DOCTORAL THESIS

Design and synthesis of proteoglycan analogues for tissue repair and regeneration

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DESIGN AND SYNTHESIS OF PROTEOGLYCAN ANALOGUES FOR TISSUE REPAIR AND REGENERATION

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Doctor of Philosophy

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March 2011

THESIS SUMMARY
ASTON UNIVERSITY

Design and Synthesis of Proteoglycan Analogues for Tissue Repair and Regeneration

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Submitted for the Degree of Doctor of Philosphy March 2011

This thesis is concerned with the design and synthesis of a novel, injectable proteoglycan analogue for tissue repair. This is of particular relevance to the restoration of disc height to a degraded nucleus pulposus of the intervertebral disc. The focus is on the use of sulfonate monomers as proteoglycan analogues, in particular sodium 2-acrylamido-2-methylpropane sulfonic acid and the potassium salt of 3-sulfopropyl acrylate.

For most biomedical applications, synthetic hydrogels need to show dimensional stability to changes in pH, osmolarity, and temperature. This is readily achieved by neutral structures however ionic sulfonate containing hydrogels are responsive to environmental change which renders them difficult to manage in most tissue replacement applications. In this case osmotic responsiveness rather than stability is desirable. Therefore sulfonate based materials possess advantageous properties. This is a result of the sulfonate becoming an ideal surrogate for the sulfate group present within the structure of natural proteoglycans.

This thesis reports polymerisation studies based on the production of a redox initiated copolymer system capable of polymerising *in situ* within a timescale of *circa.* 5-7 minutes. The rheological properties, osmotic drive, and residual monomer content of successful compositions is analysed. Properties are adapted to mimic those of the target natural tissue.

The adaptation of the material for use as an injectable intra-ocular lens, with hyaluronic acid as an interpenetrate is reported. The synthesis of a radiopaque macromer to allow visibility of the repair system once *in situ* is investigated and discussed.

The results presented in this thesis describe a suitable proteoglycan tissue analogue which is injectable, biomimetic, osmotically responsive and mechanically stable in its desired application.

Keywords: proteoglycan analogue, osmotically responsive, biomimetic

For my Family

Acknowledgements

I would like to express my sincere appreciation to my supervisor Professor Brian Tighe for all his encouragement and support during my time at Aston. In particular the opportunity to conduct a PhD within the Biomaterials Research Unit after completing an MSc project within the group. I am very grateful for the opportunities I have received during my studies including the attendance of many conferences. It has been a great privilege to work and learn from someone who is so respected and admired within his field.

I would also like to give thanks to all the members of the Biomaterials Research Unit for the assistance and encouragement they have given me along the way. In particular Dr Val Franklin for all her hard work in making the group work smoothly, and the advice and support she provided along the way. I also thank Dr Gareth Ross for his support and encouragement throughout my studies, in particular the writing up period.

Thank you also to my family and close friends who are always there to lend an ear when times have been difficult, and also to Ade whose support has been invaluable over the last three years.

I thank EPSRC and Vista Optics for their financial support for the duration of my work.

Table of Contents

List of Figures

List of Tables

Chapter One

Introduction

1.1 Introduction

This thesis is concerned with the design and synthesis of a proteoglycan analogue for tissue repair and regeneration. The initial focus of the research was to identify a polymer composition with properties similar to that of specific natural tissue such as the nucleus pulposus of the intervertebral disc and crystalline lens of the eye. This was further refined to produce a system suitable for successful injectable delivery to the target area. The concept of biomimesis was an important aspect of this research. It was important to understand the nature, composition and responsiveness of the target tissues in order to successfully design the analogue. A unique property of the natural tissues is their osmotic responsiveness, particularly under compression, which will be discussed in detail along with further mechanical properties of the analogues created.

Due to the intended use of the material as an implant of sorts, the incorporation of an X-ray contrast medium was also investigated as part of this research. It is a desirable advantage if an implant can be observed throughout its implantation/injection and subsequent lifetime. Attempts were made to synthesise a multifunctional macromer from an existing contrast agent (Histodenz) so that it would form part of the polymer structure, this was however not a major focus of this research.

It is important to mention that the concept of an injectable intervertebral disc repair system already existed prior to the research conducted in this thesis due to an EPSRC collaboration between Aston University, University of Oxford, and Robert Jones and Agnes Hunt Orthopaedic Hospital Oswestry. Where data that resulted from this collaboration has been used, it has been clearly acknowledged.

The concept of creating a synthetic proteoglycan tissue analogue is an important one because no existing hydrogel-based implant exhibits osmotically responsive behaviour. The utilisation of redox polymerisation allows the implant to be injectable as a liquid, which will polymerise *in situ,* enabling the surgical process to be minimally invasive. It also reduces the constraints of the manufacturing process on the end mechanical properties of the material.

This introduction will briefly introduce the origin of hydrogels, commonly used materials and their uses, and the role of natural hydrogels within the body. The target areas; nucleus pulposus of the intervertebral disc and structure of the crystalline lens will be discussed in further detail. There will be an overview of the methods of polymerisation relevant to this thesis, followed by the subsequent techniques used to test the resultant materials. Finally the nature of currently available X-ray contrast media will be discussed.

1.2 Hydrogels

1.2.1 History of hydrogels

Harold Ridley was an ophthalmologist who worked in the Royal Air Force during World War II. Upon removing splinters of acrylic from the eyes of pilots he noticed there was no adverse reaction to the presence of this material. This was the first suggestion that a material of this nature might not cause an adverse reaction upon implantation. Therefore he proposed the use of a similar material to treat cataracts and was the first to complete such an implant in 1949[(1)]. However it was the pioneering work of Otto Wichterle some fifty years ago that introduced interest in hydrogels and biomimesis. His work was one of the first examples of biomimesis: the use of a natural system for the production of a synthetic analogue. He developed hydrogel materials in an attempt to produce ocular implants that more closely resembled natural tissue than the rigid plastics and metals that were in use at the time. A precursor monomer (2-hydroxyethylmethacrylate) – HEMA was developed which was used to produce cross-linked water swollen polymers, which were stable to changes in pH, osmolarity, and temperature. This led to the development of HEMA based hydrogels as tissue replacement materials and soft contact lens[(2)].

1.2.2 Synthetic hydrogels

Hydrogels can be best described as cross-linked hydrophilic polymer networks that are able to imbibe large amounts of water or biological fluids but do not dissolve in them[(3)]. They are hydrophilic, three dimensional networks and due to their ability to imbibe large quantities of water or biological fluid, they are able to largely resemble natural structures[(4)]. Hydrogels contain tetrahedral sp^3 carbon atoms to form a

backbone structure which incorporates other atoms such as hydrogen, oxygen, nitrogen and sulfur[5]. The biocompatibility of hydrogels makes them interesting materials with applications in pharmaceutical delivery, wound dressings, tissue engineering, dental restoration, biomedical electrodes, and ophthalmic materials[6,7,8,9]. They occupy a unique position in the field of biomaterials due to the way in which the polymer surface properties are greatly influenced by the absorbed water. Hydrogels are flexible, non antigenic and permeable to water and metabolites. Structural variations allow for a degree of control over the water content and water binding behaviour which is arguably the most important factor in hydrogel design. Water within a hydrogel can act in several different ways:

1. A plasticiser – behaving as an internal lubricant allowing chains to rotate hence conferring flexibility.
2. A transport medium for dissolved species such as oxygen and water soluble metabolites
3. A surface energy 'bridge' between natural and synthetic systems thereby enhancing bio tolerance
4. A lubricant – reducing the coefficient of friction at the surface

Within the hydrogel structure, initially water is strongly bound to specific sites on the polymer chain such as hydroxyl and/or ester groups, this water also interacts with the crosslinking agent if used. It behaves both thermodynamically and dynamically as part of the polymer. Further water is preferentially structured around the polymer network and is weakly bound to available hydrophilic sites while any remaining water behaves in bulk. Interfacial water content will increase with increasing crosslink density. The structure is dependant on the hydrophilicity of the hydrogel which in turn depends upon the functional groups incorporated into the polymer– the order of decreasing hydrophilicity being sulfonate >> carboxylate > amide > hydroxyl.

