation of disulphide bonds within the polymer in the pH 6.8 buffer in comparison to the water uptake and gel formation of the polymers when dissolved in water. This may explain the increase in G' and G" values observed in the water samples.

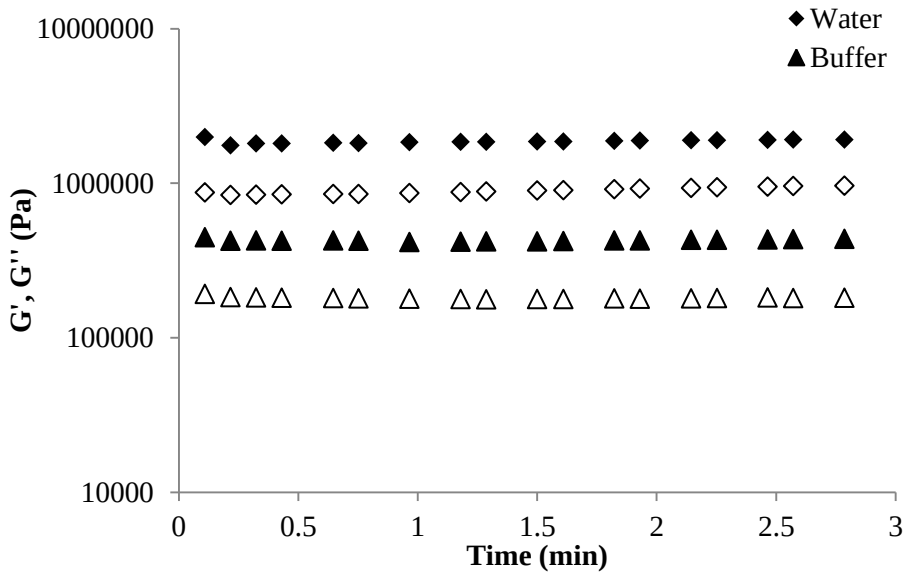

Figure 6.9 Time sweeps displaying the G' (closed symbols) and G'' (open symbols) of 1% TPAAm made in water and in buffer over the initial 3 min (n=2)

Polymer samples made in 50 mM buffer were also compared to samples mixed with mucin. Figure 6.10 shows the 2.5% TPAAm sample, which was typical of all thiolated samples. Again, the addition of mucin to the polymer samples increased both the G' and G" values in comparison to the polymer alone. After approximately 17 min, both G' and G" in the TPAAm/mucin samples increased further which indicated additional interactions occurring between the thiolated polymer and the mucin solution and the formation of a stronger gel; this may be due to the further formation of intermolecular disulphide bonds between the polymer and the mucin solution, strengthening the gel. An increase in G' and G" did not occur in the unmodified PAAm/mucin sample, and similarly the further increase in G' and G" observed in the thiolated samples also did not occur in the unmodified PAAm/mucin sample. This highlights the different types of bonds and gel structure formed within the unmodified PAA/mucin system in comparison to the thiolated PAAm/mucin system, i.e. weaker electrostatic and secondary bonding in comparison to stronger disulphide bonding.

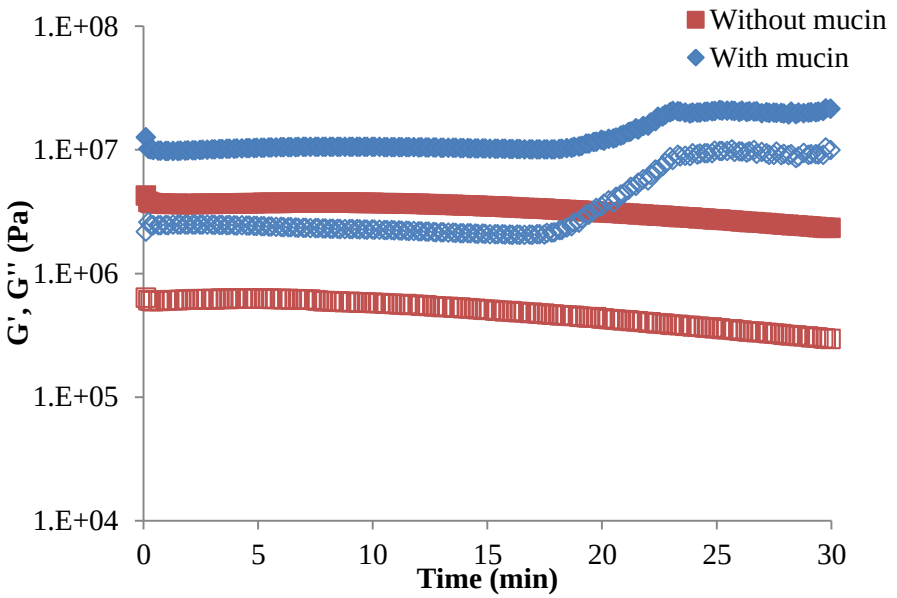

Figure 6.10 Time sweep of 2.5% TPAAm with and without the addition of mucin, displaying G' (closed symbols) and G'' (open symbols) values (n=2)

The equilibration time, prior to analysis in both the strain and frequency sweeps, was 3 min. This was in contrast to both Marschutz *et al.* (2002) and Leitner *et al.* (2003) where equilibration times were 20 min. As can be seen in both Figure 6.9 and Figure 6.10, upon mucin addition no changes in G' or G" occurred within the first 3 min, and therefore, during the equilibration time. However, changes in both G' and G" occurred within 20 min. If equilibration times conducted in this study were longer, the increase in G' and G" values after 17 min in the TPAAm/mucin samples (Figure 6.10) would not have been observed.

6.3.2.2 Scanning electron microscopy (SEM)

Unmodified PAAm with the addition of 5% gelatin and the thiolated samples, 1% TPAAm, 2.5% TPAAm and 5% TPAAm, were analysed using SEM. All samples had been freeze dried prior to analysis for a direct comparison between samples. Samples were all analysed at a magnitude of 40x and 100x.

The texture of the freeze dried thiolated PAAm was marginally different to that of thiolated gelatin and thiolated PAA. Thiolated PAAm, although it was still cotton wool-like to the eye, had a crisper texture in comparison to the other thiolated polymers, PAA and gelatin. The SEM images of the thiolated PAAm samples were,

however, similar to both thiolated PAA and thiolated gelatin. Shown in Figure 6.11 are SEM images of unmodified/5% gelatin and thiolated PAAm samples. The unmodified/5% gelatin sample had a different surface texture that the thiolated samples, but there was little variability in morphology between the thiolated samples.

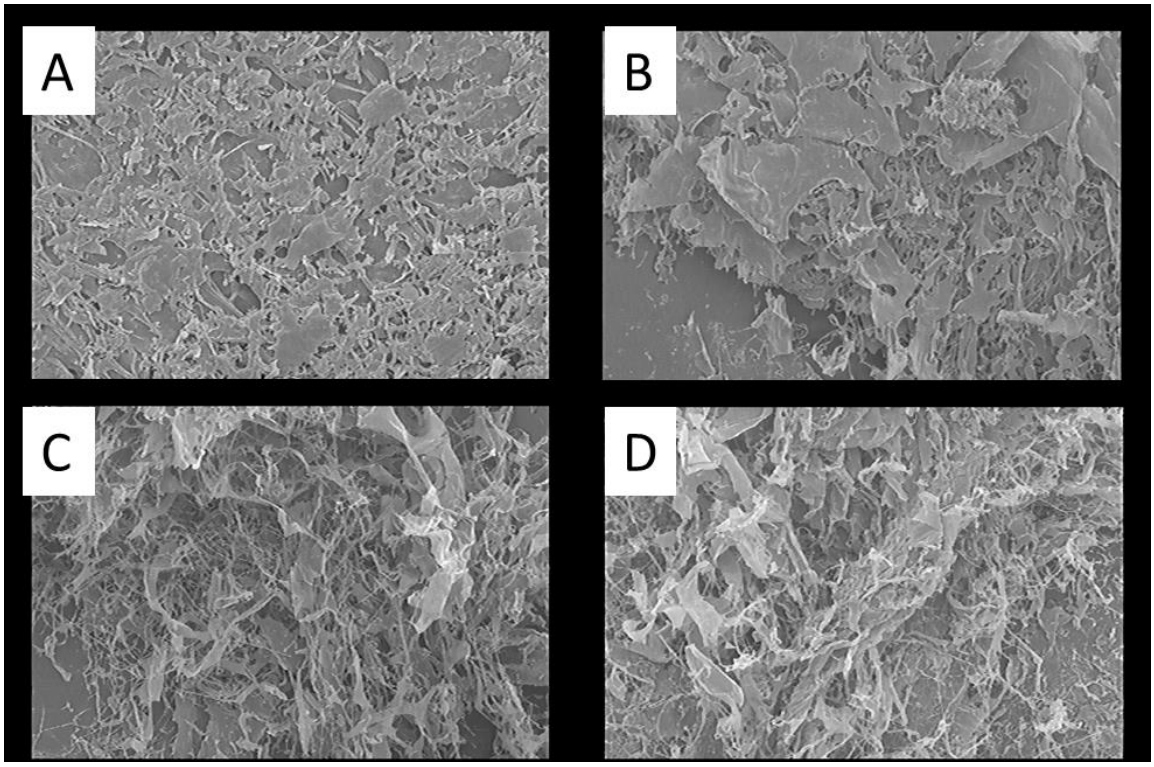

Figure 6.11 SEM imaging of (A) Unmodified/5% gelatin PAAm, (B) 1% TPAAm, (C) 2.5% TPAAm and (D) 5% TPAAm

6.3.2.3 Thermogravimetric analysis (TGA)

TGA was initially performed on unmodified PAAm and also on unmodified PAAm with 5% gelatin addition. As the unmodified PAAm used is a 15% (w/v) solution, the sample was freeze dried prior to analysis. The unmodified PAAm/5% gelatin sample was also freeze dried allowing for a more direct comparison. Figure 6.12 shows the degradation pattern of unmodified PAAm, unmodified PAAm/5% gelatin and also native gelatin. All samples displayed water loss under 100°C. Native gelatin showed up to 20% weight loss until approximately 240 °C where a final degradation occurred. Unmodified PAAm displayed a more gradual loss of weight up to 420 °C where final degradation occurred. A similar degradation pattern was observed by

Kim *et al.* (2002). As can be expected, the addition of gelatin changed the degradation pattern of the PAAm sample, displaying a pattern which appeared to be a combination of both PAAm and gelatin. Kim *et al.* (2002) examined interpenetrating polymer networks (IPN) composed of chitosan and polyallylamine. The IPN and the unmodified chitosan and polyallylamine samples were analysed by TGA and, similar to this study, noted that the degradation pattern of the IPN was composed of elements of both chitosan and polylallyamine; the derivative weight loss of the polyallylamine began at approximately 400 °C, for chitosan it began at 250 °C and for the IPN sample there were two peaks at 250 °C and 400 °C. Similarly in this study, elements of the degradation of gelatin were apparent upon the addition of 5% gelatin in to the unmodified PAAm sample and the final degradation temperature decreased from 413 °C in unmodified PAAm to 372 °C in the sample with 5% gelatin addition.