A hydrogel may consist of two types of gel: a physical gel and a chemical gel. Physical gels are entangled chains that are not linked together in any way. There may be ionic or hydrogen bonding in place but there are no covalent crosslinks, which are present in a chemical gel. The hydrogels studied in this investigation are chemical gels consisting of ionic monomers cross-linked primarily with poly(ethyleneglycol)

diacrylate (PEGDA). Cross-linking occurs in a free radically mediated process, *in situ* as part of the polymerisation reaction used to form the hydrogel.

Fig 1.2.2.1 A physical gel represented by entangled free chains and a chemical 3D network where the red lines represent cross-links

1.2.3 Synthetic hydrogels

Figure 1.2.3.1 shows some common monomers used to make hydrogels, and polymers used as interpentrants and precursors to macromers used in hydrogel formation. This is not a complete list, but it illustrates a variety of materials highlighting in particular the presence of polar hydrophilic functional groups that are a common feature of hydrogel structures.

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Fig 1.2.3.1 Examples of commonly used monomers and polymers used to make hydrogels

1.2.4 Natural hydrogels

Examples of natural hydrogels include the nucleus pulposus of the intervertebral disc which is the central gel-like part of the disc responsible for cushioning and spacing to allow passage of nerves, the cornea and articular cartilage.

1.3 Intervertebral disc (IVD)

The spine provides support for the body. It is composed of twenty-six bones that extend in a line from the base of the skull to the pelvis. Twenty-four of these bones are 'free' and known as vertebrae. The lower part of the spine known as the sacrum consists of five fused vertebrae between the hip bones and the coccyx, which itself has between three and five fused bones at the lower tip. These bones are aligned so as to provide a passageway for nerves and the spinal cord and to allow a person to stand upright, bend and twist. On the back of the vertebral body the lamina and pedicle forms a protective ring around the spinal canal. The intervertebral discs lie between the vertebrae providing a 'cushion' between vertebrae and also act as a 'spacer' to allow nerves to exit the spinal canal. The solid matrix of the intervertebral disc is composed of a gelatinous nucleus pulposus and a highly organised angle-ply laminate structure of the annulus pulposus[26]. Facet joints are located on the back of the main part of the vertebra formed from a part of the vertebra above and below it, they connect each vertebra together and permit forward and backward motion.

The position of the intervertebral disc within the spine is shown below in figure 1.3.1, followed by a close up of the annulus fibrosus and nucleus pulposus in figure 1.3.2.

Fig 1.3.1 A representation of the spinal column illustrating the position of the intervertebral disc[10]

1.3.2 The nucleus pulposus of the intervertebral disc[11]

The nucleus pulposus is an example of a hydrogel composite. It is the innate ability of the gel-like structure to retain its water content which maintains disc height during its diurnal cycle between sleeping and activity. The ability of the disc to remain hydrated while exposed to mechanical load and atmospheric dehydration is in part due to the presence of natural proteoglycans such as keratan sulfate, chondroitin 4-sulfate and hyaluronic acid. The subsequent mechanical properties of the intervertebral disc are controlled by this composition.

Up to 95% of the extracellular matrix of the disc consists of collagen, proteoglycans and water. Collagen provides the strong fibrous framework which encases, within the matrix, proteoglycans and water. Collagen has a triple helix structure and nine different forms exist in the disc. Types I, II, III, V and VI are all fibril forming collagens[12] which are present in abundance within the intervertebral disc, contributing to its fibrous structure. They have repeating glycine X-Y sequences and have a long central helical domain. The X and Y amino acids are usually proline and hydroxyproline respectively[13].

Only 1% of the composition of the disc consists of cells, these are however still important as they provide constituents which control matrix composition and cell turnover. About 70-75% by mass of the disc is water, predominantly found in the nucleus. The level of water in a disc can vary as much as 10-20% in a diurnal depending on the person. This is in part due to pressure on the disc being lowest when a person is lying down sleeping, pressure then rises and varies greatly depending on a persons daily activities. As with the water concentration, the level of proteoglycans present in the disc decreases from the centre towards the outer of the disc. The greater presence of proteoglycans within the nucleus pulposus creates a high negative fixed charged density that controls hydration levels and enables compression resistance. Figure 1.3.3 shows the structures of the most abundant proteoglycans in the IVD, chondroitin and keratan sulfate respectively

Fig 1.3.3 Chondroitin and keratan sulfate repeating units where Gal, galactose; GalNAc-6s, N-acetylgalactosamine 6-sulfate; GlcUA, D-glucuronic acid; GlcNAc-6s, N-acetylglucosamine 6-sulfate

1.3.1 Intervertebral disc degeneration

Spinal stenosis is a narrowing of spaces in the spine that results in pressure on the spinal cord and or nerve roots. It usually occurs in one or more of the following three areas of the spine:

- The canal in the centre of the column of bones, also known as the vertebral or spinal column
- The canals at the base of the roots of nerves branching out from the spinal cord
- The openings between vertebrae through which the nerves leave the spine and go to other parts of the body

It is most common in men and women over fifty years of age but can occur in those born with a natural narrowing of the spine, or after an injury.

Degenerative disc disease may be defined as changes in wear of the individual discs of the spine. It is one of the most common causes of lower back pain. However disc degeneration is a natural part of aging, but can also result from trauma and or infection. Degeneration may result in localised pain, stiffness and loss of mobility
Changes in disc height can have both local and global effects. On a local – cellular level, decreased disc height and volume results in increased load on the remaining nucleus pulposus which can lead to a decrease in cell matrix synthesis and an increase in cell necrosis and apoptosis[27]. Strenuous work/exercise or being overweight has an effect on IVD degeneration, in fact animal models have shown that overloading of the intervertebral disc can initiate disc degeneration[28,29]. Lumbar spinal stenosis is the most frequently encountered and clinically important degenerative spinal disorders in the ageing population.

When degradation has occurred and the annulus fibrosus is no longer structurally intact, this is known as a herniated disk, this has three main stages:

- A bulge -- The gel has been pushed out slightly from the disk and is evenly distributed around the circumference.
- Protrusion -- The gel has pushed out slightly and asymmetrically in different places.
- Extrusion -- The gel balloons extensively into the area outside the vertebrae or breaks off from the disk.

The stages of disc degeneration are demonstrated in figure 1.3.1.1

Fig 1.3.1.1 The stages of disc degeneration[30]

1.3.2 Current methods of disc repair

Primary management of degeneration involves the use of non-steroidal anti-inflammatory and exercise, which may be used to strengthen both abdominal and spinal muscles.

Surgical intervention becomes an option when non-operative care is no longer able to relieve pain. The most common surgery performed is a Laminectomy. If nerves have become compressed or narrowing of the spinal cord has occurred, the lamina can be removed to create space. This is represented diagrammatically in figure 1.3.2.1

Fig 1.3.2.1 A representation of laminectomy

An incision is made into the muscle and ligaments either side of the spine exposing the laminae. The lamina is then trimmed until sufficient bone is removed which frees the compressed nerve by increasing available space.

A more radical method that can be used is Posterior Lumbar Interbody Fusion, the affected disc is removed and bone graft material is inserted into the space between the two vertebrae where the disk was removed. The graft is held in place with a 'fusion cage'. The main goal of the procedure is to stimulate the vertebrae to grow together into one solid bone, creating a rigid column in the problem area. Unfortunately fusion has the potential to accelerate degradation of surrounding discs associated with the loss of mobility and flexibility.

Implants are a possible alternative yet there are none currently available on the markets that do not require extensive surgery. Due to the materials they are constructed of they are also poor mimics for the natural tissue. An example of an artificial ProDisc-L is shown in figure 1.3.2.2. This consists of two metal cobalt chrome alloy endplates which implant into vertebrae above and below the affected

disc. An ultra high molecular weight polyethylene is inserted between the two endplates and acts as a 'ball'[32].

Fig 1.3.2.2 The artificial ProDisc-L[32]

In comparison to the drastic treatment available (prostheses are implanted through the abdomen), and/or the lack of effective treatment, it is clear that the treatment of disc degeneration could be improved by the production of a minimally invasive injectable repair system designed for use in the early onset stage, this is the aim of the work described in this thesis.

The most studied nucleus pulposus replacement currently in trial is the Prosthetic Disc Nucleus or PDN marketed by Raymedica, Minneapolis. It consists of a hydrogel pellet encased in a polyurethane jacket. The implant has been shown to perform favourably in terms of biological compatibility and biomechanical testing. In one particular trial conducted in Korea, 48 patients underwent nucleus replacement surgery and the success rate was reported as 78%.

1.4 The cornea and crystalline lens

The structure of the eye is shown in figure 1.4.1. The cornea covers the transparent front part of the eye that encompasses the iris, pupil, and anterior chamber. In addition to the lens, the cornea refracts light, accounting for around two thirds of the eyes optical power. The cornea consists of Type III, IV and VII collagen plus hyaluronic acid and stromal proteoglycans[14]. The architecture of the collagen fibres coupled with an intact endothelium are the main components responsible for corneal hydration and transparency.

Fig 1.4.1 The structure of the eye[15]

The lens consists of approximately 65% water and 35% protein, it has a central thickness of ~4mm and an equatorial diameter of ~ 9mm[16]. It is totally transparent and enclosed in a thin membrane capsule that is suspended between the aqueous humour and the vitreous body by equatorial zonular fibres.

The lens of the eye, unlike any other ocular tissues, keeps growing throughout life. In lens growth new cells are laid over existing fibre cells which are then displaced towards the centre of the lens[17]. As the lens continues to grow, and cells continue to be compacted, that there is a loss of water in the lens along with the increase in protein content causing an increase in refractive index. This results in an increase in stiffness of the lens and reduces the ability of the eye to accommodate. The actual values of stiffness are believed to increase from 70Pa at birth to 2700Pa in a 65 year old[18]. Protein contents in the lens are in the range of between 17% and 38% in the outer cortex and nucleus respectively[19]. Water contents due to this alteration in protein range from 50% to 85%. Due to the alteration in protein and water content RI rises to about 1.418 but not usually higher, and appears to plateau in the centre of the lens[20].

1.4.1 Cataract formation and subsequent surgery

Cataract formation is an opacification of the natural lens which occurs due to metabolic changes of the crystalline lens fibres over time. The lens is made mostly of water and protein. The protein is arranged to let light pass through and focus on the retina. Sometimes some of the protein clumps together which can start to cloud small areas of the lens blocking some light from reaching the retina and interfering with vision. During cataract surgery the cloudy natural lens is removed and replaced with a synthetic intraocular lens to restore transparency.

Types of cataract:

- Age related
- Congenital – some babies are born with cataracts or develop them in childhood, often they do not affect vision but must be reduced anyway
- Secondary cataract – cataracts are more likely to develop in people who have certain medical problems such as diabetes. They can also be linked to the use of medications such as steroids. Long term unprotected exposure to sunlight is also believed to contribute to the development of cataracts
- Traumatic cataracts – cataracts can develop soon after an eye injury or years later[33].

With an ageing population the number of people who are suffering with cataracts is on the increase. Cataract blindness is the most common cause of blindness in the world today; the World Health Organisation(WHO) estimates that of the 45 million people who are blind, cataracts cause half.

Modern cataract surgery is today one of the most common surgical procedures performed; with millions performed each year. Most of these surgeries occur in the western world where people can afford the operation. In developing and third world countries people who suffer from cataract blindness are unable to afford the relatively simple operation which could allow them to see again. Cataract surgery consists of the clouded lens being removed from the eye and an IOL being inserted to replace the removed lens.

A summary of the surgical process is shown below[33]

1. Anaesthesia
2. Exposure of the eyeball using a lid speculum
3. Entry into the eye through a minimal incision (corneal or sclera)
4. Viscoelastic injection to stabilise the anterior chamber and to help maintain the eye pressurisation
5. Capsulorhexis- removal of the lens
6. Hydrodissection pie- removal of the lens in segments
7. Hydro-delineation- injection of fluid into the cavity
8. Ultrasonic destruction or emulsification of the cataract after nuclear cracking or chopping, cortical aspiration of the lens, capsular polishing
9. Implantation of the artificial IOL
10. Entration of IOL – usually foldable
11. Viscoelastic removal
12. Wound sealing/hydration if needed

1.4.2 Intra-ocular lenses

Intraocular lenses are artificial lenses used to replace the natural lens after cataract surgery. IOLs have been around since the 1960's although the year the FDA first approved an IOL was 1981. Traditional IOLs are monofocal- they offer vision at one

distance only. Multifocal and accommodating lenses are now available. The incision size for insertion of an IOL depends on the nature of the IOL. There are two main types – those that are foldable and those that are not. The incision size for a foldable IOL can range from 1.8mm to 3.5mm. For a rigid PMMA lens this may increase to 5-7mm. PMMA was for a long time the material of choice due to its low weight and inherent biocompatibility due to a relatively low surface energy. It does however result in corneal endothelial damage on insertion, and post -operative adhesion of inflammatory cells to the IOL structure. Attempts have been made to improve IOL materials including polishing the surface, using NVP and or HEMA, and reducing the surface energy by coating with perfluoropropane or phosphorylcholine, and or binding heparin and HA to the outer surface of the lens[34].

Foldable IOLs are usually made of silicone or acrylic material of an appropriate refractive power. They are inserted either by folding in half using special forceps or *via* the use of a special insertion device that rolls the IOL and injects it slowly into the capsular bag. Examples of currently used foldable IOLs are shown in figure 1.4.2.1

Fig 1.4.2.1 Examples of existing IOL designs[35]

The central viewing zone is called the optic, the clear round disc measures 5.5-6.5mm in diameter. On both sides haptics act like tension loaded springs to automatically centre the lens once implanted. As shown above, overall shapes vary.

The development of an injectable IOL, which would polymerise *in situ* would be advantageous, as it would reduce the incision size needed and simplify the operation, desirable for an ageing population and those patients with underlying health issues. Benefits of a small incision include:

- Less trauma to the eye
- Little discomfort during or after surgery
- Often does not require stitches
- Can aid in reducing astigmatism and provide better vision

The utilisation of an injectable IOL would also reduce the manufacturing constraints on the material, it would no longer need to withstand lathe cutting in the case of acrylics and/or moulding in the case of silicone materials. This should therefore allow a greater control over desirable mechanical properties. Hydrophilic materials, of which this analogue is based on, have been shown to be less damaging to the corneal endothelium and produce less of an inflammatory response.

1.4.3 The problem of accommodation

Accommodation is the ability of the eye to focus on near and far objects in order to produce a sharp retinal image. This is achieved *via* the contraction of the cilliary muscles which in turn contract and relax the zonular fibres. As the lens becomes stiffer with age it becomes more difficult, and eventually impossible for the muscles to alter the shape. Therefore the eye no longer has the ability to accommodate, known as presbyopia. Currently a patient who has been fitted with a standard IOL will suffer the same degree of presbyopia due to the stiffness of the IOL material, the eye cannot accommodate. The use of an injectable material with low mechanical strength has the potential to allow the eye to accommodate.