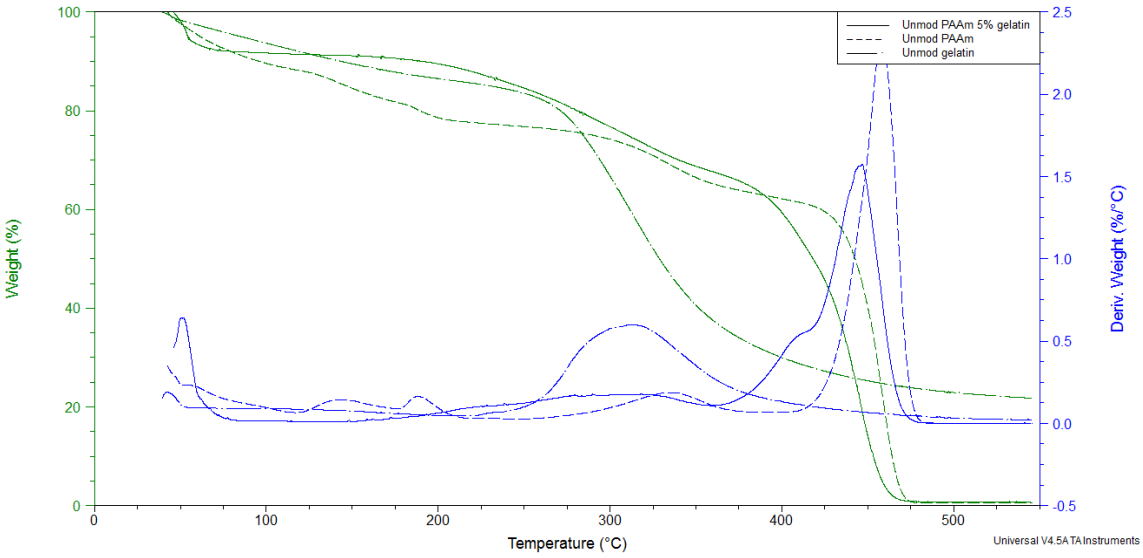

Figure 6.12 TGA of unmodified PAAm, unmodified gelatin and PAAm/5% gelatin

The degradation patterns of the thiolated PAAm samples were compared to that of unmodified PAAm which had been freeze dried. Shown in Figure 6.13 is the weight loss and derivative weight loss against temperature of the three TPAAm samples and unmodified PAAm. The thiolated samples, regardless of the ratio of Traut's reagent added, had a similar degradation pattern. This pattern differed greatly from that of

the unmodified sample. There was water loss observed in all samples below 100 °C, however, the unmodified sample continued to degrade until 200 °C, with a loss of approximately 22% at this temperature. The thiolated samples, after the initial loss of water, remained stable until approximately 230 °C before a significant loss of mass was observed up to 380 °C. The final degradation of the thiolated and unmodified polymers occurred at approximately 415 – 420 °C.

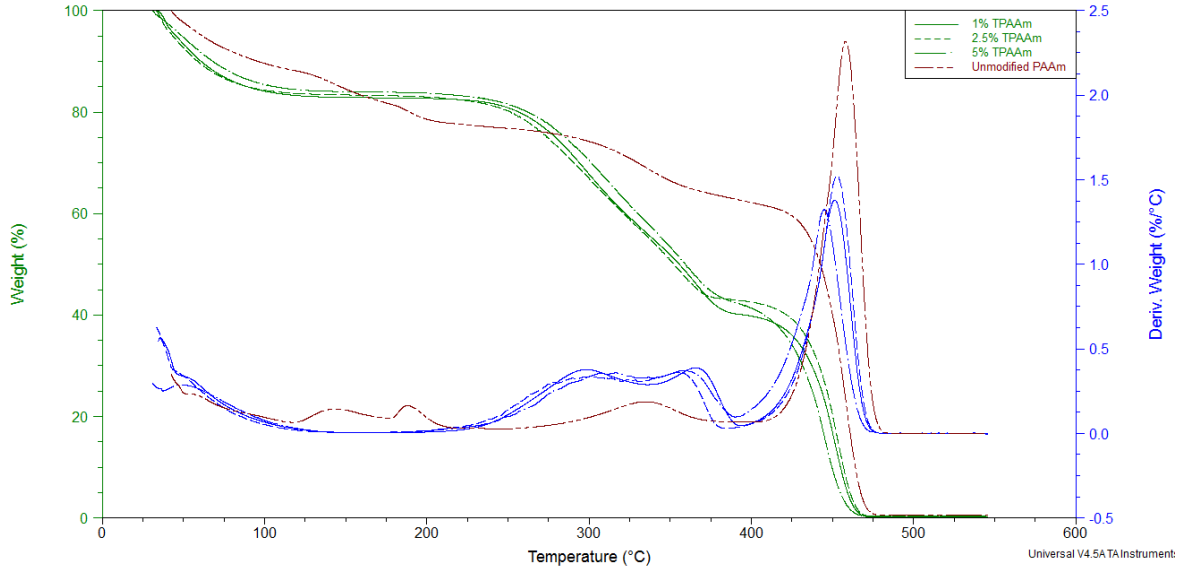

Figure 6.13 TGA of thiolated and unmodified PAAm

Thiolated PAAm samples with the addition of 5% gelatin were also analysed by TGA and compared to the degradation pattern of unmodified PAAm with 5% gelatin. All the thiolated/gelatin samples were similar in degradation but differed greatly from that of the unmodified/gelatin sample, as shown in Figure 6.14 (A); this marked difference in degradation between the thiolated and unmodified sample was also observed without gelatin addition (Figure 6.13). Figure 6.14 (B) shows the TGA thermogram of the 1% TPAAm sample with and without the addition of 5% gelatin. This highlights the slight variation in degradation that the addition of gelatin has induced on the thiolated sample, 1% TPAAm, and this thermogram was typical of all three thiolated samples when the addition of gelatin was compared to those without gelatin. The pattern of degradation was comparable in both samples, yet the final

degradation occurred at a lower temperature with gelatin addition, at 216 °C in the sample with gelatin and at 236 °C in the sample without gelatin.

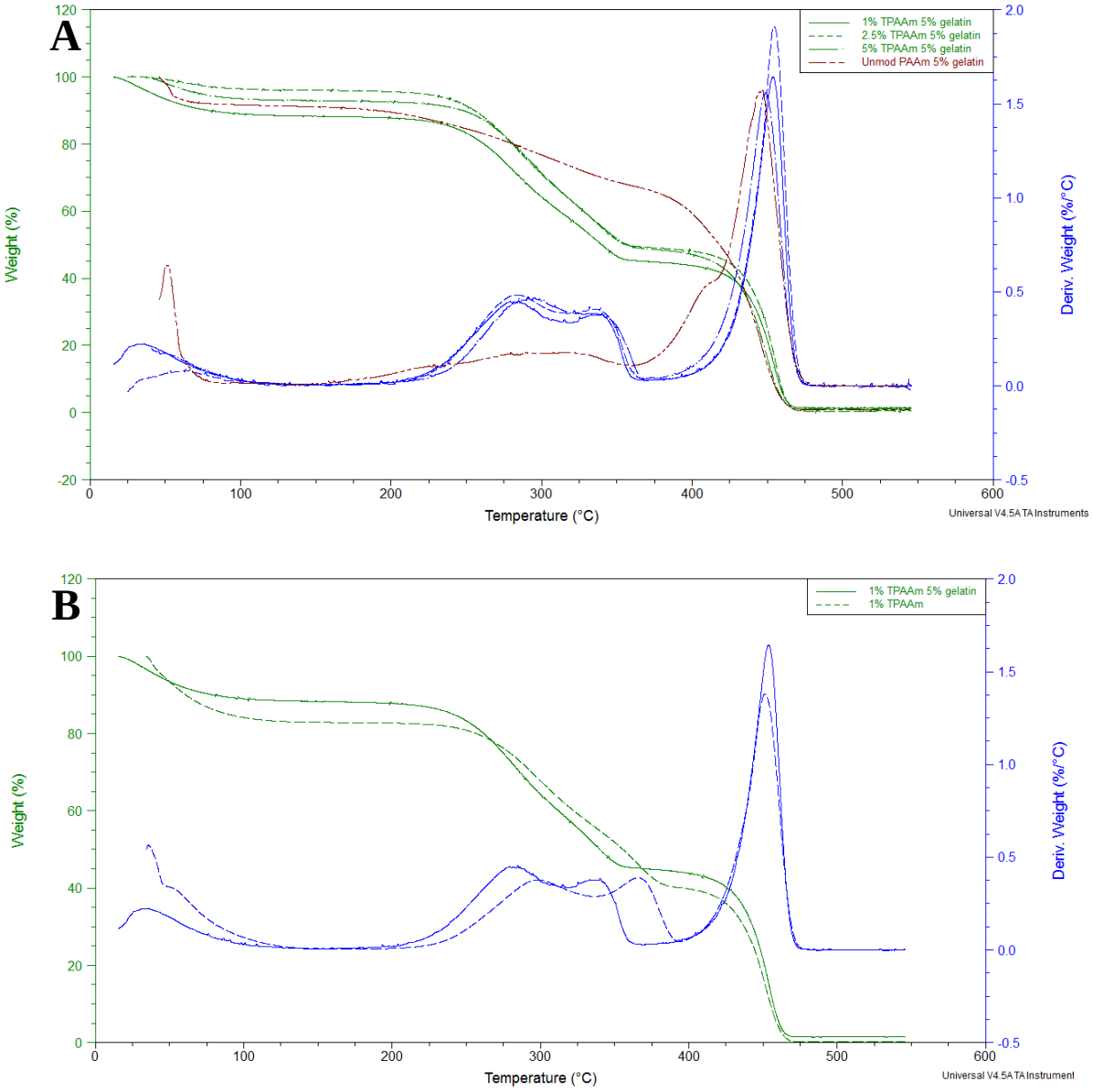

Figure 6.14 TGA of (A) thiolated and unmodified PAAm with addition of 5% gelatin and (B) 1% TPAAm with and without the addition of gelatin