1.5 Articular cartilage

Articular cartilage covers the ends of bones in syno*via*l joints and provides a shear resistant and resilient weight-bearing surface that is essential for normal joint function. It is composed of 75%-80% water with the remainder consisting of proteoglycans (87% chondroitin sulfate, 6% keratan sulfate and 7% protein) arranged around collagen fibrils[21]. Mechanically the collagen fibrils restrain excessive expansion of the negatively charged hydrophilic proteoglycans forming a compact but highly hydrated extracellular matrix[22]. The transport of nutrients, enzymes, cytokines and growth hormones, to name a few, are extremely important in the maintenance of cartilage cell structure and *via*bility. Degenerative joint disease involves an increase in water content and a decrease in proteoglycan content of the cartilage structure, thus detrimentally effecting transport of nutrients. New proteoglycans are synthesised however they do not aggregate as readily as in healthy tissue. This affects not only the mechanical stability of the matrix but also the mobility of solutes that are essential for cell *via*bility.

1.6 The role of proteoglycans(PGs) and glycosaminoglycans(GAGs) in the extracellular matrix

The extracellular spaces – particularly of connective tissue such as cartilage, tendons, skin and blood vessel walls consist of collagen and elastin suspended in a gel matrix. This matrix is made up of GAGs and PGs and is known as the extracellular matrix. The large sulfated GAG polysaccharide chain in PGs has a strong negative charge environment that binds Na^+ ions. This allows the drawing of large amounts of water into the GAG matrix by osmosis. Therefore PGs are essentially an immobilised aqueous environment that allows flow of water and its solutes subject to the negative charge generated by the sulfate group. A representation of the ECM is shown below in Figure 1.6.1

Fig 1.6.1 The ECM and the role of proteoglycans[23]

The study of the structure and functions of GAGs and PGs has greatly increased understanding of the roles they play in biological processes such as cell proliferation, differentiation and wound healing. GAGs such as heparin, heparan sulfate and dermatan sulfate serve as key biological response modifiers by acting in four different interconnecting roles.

1. Act as stabilisers, co-factors/co-receptors for growth factors, cytokines and chemokines
2. Regulate enzyme activity
3. Act as signalling molecules in response to stresses such as cellular damage due to wounding, infection and or tumorigenesis
4. Behave as targets for bacterial, viral and parasitic virulence factors for attachment, invasion and immune system invasion[24,25].

The method of polymerisation which allows the best control for an injectable application which is required for the minimally invasive methods of repair desired for the applications described in this thesis is redox. The next section of the introduction will introduce and discuss this topic.

1.7 Redox polymerisation

In order for the material to be delivered *via* injection as a low viscosity *pre-gel,* polymerisation must occur *in-situ* to give complete reliable polymerisation. Therefore there are three possible methods of free radical initiation that could be employed; thermal, UV, and redox. Due to the application as a tissue repair system thermal initiation can immediately discounted due to damage subsequent heat would cause to surrounding tissues. UV polymerisation also creates a problem as firstly, most UV based initiators are organic and could cause toxicity issues if used in the presence of bodily tissues prior to consumption. Also with respect to the disc, initiation of polymerisation would require the implantation of a UV probe which, is both impractical and defeats the object of developing an injectable repair system. Therefore this leads to the choice of redox initiation.

Redox initiators produce free radicals in an effective, mild and controlled way that is required for this application. This occurs *via* a one on one electron transfer reaction[37]. Redox initiation requires a much lower activation energy; typically 40-80 $kJmol^{-1}$ in comparison to that of thermal initiation which requires energies of 12-160 $kJmol^{-1}$. The milder conditions of redox initiation lower the possibility of side reactions occurring, producing a polymer in a good yield with a higher molecular weight than might be expected from thermal polymerisation[38]. However initiation must still be efficient, ensuring that each initiating radical produces a high enough conversion of monomer to polymer to avoid high residual monomer levels after completion.

The use of water soluble redox initiators is advantageous for use with hydrophilic monomers. The large hydration shell of the monomer allows excellent access for water soluble intiators to continually propagate the polymer chain. The level of solvation of both initiator and crosslinking agent is important as insufficiently

solvated systems tend to micellise and as a result areas of high crosslink density occur within the polymer network. Commercially available free radical initiators tend to be soluble in organic media rather than aqueous.

1.7.1 The effect of oxygen within a redox system

The presence of molecular oxygen within a redox system tends to increase the induction period, however in some cases, in particular in the use of potassium persulfate/ascorbic acid redox pair, oxygen may act as a cocatalyst with the ability to reduce the induction period. Oxygen is used at atmospheric levels favourably to assist in the degradation of ascorbic acid, however there must be control over the level of oxygen within the system so as to avoid the production of low molecular weight polymers due to oxygen acting as a chain terminator. It was discovered very early on within the polymerisation studies that although eradication of oxygen within both stock monomer solutions and initiator solutions produces a much more uniform gel, an initial nucleus of gelation is formed instantaneously rendering the application of an injectable unsuitable due to blockage of the needle.

Focusing on ascorbic acid and potassium persulfate as a redox pair, when ascorbic acid is in the presence of oxygen the following autocatalytic reactions shown in below in figure 1.7.2.1 occur.

Fig. 1.7.2.1 The oxidation reactions of ascorbic acid

Where AH_2, AH, and A represent ascorbic acid, ascorbate radical and dehydroascorbic acid respectively[38].

In aqueous solution ascorbic acid dissociates into ionic fragments and it is the monohydroascorbate ion which is mainly responsible for the strong reducing action of AA shown in figure 1.7.2.2

Fig. 1.7.2.2 The reducing action of ascorbic acid

The chain reaction involving ascorbic acid and potassium persulfate during free radical polymerisation is shown below in figure 1.7.2.3

Fig. 1.7.2.3 The reaction between ascorbic acid and potassium persulfate[37]

The action of the ascorbate radical represented by $A^{\bullet}$ is shown below in figure 1.7.2.4.

Fig 1.7.2.4 The action of the ascorbate radical

The persulfate radical generation is represented below in figure 1.7.2.5

Fig 1.7.2.5 The persulfate radical

Incorporating the monomers, the polymerisation can be represented by figure 1.7.2.6

Fig 1.7.2.6 A representation of free radical polymerisation using ascorbic acid and potassium persulfate redox pair

1.7.3 Free radical polymerisation: types relevant to this research

Initiation is the creation of free radicals capable of propagating a polymer chain. Two reactions occur, the initiator splits into two free radicals, each fragment may then react with a monomer unit initiating the polymerisation. Upon addition to the carbon-carbon bond of the monomer (breaking the pi bond), the radical with be present on the most substituted carbon atom [36].

$I \longrightarrow 2R\bullet$

$R\bullet + M \longrightarrow RM\bullet$

Propagation, the next step, involves continuous addition of monomer at the end of a free radical to regenerate the structure of the propagating species

$RM\bullet + M \longrightarrow RM_2\bullet$

$RM_n\bullet + M \longrightarrow RM_{n+1}\bullet$

Termination of a radical may occur *via* two routes, combination with another polymer radical known as bimolecular termination, or *via* disproportionation.

$RM_n\bullet + RM_m\bullet \longrightarrow R_2M_{n+m}$

$RM_n\bullet + RM_m\bullet \longrightarrow RM_n + RM_m$

I= initiator

R•= radical species

M= monomer

RM•= monomer with radical as chain end

$RM_2\bullet$= 2 monomer units with radical as chain ends

$RM_n\bullet$= n monomer units with radical as chain ends

$RM_m\bullet$= m monomer units with radical as chain ends

The kinetics of reaction were not investigated in this research, however typically in free radical polymerisation, the rate of propagation is proportional to the monomer

concentration, radical propagation, and the propagation rate constant. An assumption is made that radical reactivity is independent of chain length.

1.7.4fotopolymerisation

Ultra –violet (UV) can also be termed light induced polymerisation. The first step in a photopolymerisation reaction is the absorption of a photon. If absorption of specific energy occurs then the electron becomes excited to a higher energy state. The electronic transitions that occur may cause the molecule to split, thus generating free radicals[39]. This method of initiating polymerisation is predominantly used in the production of coatings and adhesives[40]. The properties, quality and performance of a UV cured polymer are directly related to the formulation and the curing conditions under which it was made[40]. High concentrations of photo initiator can lead to yellowing, accelerated ageing and occasionally odour issues. This has the potential to compromise overall product quality and shelf life [41].

1.8 The testing of materials: rheology

Rheology is the study of the deformation and flow of matter under the influence of externally imposed mechanical forces. There are two limiting types of behaviour. Deformation may spontaneously reverse which is termed elastic behaviour; mainly rigid solids exhibit this. The energy that is used to cause the deformation is stored, and then recovered once relaxation has occurred. Towards the other extreme, the material begins to flow and the energy to initiate this is non recoverable. This is viscous behaviour and is characteristic of a liquid. The viscoelastic properties of the hydrogel are measured *via* the application of a torque and deforming the gel with an applied stress, which results in a strain, measured as a function of frequency. Varying the applied frequency (5-25Hz) applies an oscillating force to the sample and a sinusoidal strain is generated. Measurement of the amplitude of deformation at the peak of the sine wave, and the log between the stress and strain waves allows the calculation of complex, viscous, and elastic modulii. Division of the viscous modulus by the elastic modulus calculates the tangent of the phase shift. Where the phase shift is the difference between the shear stress sine wave and the shear strain sine wave.

The modulus calculated *via* this form of testing is not to be confused with the Youngs Modulus that is calculated from the slope of a typical stress strain curve.

$$\text{Tan}\delta = \frac{G''}{G'}$$

G" = Viscous modulus
G'= Elastic modulus
Shear stress =Force/Area
Shear rate = Change in shear strain/change in time
Shear strain= gel displaced/gap height
Viscosity= shear stress/Shear rate ($Nm^{-2}s$ or Pas)

Parallel plates are used to test under compression if the sample is primarily elastic. If the sample is of a viscous or liquid nature and the angle is very small then a cone and plate is used at 1^o or 4^o to allow the generation of a homogenous strain across the sample.

Ideally materials that exhibit elastic behaviour will yield a linear response where the modulus is independent of load and loading rate. When the material becomes placed under tension, the linear region is followed by a curve that is caused by necking of the sample and its subsequent drawing out

Molecularly the dashpot in the spring and dashpot model represents the resistance of the chains to uncoiling. The spring represents the thermal vibration of chain segments that seek the lowest energy arrangement. At low frequencies the resultant curve is observed to be fairly flat and it can be said that the sample is exhibiting Newtonian behaviour. At this low frequency viscosity is dependent more on molecular weight than it is on the application of a strain. As the testing frequency is increased the sample begins to behave in an increased elastic fashion and is no longer behaving in a Newtonian manner. At some point increasing the frequency will begin to irreversibly degrade the polymer by actually breaking chains.

1.9 A brief insight into radio-opaque contrast media, types and uses

Contrast media are by default known as drugs. This makes their use in new materials for biological applications a tricky one. Their primary use is to enhance the diagnostic information provided by medical imaging systems. The structures represented in figure 1.6.1 demonstrate the most common contrast media that are used, or modified to form the basis of contrast media.

Fig 1.9.1 Diatrizoic acid, metrizoic acid, and iothalmic acid, the basis of most modern contrast media[42].

Current contrast media make opacification of practically any vascular and parenchymal structure possible with relatively low levels of toxicity[43]. One of the drawbacks of iodinated contrast media is that in order to be visible they must be used in rather high concentrations so as to maximise their iodine content. This results in a solution with a very high osmolarity, often six or seven times greater than that of natural tissue.

The use of non-ionic contrast media (water soluble) allows the preparation of solutions with a much lower osmolarity. The compound focused on in this research was iohexol (Histodenz), structure shown below in figure 1.9.2 this has a molecular weight of 821 with iodine comprises 46.4% of this atomic mass. A 300mgI/ml solution results in an osmolarity of 690 mOsm, almost twice that of natural tissue but relatively low for contrast media.

Fig 1.9.2 Iohexol (Histodenz)

1.10 The scope and objectives of the work presented in this thesis

The aim of the work reported in this thesis was to design and develop a novel intervertebral disc repair system. The requirements are that it should be:

- Injectable
- Polymerise *in situ*
- Form a complete gel within 3-7 minutes
- Biomimetic, based on the natural sulfate in proteoglycans
- Osmotically responsive to restore disc height
- Mechanically stable with properties similar to the nucleus pulposus
- Must not degrade
- Must not be cytotoxic to surrounding tissues

The technology can be further adapted to the production of an injectable intraocular lens. In addition to the synergistic requirements above the material must fill the cavity without damage to the capsular bag, and mimic the mechanical properties of the natural lens to restore the ability of the eye to accommodate.

Chapter Two

Materials and Methods

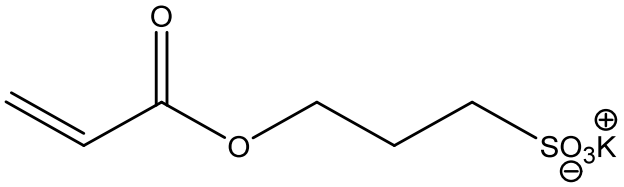
2.1 Reagents

2.1.1 Initiators

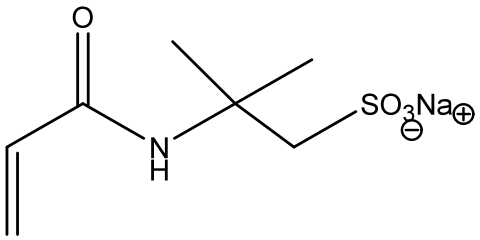
Table 2.1.1 Initiators

Reagent	**Formula**	**Supplier**
L-ascorbic acid (AA)	$C_6H_8O_6$	Sigma Aldrich
Oxone	$2KHSO_5.KHSO_4.K_2SO_4$	Sigma Aldrich
Potassium persulfate	$K_2S_2O_8$	Sigma Aldrich
Iron II lactate hydrate	$C_6H_{10}FeO_6aq$	Fluka
Iron II gluconate	$C_{12}H_{22}FeO_{14}aq$	Fluka
Dimethyl paratoluidine (DMT)	$C_9H_{13}N$	Sigma Aldrich
2,2 azo bis (2-methyl propionamide) dihydrochloride	$C_8H_{18}N_6.2ClH$	Sigma Aldrich
N'N'N'N tetramethylene diamine (TEMED)	$C_6H_{16}N_2$	Sigma Aldrich
Benzoyl peroxide (BP)	$C_{14}H_{10}O_4$	Sigma Aldrich
Tertiary-butyl hydroperoxide	$C_4H_{10}O_2$	Sigma Aldrich
Ammonium persulfate	$(NH_4)_2S_2O_8$	Sigma Aldrich
Hydrogen peroxide	H_2O_2	Sigma Aldrich

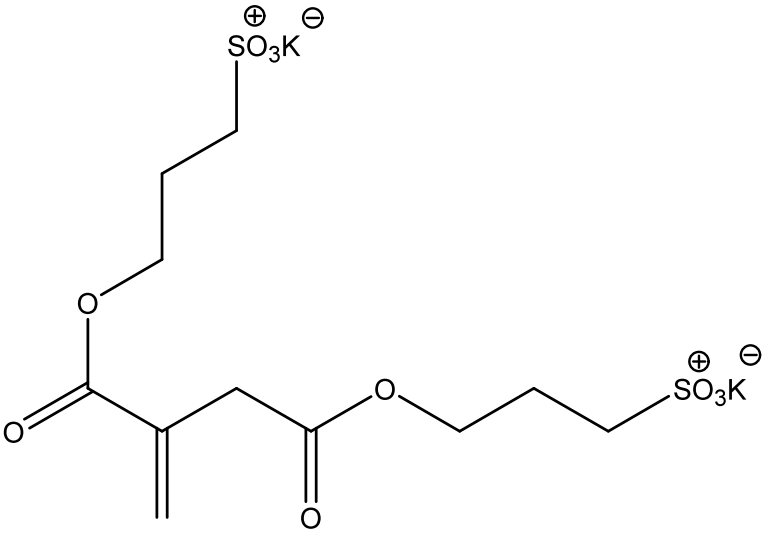
2.1.2 Monomers and crosslinking agents [supplier in brackets]