6.3.2.4 Differential scanning calorimetry (DSC)

DSC analysis was conducted on unmodified PAAm, unmodified PAAm with the addition of varying ratios of gelatin, on thiolated PAAm samples and on thiolated

PAAm samples with the addition of 5% gelatin. Samples were analysed using both conventional and modulated DSC.

Similar to both PAA and gelatin samples, DSC was conducted using a heat-cool-heat cycle. In the first heating cycle, a large endothermic peak was observed at approximately 100 °C indicating water loss which masked the T_g of the unmodified PAAm sample, as shown in Figure 6.15. Prior to DSC analysis, the unmodified PAAm solution was freeze dried. The T_g was observed in the second heating cycle and was measured at 43.5 °C. From the literature, the T_g of polyallylamine alters vastly between papers; Kuo *et al.* (2005) synthesised polymer hybrids based on polyallylamine and polysiloxane. PAAm of MW 10 kDa was used, and in DSC analysis, two T_g's were observed. Kuo *et al.* surmised that the initial T_g, measured at 39.9 °C, was the T_g of PAAm while the second T_g was due to the matrix hybrid as a whole. This T_g for PAAm is in agreement with the measured T_g in this study. Other measurements were, however, in contrast to this study. DSC analysis was performed by Kim *et al.* (2002) on interpenetrating polymer network systems composed of chitosan and polylallylamine. In the study, the T_g of polylallylamine was measured at -26 °C. However, it was not stated whether the polyallylamine used was PAH or PAAm, nor did it refer to the MW of the polymer. Similarly, DSC analysis of unmodified PAAm was conducted by Cai *et al.* (2008) in which the T_g was measured at 93 °C. PAAm was synthesised from the monomer and the MW of the polymer was not given. The T_g measured by Ibie *et al.* (2015) was -12 °C; Ibie *et al.* reacted PAH with NaOH to yield the free base form of PAAm which may have affected the T_g.

Unmodified PAAm showed two endothermic peaks, at 141 °C and 181 °C as shown in Figure 6.14. Both peaks were non-reversible events and were only present in the first heating cycle of analysis. Endothermic peaks in unmodified PAAm have also been observed by Kim *et al.* (2002) and Ibie *et al.* (2015), measured at 110 °C and 138 °C, respectively. The initial peak observed at 141 °C may be the loss of bound water within the polymer matrix. The second endothermic peak coincides with weight loss in the TGA thermogram.

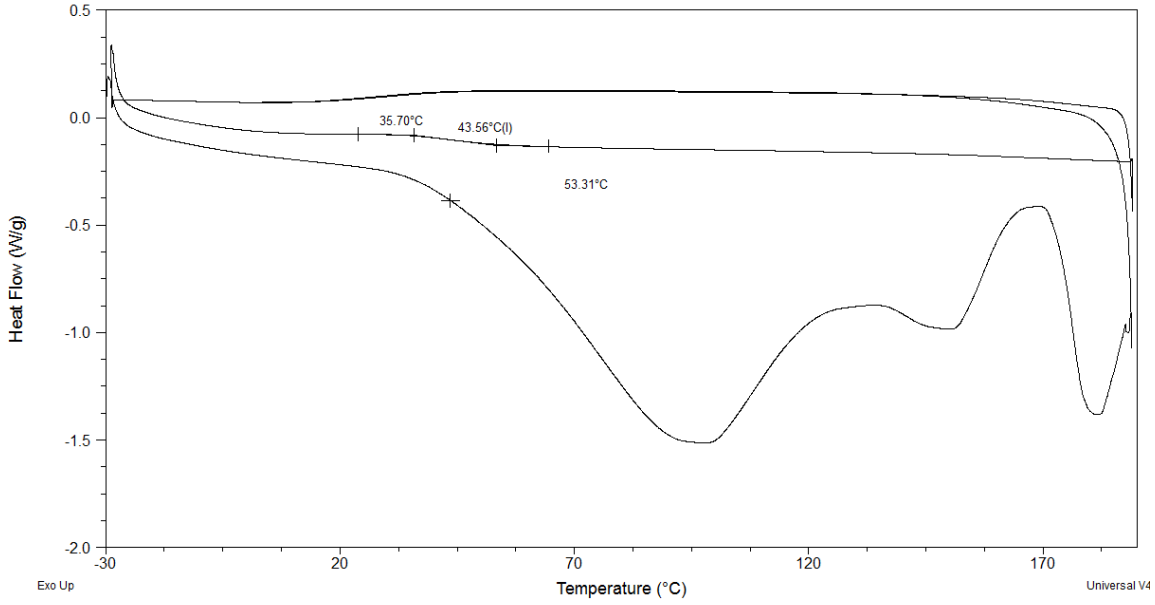

Figure 6.15 DSC thermogram, displaying the heat-cool-heat-cool cycles of unmodified PAAm

The effect combining two or more polymers has on the T_g of the resulting mixture has been investigated and a number of equations, including the Gordon - Taylor equation (Gordon and Taylor, 1952) and the Fox equation (Fox, 1956), allow for the prediction of the T_g of that mixture. These equations are based on the properties of the single components of the mixture, namely both the T_g and the concentration, often resulting in a T_g which is an intermediate of both single components. Both the Fox and Gordon – Taylor equations do have limitations; for example, the Gordon - Taylor equation is based on two assumptions: of an ideal volume of mixing and also a linear increase in volume with increasing temperature (Pinal, 2008). Because of this ideal behaviour, a number of equations have been created which are modified versions of the Gordon - Taylor equation which account for the interactions occurring between components of the mixture, as well as the concentration of water or the presence of a plasticiser. Investigating the theoretical T_g of the sample can give an insight into the interactions occurring within that sample mixture and can also give an indication as to whether the concentrations of the mixture are compatible/stable. Therefore, investigating the predicted T_g using an equation such as the Gordan-Taylor could be highly advantageous in the creation of a co-polymer/bi-polymer matrix, and may allow for the creation of a highly stable material.

Shown in Figure 6.16 is the DSC thermogram of unmodified PAAm with added ratios of gelatin acting as an excipient. Similar to the TGA data in which the degradation pattern of PAAm was altered upon the addition of gelatin, the T_g also changed with gelatin addition. The T_g of native gelatin 4569 in chapter 4 was measured at 203.73 °C whereas the T_g of unmodified PAAm was measured at 43.56 °C. The resulting T_g of the mixture of both of these components resulted in an intermediate T_g and as the concentration of gelatin added to the unmodified PAAm samples increased, the T_g also increased (Figure 6.16). This, as outlined by Gordon-Taylor (1952), was due to the increase in concentration of the component of the mixture which had the higher T_g, i.e. gelatin, which in turn increased the T_g of the mixture. As was also observed in chapter 4, the modification of gelatin decreased the measured T_g and Traut's reagent was deemed to be inducing plasticising effects on gelatin sample. Here, the addition of gelatin increases the T_g of the unmodified PAAm sample and, therefore, demonstrates an anti-plasticising effect (Teja *et al.*, 2013).

Also seen in both the 5% gelatin and 10% gelatin samples on the first heating cycle, is an endothermic peak which occurs after water evaporation and the T_g. This endothermic peak was also seen in the unmodified gelatin samples, as discussed in chapter 4, section 4.3.4.3. It is classified as an endothermic relaxation peak, and may be due to the addition of gelatin into the matrix.