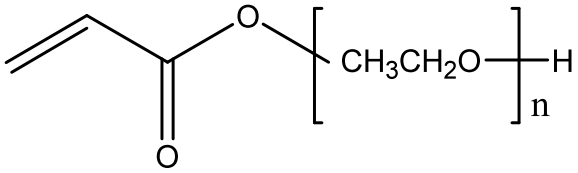
Potassium salt of 3-sulfopropyl acrylate (KSPA), [Raschig GmbH]



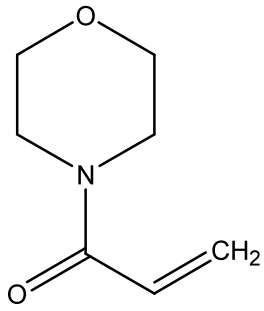
Sodium 2-acrylamido 2-methylpropane sulfonic acid (NaAMPs), [58% solution supplied by Lubrizol, 50% solution Sigma-Aldrich]



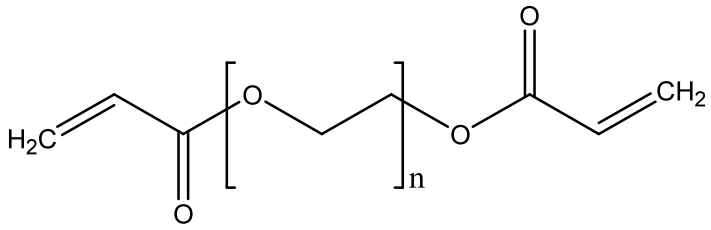
Dipotassium salt of bis (3-sulfopropyl) itaconate (KSPI) [Raschig GmbH]



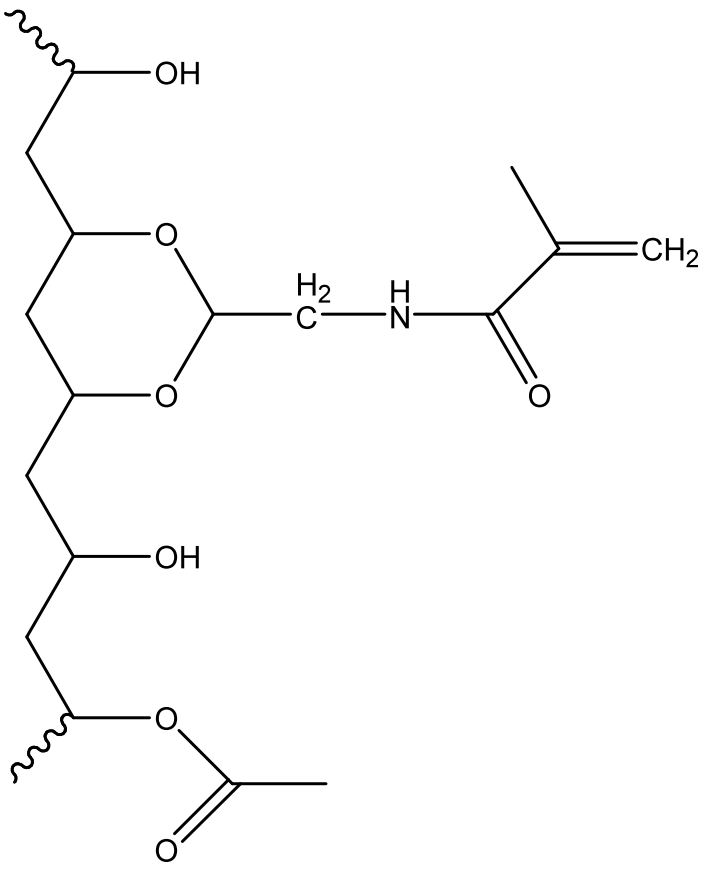
Poly(ethyleneglycol) acrylate (PEGA), [Sigma-Aldrich]



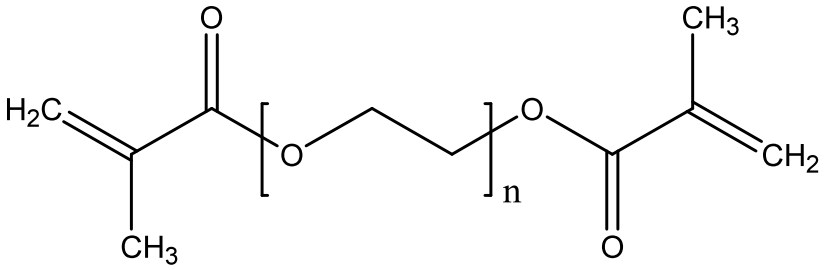
Acryloylmorpholine (ACMO), [Sigma-Aldrich]



Poly (ethyleneglycol) diacrylate. typically n~575, [Sigma- Aldrich]



Nelfilcon - modified PVA [Cibavision]



Poly (ethyleneglycol) dimethacrylate, typically n= 1000, [Sigma-Aldrich]

2.2 Methods

2.2.1 Typical gel procedure

For example a typical composition consists of:

Gel	**NaAMPs (50%)**	**KSPA**	**Water**	**Crosslinking agent PEG575DA**	**Ascorbic acid 0.1M**	**Oxone 0.1M**
Rf1	3.7 1.6 x 10^{-3} mol	1.4g 0.6 x 10^{-3} mol	3.6g 0.2 mol	0.2g 3.5 x 10^{-4} mol	0.65g 6.5 x 10^{-5} mol	0.65g 6.5 x 10^{-5} mol

For the injection procedure to take place, the components need to form two pregel solutions, for example:

Redox A:

- Monomer 1
- Water
- Redox initiator A

Redox B:

- Monomer 2
- Water
- Crosslinking agent
- Redox initiator B

So for the composition above, stock solutions of initiators were prepared, a 0.1M solution of ascorbic acid and Oxone was prepared from the solid material to form a 10ml standard solution. This was then vortexed to ensure complete solubilisation.

In two separate *via*ls the two redox A and redox B solutions are weighed out, the water component is split between the two stock solutions to create equal volumes.

Redox A
3.7g 50% NaAMPs
0.65g of AA 0.1M stock solution
0.75g distilled water

Redox B
1.4g KSPA
0.2g PEG575DA
0.65g of Oxone 0.1M stock solution
2.85g distilled water

Each stock solution was vortexed to ensure all components were thoroughly mixed.

If at this point the removal of oxygen was required, each pregel solution had nitrogen bubbled through for 10 minutes.

To form a gel, measures of each stock solution, Redox A and Redox B (equal measures for this composition) were placed into separate sides of a dual syringe, and injected into the required cavity typically *via* a 19 gauge needle.

2.2.3 Swelling procedure

For free swelling experiments, discs of gel of diameter 20mm were cut from the gels prepared within the Petri dishes using a number 13 cork borer. The discs were weighed and then placed into a jar with an excess of the swelling medium, which was typically phosphate buffered saline solution or distilled water. The discs of gel were removed at selected time intervals, the surfaces dabbed with filter paper to remove

any surface liquid, and then reweighed. The percentage increase in mass per allocated time was used as a measure to determine swell capacity.

Swell procedure for compression testing

Typical pre-gel compositions (volume circa 3ml) were injected into dialysis tubing (12,000 mol wt cut off) using the dual syringe method. The dialysis bags were sealed with clips and the polymers were allowed to cure. The dialysis bags were then placed into PEG solutions of different osmolarities (ranging from 300-1500mOsm) and allowed to swell under osmotic pressure at room temperature for 72 hours Compression tests were carried out on the dialysis bags containing the swollen polymers using a Hounsfield S-series H10KA dual column bench top tensiometer. The thickness of each sample is measured and corresponding displacement set to between 5 and 50%; tests were carried out at a speed of 1.0mm/min.