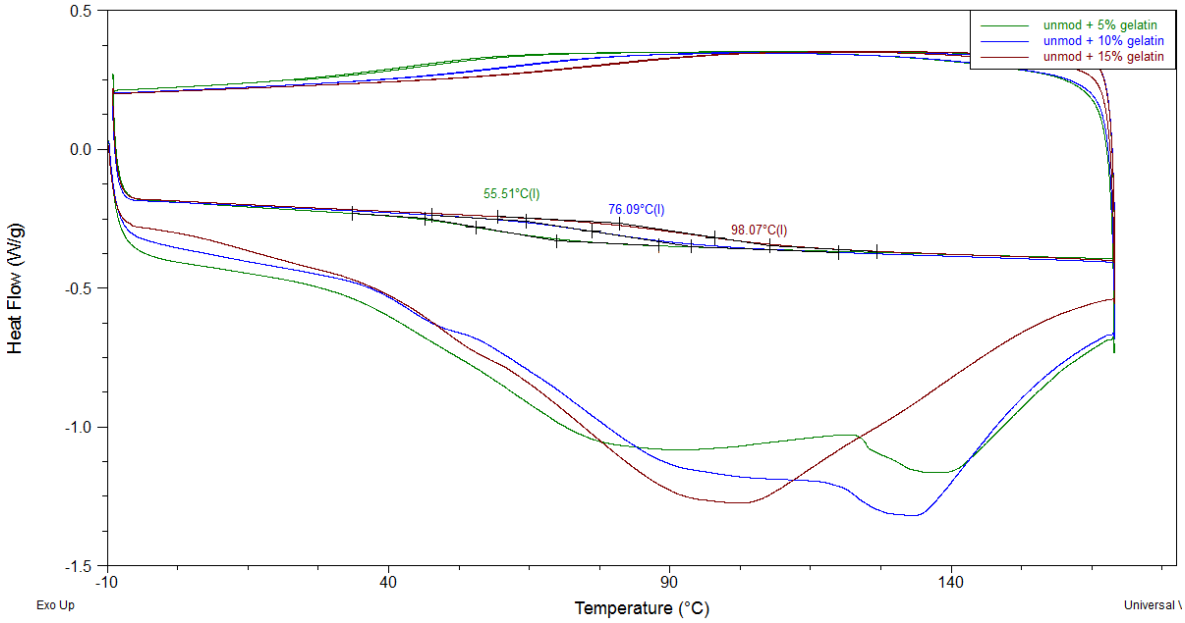

Figure 6.16 DSC analysis of unmodified PAAm with the addition of gelatin at different ratios

Thiolated PAAm samples and thiolated PAAm samples with the addition of 5% gelatin were also analysed with DSC. In convectional DSC, no T_g was observed in any thiolated sample. Because of this, the thiolated samples, with and without the addition of gelatin, and also TPAAm samples with CPM incorporated into them (TPAAm-CPM conjugates) were analysed using modulated DSC (MDSC).

6.3.2.4.1 Modulated DSC (MDSC)

As the reverse heat flow of a sample can be isolated with MDSC, the T_g of that sample can be identified. Shown in Figure 6.17 is the MDSC thermogram of the 1% TPAAm sample, showing the total heat flow, and the reversing and non-reversing heat flows. There was no endothermic step observed in the reversing heat flow of the sample, which signifies the T_g, therefore, no T_g was observed in the thiolated samples. Ibie et al. (2015) conducted conventional DSC on thiolated PAAm samples at a heating rate of 20 °C/min from -90 °C to 370 °C but did not measure a T_g in the thiolated samples. The increase in heating rate would increase the sensitivity of the DSC analysis, often making a T_g more visible, and it was surmised that the T_g was too subtle to be measured. In this study, there was an exothermic step observed in the MDSC thermograms, as highlighted in the 1% TPAAm sample in Figure 6.17 (A);

this exothermic step was present in all three thiolated samples, as displayed in Figure 6.17 (B) and also in the thiolated samples with the addition of 5% gelatin.

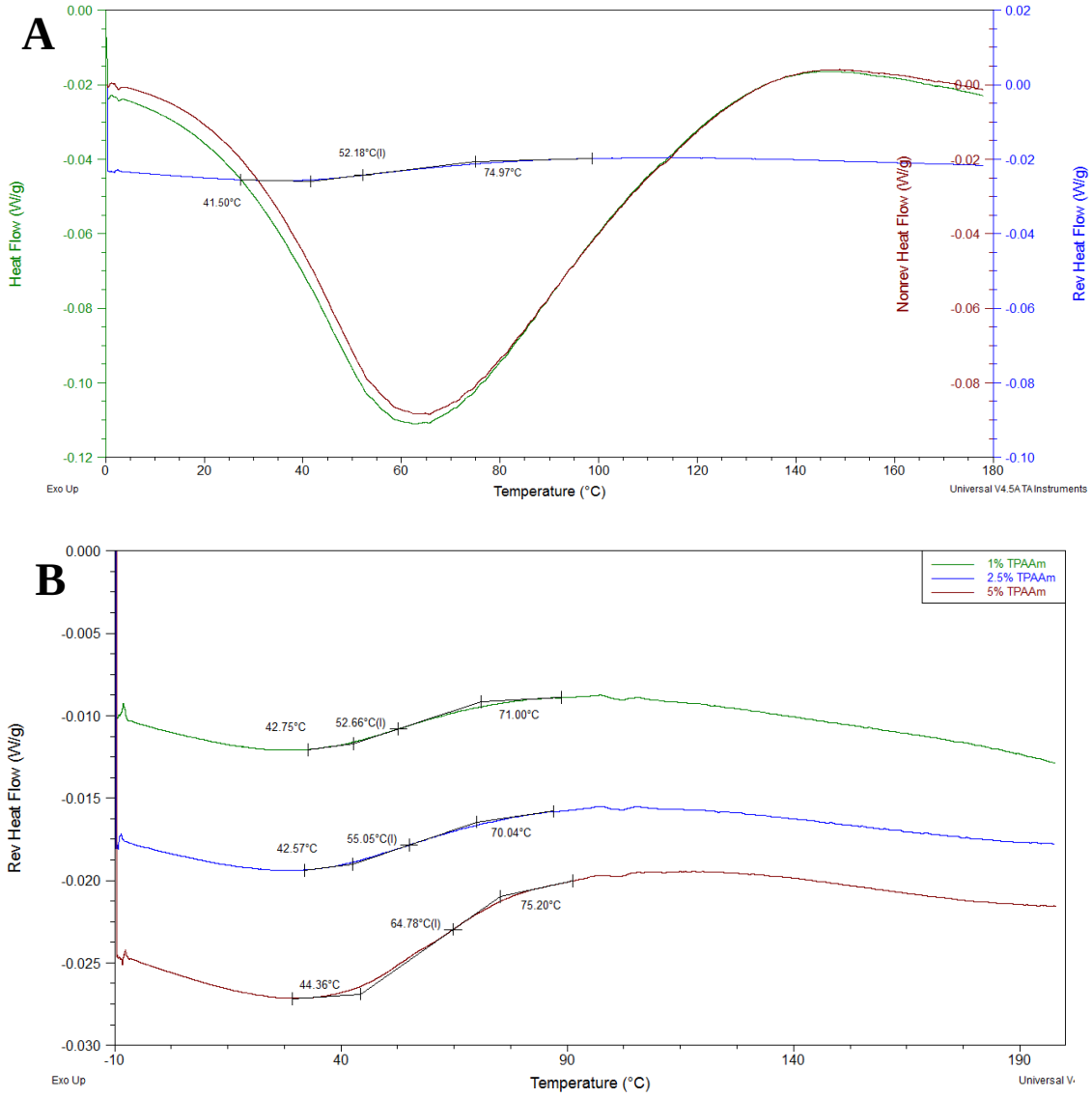

Figure 6.17 MDSC thermogram of (A) 1% TPAAm displaying the heat flow and reversing and non-reversing heat flows and (B) the reversing heat flow of TPAAm samples, with exothermic peak highlighted

Table 6.6 displays the measured exothermic step of all the thiolated PAAm samples, with and without the addition of gelatin. With the increase in thiol content, the temperature of the exothermic step also increased, as is shown in Table 6.6. This exothermic step is occurring 10 – 20 °C higher than the T_g of the unmodified PAAm sample. It may, in fact, be the T_g of the thiolated samples, however, the reverse heat

flow values of these samples are so small, that it is not possible to state this. With gelatin addition, the values of this possible T_g were marginally lower in comparison to the samples without gelatin but the trend of increasing exothermic step temperature with increasing Traut's reagent concentration remained.

Table 6.6 Exothermic step temperatures of thiolated PAAm with and without the addition of gelatin

Sample	Without gelatin (°C)	With gelatin (°C)
1% TPAAm	52	52
2.5% TPAAm	55	68
5% TPAAm	66	57

MDSC was also conducted on thiolated samples and unmodified PAAm/5% gelatin controls with the addition of CPM. The addition of CPM into the PAAm matrix allowed for a clear T_g to be measured, as shown in Figure 6.18. The T_g temperatures for all thiolated and control samples are displayed in Table 6.7. The addition of CPM in the unmodified/5% gelatin control increased the T_g from 56 °C to 121 °C. This also occurred in chapter 2 in the unmodified PAA sample where the T_g increased from 130 °C to 134 °C with the addition of CPM. This was in contrast to the thiolated PAA samples in chapter 2 where CPM was noted to act as a plasticiser, decreasing the T_g from 158 °C to 145 °C for example in the pH 6.5 PAA sample. This would imply that the interactions occurring between CPM and the unmodified polymers (both PAA and PAAm) are different than those occurring in the thiolated samples of both polymers. CPM appears to be exhibiting anti-plasticising effects (increasing the T_g) in the unmodified samples but plasticising effects (decreasing the T_g) in the thiolated samples. The plasticising effects of CPM were also observed by Wu and McGinity (1999) and Zhu *et al.* (2002). If, similar to the thiolated PAA samples, CPM is acting as a plasticiser in the TPAAm samples, the T_g of the TPAAm samples may be higher than the analysis temperature used in this study. However, as mentioned above, Ibie *et al.* (2015) also did not observe a T_g in the thiolated samples having analysed to a temperature of 370 °C in conventional DSC. It is possible that the modification of PAAm through thiolation has fundamentally

affected the polymer structure, not allowing it to transition into glass like structure. Alternatively, the T_g may be too subtle to measure it.

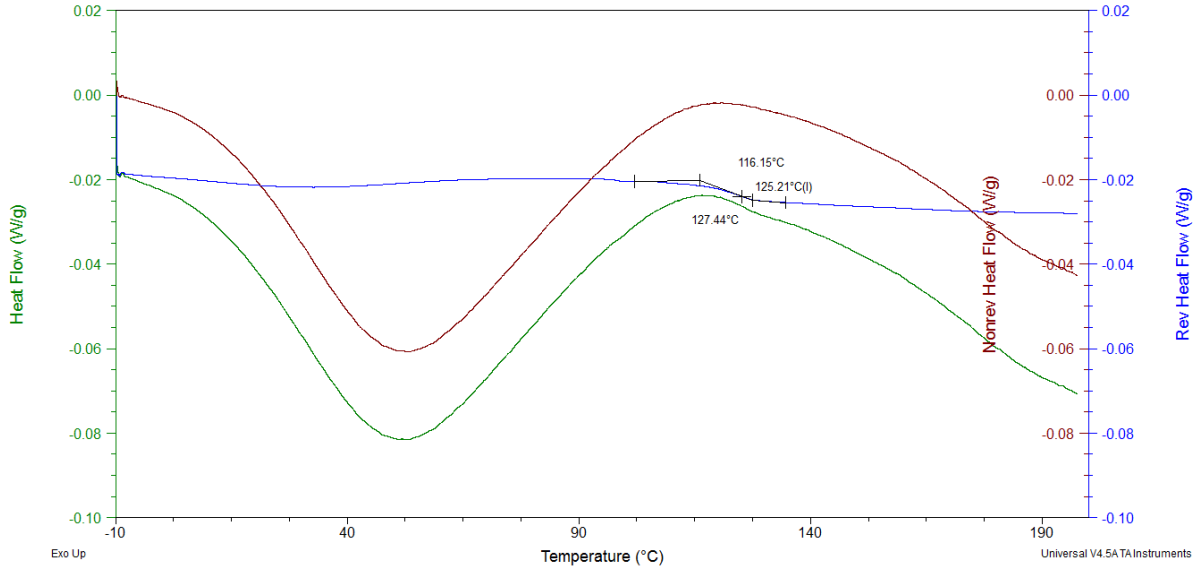

Figure 6.18 MDSC thermogram of 5% TPAAm with the addition of CPM

Table 6.7 Tg temperatures of samples with the addition of CPM

Sample	Measured T_g (°C)
Unmodified PAAm/5%gelatin + CPM	121
1% TPAAm + CPM	124
2.5% TPAAm + CPM	133
5% TPAAm + CPM	125

6.3.3 Antimicrobial testing: well diffusion studies

Due to the cationic nature of polyallylamine, the polymer has been shown to be toxic to both cells and microbes; this is due to the ability of the polymer to interact with and disrupt the cell wall (Boussif *et al.*, 1999; Vigl *et al.*, 2009; Iarikov *et al.*, 2013; Andrews *et al.*, 2011). Therefore, antimicrobial testing was conducted on unmodified PAAm and the three thiolated PAAm samples. Aqueous solutions of all samples were made at three different concentrations, 0.5 mg/mL, 1 mg/mL and 2 mg/mL, and the polymers samples were compared to the positive control, novobiocin, and to a negative control, water. Novobiocin can act as both a bacteriostatic or bactericidal antibiotic against Gram-positive bacteria (Smith and Davis, 1967), and although it is

active against Gram-negative bacteria, it is less effective. This may be due to the inability of the compound to penetrate the cell membrane of the Gram-negative bacteria (Barrett-Bee and Pinder, 1994). The less effective action against Gram-negative bacteria was also observed in this study. In Figure 6.19, the antimicrobial testing of unmodified and thiolated PAAm samples at a concentration of 0.5 mg/mL is shown. In Figure 6.19, the thiolated PAAm samples are referred to as 20, 50 and 100 which correlate to 1% TPAAm, 2.5% TPAAm and 5% TPAAm respectively. The zone of inhibition caused by the positive control, novobiocin, is highlighted for each bacteria in Figure 6.19. There is a larger ring of inhibition due to the antibacterial nature of novobiocin occurring in the *S. aureus* samples in comparison to the *E. coli* samples, which highlights the increased activity novobiocin has against Gram-positive bacteria in comparison to Gram-negative bacteria. Water, the negative control, displayed no bacterial inhibition.

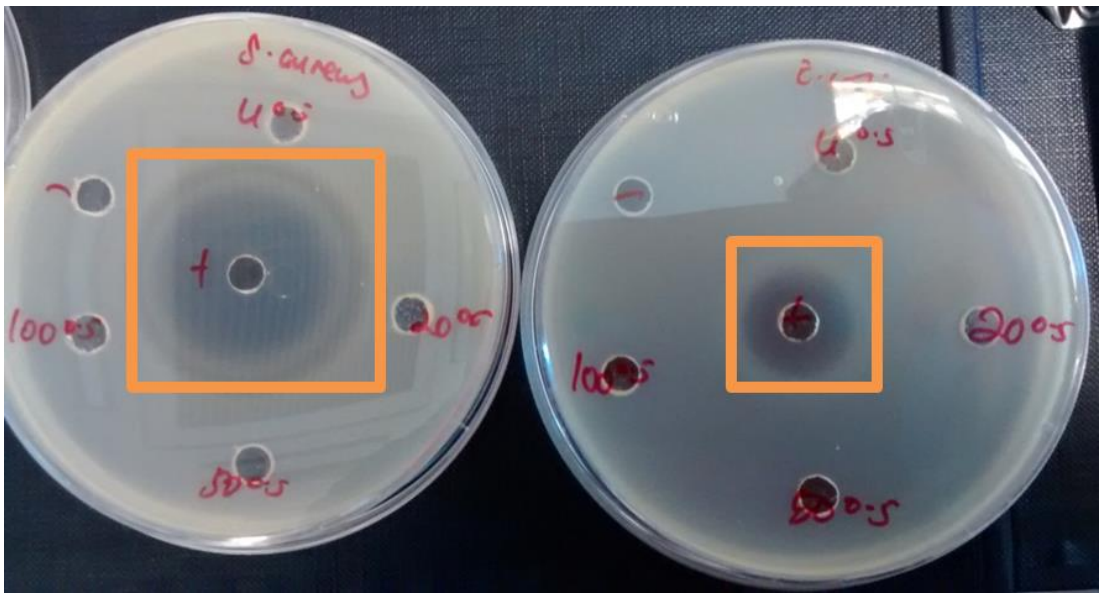

Figure 6.19 Antimicrobial testing against *S. aureus* (left) and *E. coli* (right) using unmodified and thiolated PAAm samples at a concentration of 0.5 mg/mL. Antibacterial activity of novobiocin is highlighted

Focusing on the antibacterial action of the polymers, at a concentration of 0.5 mg/mL, no polymer sample, unmodified or thiolated, displayed antimicrobial activity against either bacterium, as shown in Figure 6.19. As the concentration increased to 1 mg/mL, the unmodified PAAm sample began to display its

antibacterial activity with a small ring developing around the well of the sample; this occurred against both *E. coli* and *S. aureus*. The thiolated samples at this concentration did not display inhibition of bacterial growth. At a concentration of 2 mg/mL, again the unmodified PAAm samples displayed antibacterial action against both bacteria. At this concentration, all three thiolated samples, 1% TPAAm, 2.5% TPAAm and 5% TPAAm, displayed zones of inhibition against S. *aureus*, however, the inhibition zone produced was not as great as that produced by the unmodified sample, as is shown in Figure 6.20. The inhibition produced by the thiolated samples was not observed against *E. coli*. As *S. aureus* is a Gram-positive bacterium, its cell wall is much less complex than Gram-negative organisms, e.g. *E. coli*, with only one phospholipid membrane layer in their cell wall in comparison to the two membrane layers within Gram-negative cell walls. It is because of this that the thiolated samples were more effective against *S. aureus* than against *E. coli*. The greater effect antimicrobial activity of polyallylamine had on Gram-positive bacteria was also observed by Iarikov *et al.* (2013). Using both PAAm and PAH (polyallylamine hydrochloride), Iarikov *et al*. created films for use as antimicrobial surfaces. Varying MWs of 15 kDa or 58 kDa were used to synthesise the films. These films were then bound to glass by either covalent binding or electrostatic adsorption, and were tested for antimicrobial activity. Both Gram-negative and Gram-positive bacteria were used, and similar to this study, greater activity was observed against *S. aureus* and *Staphylococcus epidermidis*, both Gram-positive bacteria, with a kill efficiency of 97%. This is in comparison to 88% killing efficiency against the Gram-negative *Pseudomonas aeruginosa*.

It has been noted that the MW of the polymer may have an influence on its antibacterial properties, increasing them with increasing MW (Klibanov, 2007; Malmsten, 2011). In this study, PAAm of MW 15 kDa was used and the decrease in antimicrobial activity of the thiolated PAAm samples observed may change if a polymer of higher MW was used. Iarikov *et al.* (2013) investigated the antibacterial properties of PAAm with MW of 15 kDa and 58 kDa; against the Gram-positive *S. aureus*. There was greater killing efficiency of 97% with the 15 kDa sample in comparison to the 58 kDa sample with 90%. The zones of inhibition produced by the three thiolated polymers in this study were small in comparison to the unmodified PAAm sample. Thiolation of a higher MW PAAm may result in lower antimicrobial

activity than the thiolated PAAm synthesised in this study. The decrease in antimicrobial activity of the thiolated PAAm samples may be advantageous, particularly when targeting the intestinal tract for mucoadhesive drug delivery. The intestinal tract is home to millions of natural bacteria which assist with the digestion of food (Pratt and Cornely, 2011). If, when targeting the small intestine for mucoadhesive drug delivery, the polymeric tablet was to kill the intestinal bacteria, it may have an adverse effect on the patient, leading to digestive problems.