2.2.4 Rheology

To assess the *via*bility of the gels mechanically, the elastic and viscous modulii were recorded using a Bohlin CVO Rheometer. Initial Stress values were calculated following amplitude sweeps from 2.66Pa-20kPa carried out at 0.5 Hz and 30Hz. An initial stress was chosen that lay within the linear region for both the high and low frequency runs. This ensured that the material can be continuously excited without exceeding the strain value that destroys its structure. The applied stress is continuously adjusted so that the resultant strain is kept at a specific value.

Gels were prepared within Petri dishes allowing a flat circular structure from which discs of 20mm diameter and approximately 2mm thickness were cut using a no 13 cork borer. These 'discs' were then placed onto the bohlin rheometer using a parallel plate method commonly used for samples displaying elastic behaviour. All tests were carried out at 37^{o}C to mimic body temperature. Samples were then subjected to an oscillating frequency between 0.5 and 25Hz (the frequency required to complete a sine wave) to observe the ability of the material to maintain its structural integrity. The normal force was set at 100g. All tests were repeated to ensure reproducibility. The storage modulus (G') and the loss modulus (G'') corresponding to the elastic and

viscous components respectively were measured. The loss tangent (tan δ) which is the phase angle was calculated as the ratio between the G'' and the G'

2.2.5 Residual monomer analysis

Gels were prepared within Petri dishes, and 10mm discs of gel were cut out. The discs were then placed in 10ml of HPLC grade water for 2weeks. Standards of NaAMPs and KSPA were prepared from 0.0001% concentration to 5% to allow a calibration to be made. After the time had elapsed the liquid was filtered and analysed *via* the following techniques. Residual monomer levels were assessed typically by ion chromatography (Dionex DX600 system with GP50 gradient pump, EG50 eluent generator, PDA-100 photodiode array and ED50 electrochemical detectors); or refractive index (Index Instruments automatic refractometer GPR 11 – 37X).

2.2.6 FTIR

Infra-red analysis was conducted on samples as either solid, liquid, or gel using a Nicolet-380 FTIR Spectrophotometer with a diamond attenuated total reflectance attachment. The required amount of sample ~ 0.1g was placed on the plate above the laser and held in place by screwing down the relevant tip which is adjusted according to the nature of the sample, to provide maximum surface contact. If sampling a liquid, a volatile cover is placed over the sample instead of the tip. Each spectra is set to run 60 scans per sample. A typical spectra for water is shown in figure 2.2.6.1

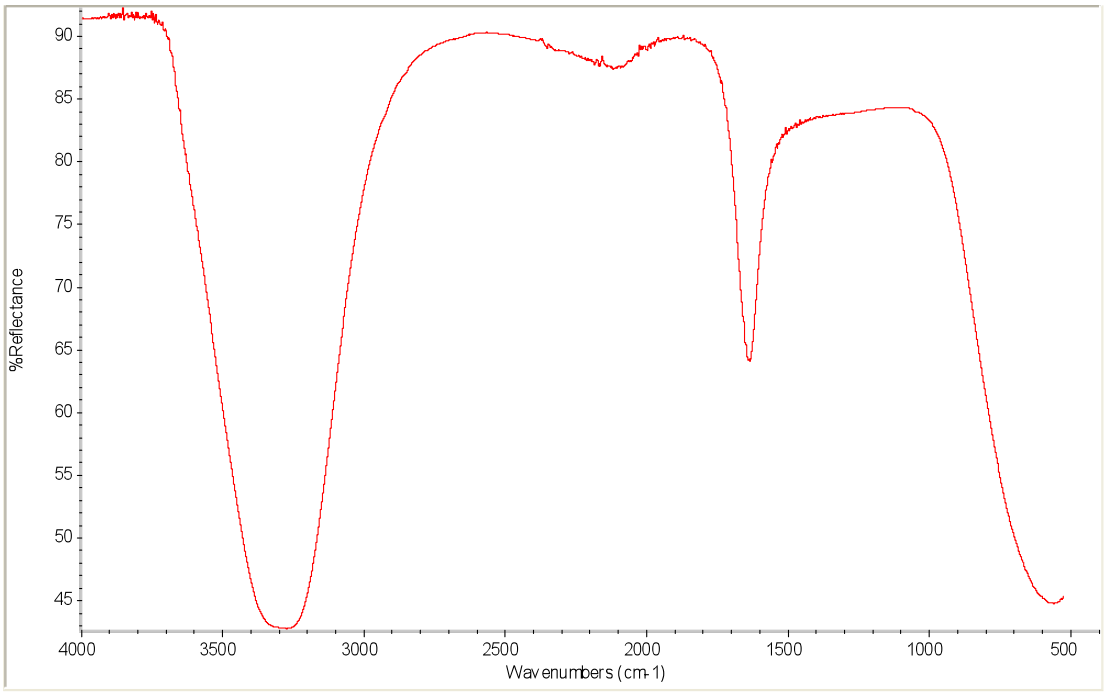


Fig 2.2.6.1 A typical FTIR spectra for water

2.2.7 Medac CHN analysis

Samples based on functionalised histodenz discussed in chapter seven were sent to Medac for CHN elemental analysis to assess for iodine content. The method is based on combustion analysis as a method of determining sample purity. Typical sample size was 5mg of solid material. Results are supplied in the format below in table 2.2.7.1.

Table 2.2.7.1 Medac analysis results

ELEMENT	C	H	N	S	Cl	Br	I	O	Na
% Theory									
% Found 1	2.68	11.68	0.46	0.68			1.24		0.15
% Found 2	2.91	11.65	0.51	0.63			1.28		0.17

2.2.8 NMR

NMR was conducted on histodenz samples introduced in chapter seven using a Bruker 300MHz NMR instrument. For this application the method used was based on the WATERGATE water suppression technique which is facilitated by gradient tailored excitation. This removes resonance exhibited by the solvent but retains that of the sample.

2.3 Synthetic routes to sulfonated monomers

2.3.1 The synthesis of KSPA

This process is for the commercial production of KSPA and was not reproduced within the laboratory.

Salts of sulfoalkyl acrylate can be produced in high purity by reacting salts of acrylic acid (produced by neutralising the acid in this case with potassium hydroxide) with 1,3 propane sultone at 110-120^{o}C, in the presence of acrylic acid [(1)]. 1,3 propane sultone is used as a chemical intermediate to introduce the sulfopropyl group into molecules and also to provide or improve water solubility along with ionic character. Although a suspected carcinogen, it is used as an intermediate in the production of fungicides, insecticides, cation exchange resins, dyes, vulcanisation accelerators and a variety of other chemicals[(45)(46)]. The product salts out of the reaction solution and is collected *via* filtration and washing with acetone. Yield is typically ~ 99% with purity ~ 98% [(44)]. The reaction scheme for which is shown on the following page in figure 2.3.1.