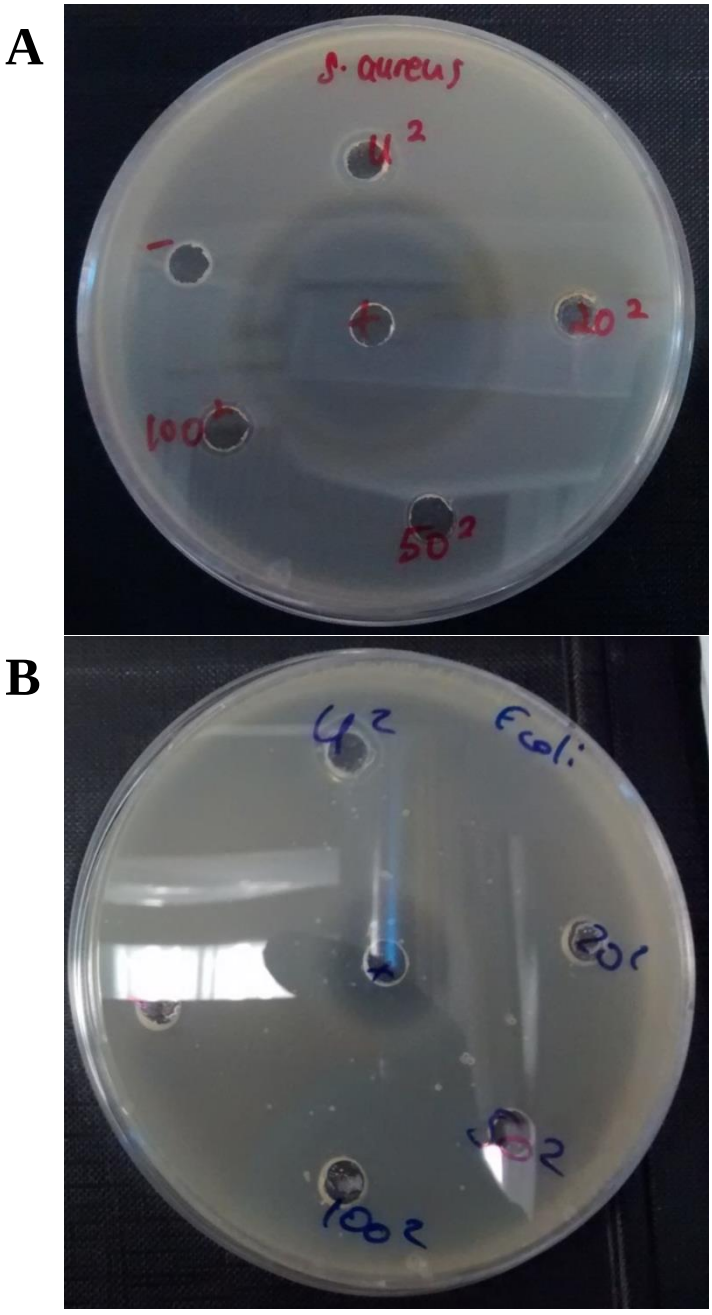

Figure 6.20 Antimicrobial testing against *S. aureus* (A) and *E. coli* (B) using unmodified and thiolated PAAm samples at a concentration of 2 mg/mL.

The thiolation of PAAm decreased the levels of toxicity towards microbes due to a decrease in amine groups and, therefore, decreased cationic nature of the polymer backbone. As discussed in chapter 1, the cytotoxicity of polyallylamine can also be reduced upon modification. The use of polyallylamine in gene transfer has been investigated using both PAH (Boussif *et al.*, 1999) and PAAm (Oskuee *et al.*, 2015). Without modification, the polymer was highly toxic and was not suitable for use; it also had low transfection efficiency for the delivery of DNA. To improve gene

transfer and to decrease the polymer's toxicity, Boussif *et al*. and Oskuee *et al*. modified polyallylamine, by glycolylation and by addition of acrylate groups, respectively. Both papers displayed improved gene transfer upon modification and, more importantly, a decrease in toxicity was also observed in comparison to unmodified polyallylamine.

In using PAAm as a mucoadhesive drug delivery system, it is vital that the polymeric matrix is not toxic to cells and the thiolation of PAAm has been seen to decrease the cytotoxic properties of PAAm. Vigl *et al.* (2009) thiolated PAH with N-acetyl cysteine and EDC, resulting in a thiol content of 77.6 µmol/g when a 15 kDa polymer was used and 83.1 µmol/g when the MW of PAH was 70 kDa. A previously crosslinked 70 kDa PAH sample was also thiolated, resulting in 162.5 µmol/g thiol content. Cytotoxicity assays were conducted on the three thiolated PAH samples. It was observed that MW had a strong influence on the cytotoxicity levels of the polymer; the unmodified 15 kDa PAH sample displayed 100% cell death whereas the unmodified 70 kDa sample displayed decrease cell death at approximately 92%. Similarly, once thiolated, the 15 kDa thiolated PAH sample had higher levels of cytotoxicity than the thiolated 70 kDa sample, and percentage cell death values of the thiolated 15 kDa sample were similar to the 70 kDa unmodified PAH sample. However, it was noted that thiol content of the samples was low and that an increase in thiol content may decrease the cytotoxicity. The influence of thiol content was highlighted further, as the crosslinked 70 kDa sample, which had the highest degree of thiolation, showed the lowest levels of cytotoxicity.

Using an MTT assay, Ibie *et al.* (2015) investigated the cell viability of Caco-2 cells having treated them with unmodified and thiolated PAAm samples. The thiolated samples were thiolated with either Traut's reagent or N-acetyl cysteine/EDC, resulting in a total thiol content (free thiol and disulphide bond contents) of 1080 µmol/g and 340 µmol/g, respectively. The unmodified PAAm sample showed the highest level of cell toxicity, due to its cationic backbone. Upon thiolation, cell toxicity reduced. The samples thiolated with Traut's reagent were observed to have lower levels of toxicity towards cells and it was thought that this was due to the higher levels of thiolation and, therefore, lower free amine groups along the polymer backbone.

6.4 Conclusion

As discussed in chapter 5, the thiolation of PAAm with Traut's reagent resulted in a range of products depending on the amount of Traut's reagent added; thiol contents of 133.63 ± 43.27 µmol/g, 329.53 ± 13.39 µmol/g and 487.43 ± 18.26 µmol/g were achieved in the 1% TPAAm, 2.5% TPAAm and 5% TPAAm samples, respectively. In this chapter, the thiolated PAAm samples were characterised by drug release of the model drug, chlorpheniramine maleate (CPM), by rheology, by SEM and thermal analysis and they were compared to unmodified PAAm control. Antimicrobial studies were also conducted on the samples.

As shown in chapter 5, the modification of PAAm by thiolation improved the swelling ability of the samples in comparison to the unmodified control, and the degree of thiolation influenced the swelling rate. This, in turn, had an effect on the drug release profile of CPM from the thiolated samples. The unmodified PAAm sample with the addition of 5% gelatin quickly dissolved in phosphate buffer during the swelling studies; with the addition of CPM into the matrix, full disintegration of the unmodified polymer also occurred within 30 s, thereby releasing CPM. This was in contrast to the thiolated PAAm samples. The thiolation of PAAm created a more cohesive polymer, which allowed the thiolated polymer to swell, slowing the release of CPM. In contrast to the results of the swelling studies, in which the 5% TPAAm sample swelled to the highest degree, the 5% TPAAm sample had a slower rate of drug release in comparison to the 1% TPAAm and 2.5% TPAAm samples. The 5% TPAAm had the highest levels of thiolation, with a thiol content of 487.43 ± 18.26 µmol/g, which may have allowed for the slower release of drug. Complete drug release from all thiolated PAAm samples was achieved after 2 h, which was faster than the release of CPM from the thiolated PAA samples in chapter 2; the PAA samples achieved complete release over 8 h. The swelling rate of the PAAm samples was markedly higher than that of the thiolated PAA samples, and this influenced the rate of drug release over time.

Rheological studies gave further insight into the swelling behaviour, and importantly, the mucoadhesive properties of the PAAm samples. Unmodified PAAm, without the addition of gelatin, was compared to the three thiolated samples. An 8% mucin solution was also mixed with the polymers to assess the mucoadhesive

behaviour. Strain sweeps, frequency sweeps and times sweeps were conducted on all PAAm samples, with and without the addition of mucin. In general, the storage modulus (G') was higher than the loss modulus (G") in all samples. This indicated that the polymers, unmodified and thiolated, had a predominantly solid-like response. In all tests, the addition of mucin increased the response values of G' and G" in comparison to the polymers alone. This suggests an increase in both gel strength and in bond formation in the polymer/mucin mix, indicative of a mucoadhesive polymer. Time sweeps of the unmodified and thiolated PAAm samples were conducted over a period of 30 min. Similar to the strain and frequency sweeps, the addition of mucin increased G' and G" in comparison to polymers samples. After approximately 15 min in the polymer/mucin samples, a further increase in G' and G" was observed, suggesting an increase in entanglement or bonding occurring in the polymer/mucin samples. This increase did not occur in the unmodified PAAm/mucin sample.

TGA studies on unmodified PAAm showed that the addition of gelatin altered the degradation pattern of the polymer, and a pattern similar to both unmodified PAAm and native gelatin was observed. Modification of the polymer backbone by thiolation also altered the degradation pattern of the polymer, as had occurred with both PAA and gelatin. The unmodified PAAm sample had a stepwise degradation pattern up to approximately 430 °C which was in contrast to the thiolated samples, which had a sharp decline in weight at 230 °C. The degree of thiolation in the TPAAm samples did not appear to change the rate of degradation, and comparable degradation rates were measured between the three TPAAm samples. The addition of 5% gelatin into the thiolated matrix increased the degradation rate of the thiolated samples, while the overall pattern was maintained.

In DSC analysis, the literature lists varying glass transition (T_g) temperatures for unmodified PAAm. In this study, a T_g of 43.5 °C was measured for the unmodified PAAm sample. The addition of gelatin into unmodified PAAm raised the glass transition temperature (T_g) and, as the ratio of gelatin added increased, the T_g also increased. The thiolated PAAm samples, with and without the addition of 5% gelatin were analysed with DSC. No T_g was observed using conventional DSC and therefore modulated DSC (MDSC) was used. Again, no clear T_g was observed in the thiolated samples using MDSC; an exothermic step was measured in the reversing heat flow

of the thiolated PAAm samples, and the temperature at which this step occurred increased with increasing thiol content. This exothermic step was also observed in the thiolated samples which had the addition of 5% gelatin into the matrix. Upon the addition of the drug, CPM, into the thiolated polymers, a clear T_g was then observed between 123 – 135 °C. If CPM was acting as a plasticiser in these thiolated PAAm samples, it may be that the T_g of the thiolated samples was above this temperature and was too small to be measured.

Polycationic polymers, such as PAAm, are known antibacterial agents (Iarikov *et al.*, 2013). Antibacterial testing was conducted on the unmodified and thiolated PAAm samples, using both Gram-positive, *Staphylococcus aureus*, and Gram-negative, *Escherichia coli*, bacteria. Well diffusion tests were conducted on the polymers at three different concentrations, 0.5 mg/mL, 1 mg/mL and 2 mg/mL. At a concentration of 0.5 mg/mL, no zones of inhibition were observed in the unmodified PAAm samples, but when concentration was increased to 1 mg/mL, zones of inhibition were then observed; this increased further when the concentration was increased to 2 mg/mL. Modification of the PAAm backbone has also been shown to alter this antibacterial activity; in this study, thiolation of PAAm was shown to lessen the activity of the polymer against both *Staphylococcus aureus* and *Escherichia coli* in comparison to the unmodified PAAm. Similar to the unmodified PAAm sample, no zones of inhibition were observed at a concentration of 0.5 mg/mL in the thiolated PAAm samples; neither was it observed at a concentration of 1 mg/mL, which was in contrast to the unmodified PAAm sample. Once the concentration of the TPAAm samples was increased to 2 mg/mL, a zone of inhibition was observed against S. *aureus* but was not observed against the more complex bacteria of *E. coli*. The inhibition produced by the 2 mg/mL TPAAm samples was not as great as produced by the unmodified sample, again highlighting the influence that thiolation of PAAm has had on the antibacterial properties of the polymer.

Throughout chapters 5 and 6, the synthesis and characterisation of the novel thiolated and mucoadhesive PAAm have been examined. Full mucoadhesive characterisation of thiolated PAAm, using swelling studies, mucoadhesive testing on the porcine intestinal tissue and rheology, has not previously been conducted prior to this study. Cohesive and mucoadhesive properties were greatly increased by the

thiolation of the PAAm backbone and the rheological studies confirmed the strong interactions occurring intramolecularly in the TPAAm samples in comparison to unmodified PAAm. Rheology also showed the strong intermolecular bonding and interactions between the TPAAm samples and mucin, which were comparable to the known mucoadhesive PAA, thus highlighting the potential thiolated PAAm has as a mucoadhesive drug delivery system. Using thiolated PAAm as a mucoadhesive drug delivery system will allow for prolonged and strong adhesion to the site of interest combined with controlled drug release over a 2 h period.

6.5 References

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Chapter 7 Conclusions and future work

7.1 Conclusions

The thiolation of three polymers, synthetic and natural, was conducted within this study. Varying methods of thiolation were utilised and the resulting thiolated products were tested for swelling ability, cohesive and mucoadhesive properties, as well as characterised by drug releasing ability, rheology, SEM and thermal analysis. The synthetic polymer polyacrylic acid (PAA) is a well-established and well characterised polymer used in the creation of thiolated mucoadhesive polymers (Palmberger *et al.*, 2007; Bernkop-Schnürch and Steininger, 2000; Marschütz and Bernkop-Schnürch, 2002; Hornof *et al.*, 2003; Iqbal *et al.*, 2012) and as such, the cohesive and mucoadhesive properties of PAA could be used as a direct comparison to the novel thiolated polymers of gelatin and polyallylamine (PAAm). In contrast to both native gelatin and unmodified PAAm, unmodified PAA showed inherent cohesive and mucoadhesive properties. Therefore, the thiolation of PAA was to improve mucoadhesion as opposed to the thiolation of both gelatin and PAAm which was to create mucoadhesion.

PAA was thiolated with the addition of L-cysteine in the presence of the crosslinker, EDC. Within the literature, the degree of thiolation was increased by increasing the concentration of EDC added to the reaction or by increasing the time period the EDC activated the PAA backbone prior to the addition of L-cysteine. In this research, it was concluded that pH played an integral part in the PAA-EDC reaction and, by monitoring the pH profile of the PAA-EDC reaction, a specific and controllable level of thiolation could be achieved. Varying the pH at which cysteine was added resulted in the creation of thiolated PAA samples with thiol content ranging from 400 – 1000 µmol/g.

The resulting thiolated PAA samples were analysed for cohesive and mucoadhesive properties and swelling ability, whilst being compared to unmodified and thiolated control PAA samples. Thiolation vastly improved the cohesive and mucoadhesive properties of PAA. Thiol content was seen to be of extreme importance, with swelling behaviour and mucoadhesive properties differing with increasing thiol content. Samples with a thiol content of 1000 µmol/g displayed a higher degree of swelling ability in comparison to other PAA samples with lower thiol content.

Mucoadhesion was also affected by this increased swelling ability as the bond formation and interactions between the thiol groups of the polymer and the mucosal layer may have been influenced by the increased ability to swell. This suggests that too high a thiol content may decrease the mucoadhesive properties of a thiolated polymer.

As a natural polymer, gelatin has many advantages over both PAA and PAAm, including non-toxic effects, biocompatibility and biodegradability. In a novel two-step reaction process, thiolated gelatin was created. This two-step reaction involved initially aminating the carboxyl groups along the gelatin backbone by reacting native gelatin with ethylene diamine in the presence of EDC. In the second reaction step, the aminated gelatin was then thiolated with 2-iminothiolane (Traut's reagent), creating a highly thiolated gelatin product. An increase in thiolation levels of up to 10-fold was achieved following the two-step reaction in comparison to the direct thiolation of native gelatin samples. Product yields were poor after both the amination and the thiolation reactions and the degree of thiolation of gelatin was inconsistent. A number of changes to the parameters of the amination reaction were made in an attempt to increase product yield including altering the concentration of EDC added and changing the pH at which the amination reaction was conducted. The concentration of ethylene diamine added did not affect amine content or the percentage yield of product. pH could influence both EDC efficiency and protonation of ethylene diamine during the amination, and this was observed with an increase in the product yields with increasing pH.

A number of gelatin samples of differing molecular weights (MWs) were analysed and, similar to PAA (Leitner *et al.*, 2003), MW was observed to play an integral role in the creation of a highly thiolated and mucoadhesive material. All samples of varying MW showed improved thiol content following the two-step reaction in comparison to thiolated native samples. However, depending on the MW of the gelatin sample used, swelling ability and mucoadhesive properties varied. By comparing thiolated gelatin samples with similar thiol content but differing MW, it was observed that gelatin 4569, with a MW of 20 - 25 kDa, was shown to have the most potential for mucoadhesive drug delivery; gelatin 4569 displayed excellent

swelling abilities, cohesion and mucoadhesion. Gelatin samples of higher MWs ranging from 40 - 100 kDa, gelatins 4567, 4568 and 4571, had decreased swelling abilities, decreased cohesion and decreased mucoadhesive properties. Thiolated gelatin 4569 showed the most potential as a mucoadhesive drug delivery system. Although the cohesive and mucoadhesive properties were lower than the thiolated PAA samples, as a natural polymer gelatin offers the advantages of biocompatibility over the synthetic polymer. As mucoadhesion was improved in comparison to the unmodified gelatin sample, a drug delivery device could be created which allows for the fast release of drug to a specific site using thiolated gelatin 4569.

The thiolation of a second synthetic polymer, polyallylamine (PAAm), was conducted. Similar to gelatin, PAAm was also thiolated with Traut's reagent. PAAm was reacted with varying concentrations of Traut's reagent with the aim to thiolate 1%, 2.5% and 5% of the polymer backbone. This resulted in three thiolated samples with varying degrees of thiolation. As a control, unmodified PAAm was mixed with 5% gelatin to aid compression. Similar to native gelatin, the cohesive and mucoadhesive properties of the unmodified control were poor, and the tablets disintegrated quickly. The swelling rates of the TPAAm samples were rapid; all samples displayed an increase in mass of up to 1500% within the first minute of the test. The thiol content, and indeed the disulphide bond content of the samples, did influence the swelling behaviour and the 5% TPAAm samples displayed the highest levels of swelling. The overall swelling profiles of the three thiolated samples were, however, comparable. Both the cohesive and mucoadhesive properties of PAAm were vastly improved upon thiolation in comparison to unmodified control samples. The degree of thiolation appeared to have little influence on the mucoadhesive properties of the thiolated PAAm (TPAAm) samples, with slight variations in adhesion times.

Drug release studies were conducted by incorporating the anti-histamine drug, chlorpheniramine maleate (CPM), into thiolated and unmodified/control samples of PAA, gelatin and PAAm. The thiolated PAA sample was observed to release CPM in a slower and more controlled fashion than the unmodified sample, and

demonstrated equivalent release to thiolated PAA samples in the literature (Hornof *et al.*, 2003). This was in vast contrast to the drug release of CPM from the thiolated gelatin 4569 sample in which controlled drug release was not achieved. Drug release from the thiolated gelatin sample was poor and showed a similar release pattern to the control sample. The thiolated gelatin sample used displayed little cohesive nature and the quick release of drug from the polymer matrix may be due to the lack of cohesion. When comparing the drug release of thiolated PAAm to thiolated PAA, controlled release from the thiolated PAAm samples was achieved, unlike gelatin, but it too was inferior to the thiolated PAA samples. Release of CPM from the all TPAAm samples was achieved over a 2 h period. This is in comparison to the 8 h release achieved by thiolated PAA. The thiol content and swelling abilities affected the drug release profile of the three TPAAm samples. Unlike the swelling studies, however, the 5% TPAAm displayed the slowest rate of drug release. Slow release to specific sites, such as the GI tract, is not always necessary; the improved mucoadhesive properties demonstrated by the thiolated PAAm can offer fast release to mucosal sites.

Rheology was conducted on both thiolated PAA and thiolated PAAm, and the results mirrored the swelling and mucoadhesive properties of the two thiolated polymers. The storage modulus (G') and the loss modulus (G") of the thiolated PAAm were markedly higher than the thiolated PAA samples, suggesting the increased ability of the thiolated PAAm to swell. Upon mucin addition, G' and G" values of both thiolated polymers increased, which has been shown to indicate mucoadhesion (Marschütz and Bernkop-Schnürch, 2002). The G' and G" values of the two thiolated polymer/mucin mixes were comparable, suggesting the bonding with mucin within those systems was similar. This once more highlights the potential the more novel thiolated PAAm has as a mucoadhesive polymeric drug delivery system. The addition of mucin to the unmodified PAA and unmodified PAAm samples again showed the mucoadhesive nature of PAA, as G' and G" values increased in the unmodified PAA sample. This was in contrast to the unmodified PAAm sample which showed no change in G' or G" upon mucin addition.

Thermal analysis was conducted on all thiolated and unmodified/control samples of PAA, gelatin and PAAm. The thiolation of PAA was observed to alter the degradation pattern of the polymer, decreasing the initial degradation step, as observed by TGA. DSC analysis showed that the T_g increased in thiolated samples in comparison to the unmodified and control samples. This was in contrast to the thermal analysis of gelatin. In TGA, the overall degradation pattern of the native, aminated and thiolated gelatin samples were comparable, with slight variations in weight loss. However, in DSC analysis, with each reaction step in the thiolation of gelatin process, the polymer was modified and this was observed as a decrease in T_g after each step. The morphology changes between aminated and native samples were also observed by SEM. Thermal analysis of the three TPAAm samples 1% TPAAm, 2.5% TPAAm and 5% TPAAm, were comparable. There was a marked difference between the degradation pattern of the thiolated samples and the unmodified PAAm sample which had a more step-wise degradation. Upon the addition of 5% gelatin into the unmodified matrix, the degradation pattern again shifted, displaying elements of both gelatin and PAAm degradation; however, the overall degradation patterns between the gelatin incorporated and non-gelatin incorporated samples were comparable. The T_g of the unmodified PAAm sample was measured at 43.5 °C; however, no T_g was observed in the TPAAm samples when analysed by either conventional DSC or Modulated DSC methods.

7.2 Future works

7.2.1 Gelatin

Rheological studies were not conducted on the aminated and thiolated gelatin samples, as the samples were used up. A better insight into the swelling behaviour and mucoadhesive properties of the aminated and of the thiolated samples may be achieved. Similarly, a direct comparison of gel strengths and viscoelastic properties could then be made between PAA, PAAm and gelatin.

Although gelatin is a natural and biodegradable polymer, as the polymer has been modified through the introduction of amine and thiol groups, cytotoxicity testing

using cell culturing techniques should be conducted to ensure the safety of the aminated and thiolated products. Cell viability was carried out by Kommareddy and Amiji (2005) on thiolated nanoparticles which had been thiolated with 2-iminothiolane. Cytotoxicity was observed to increase with increasing thiol content, with 92 % cell viability observed with a thiolated nanoparticle which had 20 mg/g of 2-iminothiolane added and 79 % relative cell viability observed when 100 mg/g of 2-iminothiolane was added (there was an 8-fold increase in thiolation between both samples). Native gelatin showed 100 % cell viability.

Continued work on the reaction process of amination and thiolation is required as reproducible results have not been achieved. The inherent variability of gelatin was highlighted throughout chapters 3 and 4, and it was thought areas of high and of low amine content, and consequently areas of high and low thiol content, could be potentially occurring during the modification process, and thus effecting results. To investigate whether such areas occur throughout the polymer backbone, the aminated gelatin could be fluorescently tagged using a N-hydroxysuccinimide (NHS) ester activated amine reactive dye which, when examined under the fluorescent microscope, may show the areas of amination. Equally, the thiol groups could be fluorescently labelled using a maleimide activated reactive dye to show areas of thiol content. This method may give an insight into the both the 2-step reaction process and also the variable nature of gelatin.

7.2.2 Polyallylamine

The swelling ability of the thiolated PAAm samples was altered with the addition of 5% gelatin into the polymer matrix; gelatin addition increased the initial swelling of the 1% TPAAm samples but reduced the initial rapid swelling rate of the 5% TPAAm sample. Although gelatin addition did lessen the mucoadhesive properties of the samples, it may have a positive influence on the drug release profile of the model drug, CPM. Drug release from the TPAAm samples occurred over a 2 h period; the addition of gelatin into the matrix could have the potential to decrease the rate of release, making the polymeric system more comparable to thiolated PAA.

Similar to the gelatin samples, cytotoxicity was not conducted on the TPAAm samples. In contrast to gelatin, however, PAAm is a known cytotoxicity agent.

Thiolation of the polymer has been shown to decrease the cytotoxic properties of PAAm (Vigl *et al.*, 2009; Ibie *et al.*, 2015). Increased thiol content has been shown to increase cell viability when treated with thiolated PAAm samples. Testing the 1% TPAAm, 2.5% TPAAm and 5% TPAAm samples, at varying concentrations, would give more of an insight into the influence of thiol content on cytotoxic properties.

MW of polymers can have an important effect on cohesive and mucoadhesive properties, as was shown in this study with both PAA and gelatin. Optimal MW for a mucoadhesive polymeric system is also unique to the polymer (Andrews *et al.*, 2009). In this study, only PAAm of MW 15 kDa was investigated. Although cohesive and mucoadhesive properties were vastly improved by thiolation in comparison to the unmodified control, increasing the MW of the polymer used may further improve the mucoadhesive properties of the sample. MW was shown to alter the antimicrobial and cytotoxic properties of unmodified PAAm, and, therefore, thiolating a longer chained polymer may be advantageous.

7.2.3 Thiolation of polymers

Utilising the novel two-step reaction method used in the thiolation of gelatin, thiolation of other proteins could be investigated. The protein of interest is β-lactoglobulin, a protein found in milk as part of the structure of whey proteins. β-lactoglobulin is found to have a MW of 18 kDa (Eigel *et al.*, 1984), is similar in structure to gelatin, however, it already has free thiol groups and disulphide bonds within its amino acid structure due to the presence of cysteine (Withers *et al.*, 2013; Livney, 2010). Withers *et al.* (2013) investigated the binding of two native milk proteins, casein and β-lactoglobulin, to the oral mucosa. Using rheological measurements, β-lactoglobulin was observed to have a better force of adhesion to oral mucosa than casein which may have been due to the presence of thiol groups in β-lactoglobulin due to cysteine. Research has been conducted involving food based products, looking into the delivery of bioactives through the means of proteins, utilising milk based proteins for drug delivery and food supplements and characterisation of those proteins (Kehoe and Foegeding, 2014; Livney, 2010). However, little work has been conducted involving the modification of these proteins or their potential in mucoadhesive drug delivery. Once modified using the

amination/thiolation procedure, the protein β-lactoglobulin has potential as a mucoadhesive natural polymeric tablet.

7.2.4 Pharmaceutical uses for thiolated polymers

Thiolated polymers have great potential for use in targeted drug delivery allowing for the controlled release of drug over time. Thus far, thiolated polymers have been investigated in the delivery of drugs through a number of different routes including: nasal (Wang *et al.*, 2009), ocular (Hornof *et al.*, 2003), buccal (Langoth *et al.*, 2003), gastrointestinal (Kremser *et al.*, 2008) and cervicovaginal (Friedl *et al.*, 2013).

Due to the mucoadhesive nature of thiolated polymers, utilising them for the treatment of an area which is not the site of adhesion would also be advantageous; for example, nasal administration for drug delivery to the brain. As the nasal cavity is highly vascularised, it has been used in the delivery of proteins and steroids (Shahnaz *et al.*, 2012). Nasal administration can by-pass the blood brain barrier (BBB) (Dhuria *et al.*, 2010), a major barrier in the administration of drugs to the brain, further work has also been conducted for the treatment of central nervous system (CNS) diseases such as Alzheimer's disease (Wu *et al.*, 2012) and Parkinson's disease (Ugwoke *et al.*, 1999) through nasal administration. With the increase in the life expectancy and an aging population throughout the world, utilising mucoadhesive nasal delivery for the treatment of such diseases may have great potential.

Similarly, drug delivery to the eye is an area which may hold significant promise for mucoadhesive polymers. As mentioned previously, Hornof *et al.* (2003) designed a mucoadhesive PAA-based ocular insert and tested it on volunteers; results showed low levels of irritation within the volunteers and the inserts themselves displayed a controlled release of drug over time. Similar to the increase in prevalence of CNS diseases due to an aging population, the occurrence of posterior ocular diseases and dry eye syndrome is also on the rise. A product which contains thiolated chitosan will be release into the European market in the coming months (Bonengel and Bernkop-Schnürch, 2014); called Lacrimera ®, these eye drops will be used for the treatment of dry eye syndrome, and further investigations in the thiolation of

hyaluronic acid for the use in ocular treatments are also underway (Bonengel and Bernkop-Schunurch, 2014).

The use of mucoadhesive polymers in the treatment of diseases such as diabetes is also an area of extreme importance, with both financial and medical benefits to be obtained. Once enterically coated, thiolated polymers could allow for the oral delivery of insulin by providing protection against enzymatic degradation. A number of publications have investigated this area utilising PAA, chitosan and PAAm as the mucoadhesive polymer (Grabovac *et al.*, 2008; Wang *et al.*, 2009; Zhang *et al.*, 2012; Ibie *et al.*, 2015). Incorporating other peptide and protein based drugs into thiolated matrices may potentially allow for more patient-friendly treatments for certain cancers, GI diseases, such as Crohn's disease, and ocular diseases.

7.3 References

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