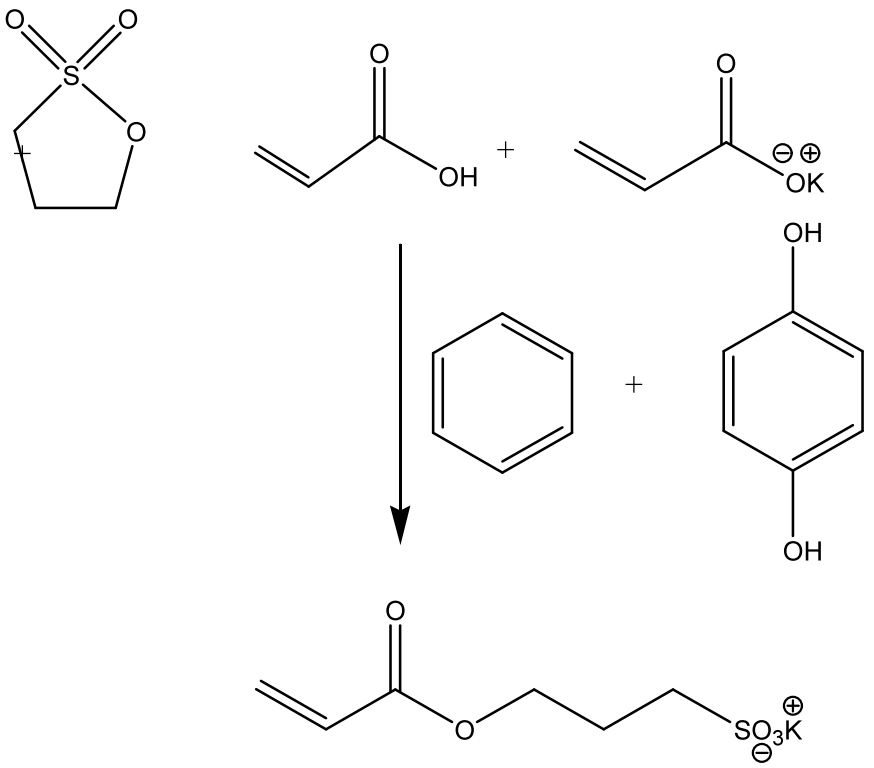


Fig 2.3.1 The production of potassium salt of 3-sulfopropyl acrylate

Alternative methods exist utilising the esterification of acrylic acid or the reaction of an acrylic halide, with a hydroxyalkanesulfonate. These reactions have disadvantages in the solubility and undesirable halide reactions respectively.

2.3.2 The synthesis of NaAMPs

This describes a commercial process and was not reproduced in the laboratory.

The synthesis of sodium 2-acrylamido 2-methylpropanic sulfonic acid (AMPA) requires the combination of acrylonitrile and isobutylene with fuming sulfuric acid. The reaction mixture is initially maintained at approximately -20^{o}C for 2 hours before being allowed to reach room temperature. After ~ 2 hours a dense white solid is formed AMPA, this is filtered off and washed with acetonitrile. Typical yields are usually ~86%[47]. The resultant acid AMPA, is then neutralised with sodium hydroxide to provide the sodium salt. The mechanism is shown in figure 2.3.2

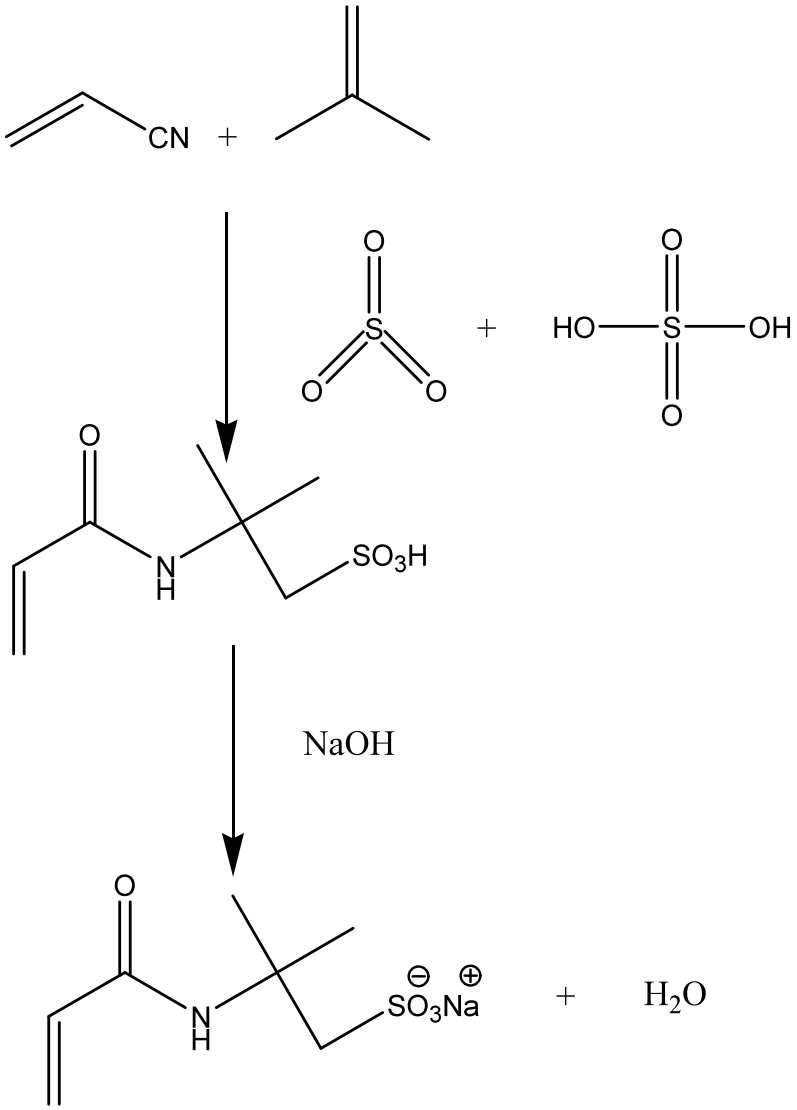


Fig 2.3.2 The production of sodium 2-acrylamido-2-methylpropane sulfonic acid

Chapter Three

The Design and Synthesis of a Proteoglycan Analogue: Experimental Considerations

3.1 Introduction

To design a material suitable material for use as a proteoglycan analogue for use in applications such as intervertebral disc (IVD) repair or as an injectable intraocular lens (IOL) the material must meet several requirements, which will be discussed in this chapter.

In order to be feasible as a novel injectable material, it is important that the components are readily available, and advantageous if they have previously demonstrated compatibility in a biological environment with no cytotoxic response. The materials must be capable of polymerising under conditions that are suitable for the end use of the material, allowing it to be injected in to the respective cavity as a liquid that will then polymerise completely *in situ.* In addition to this, the method of polymerisation used must result in complete reproducible polymerisation with minimal residual monomer remaining, the method employed must also cause no adverse effects to the cavity in which it is to be used. Finally, the materials used must create a highly hydrophilic network capable of imbibing water or biological fluids, have mechanical strength similar to that of the target natural structure and maintain integrity whilst under compression. The full requirements of the system are:

- Deliverable *via* injection
- Low viscosity to enable use of a small diameter discography needle
- To provide complete gelation *in situ*
- Have appropriate mechanical properties to match that of target tissue
- Stable to enzymatic and hydrolytic degradation

This chapter will begin by discussing monomer selection criteria and investigations, followed by the method and nature of free radical polymerisation to be employed. The choice of cross-linking agent will be discussed, and the subsequent affect this has on the end polymer. Finally variables which have the potential to influence the polymerisation, delivery, stability, and lifetime of the material will be discussed.

3.1.1 Monomer selection- the rationale for the use of sulfonates

Conventional neutral synthetic hydrogels such as those based on hydroxy ethyl methacrylate and *N*-vinyl pyrollidone for example have been proved to be very effective soft tissue biomaterials for a range of applications. However these materials fail to match the behaviour of natural tissue in its ability to retain water when compromised. Natural tissue such as cornea, cartilage or intervertebral disc show a remarkable ability to retain water content whilst compromised. Conventional hydrogels in comparison, lose water relatively rapidly. The exception to this is found in synthetic hydrogels which contain sulfonate groups, which show an ability to retain their water content.

For most biomedical applications, hydrogels need to show dimensional stability to changes in, osmolarity and temperature. PolyHEMA is successful as a material because it behaves well in these conditions. Sulfonate-containing materials, in comparison, are responsive to environmental changes which render them unsuitable or at most difficult to manage for most tissue replacement or tissue augmentation applications.

The biomimetic approach of the work detailed in this thesis will be based on the use of the synthetic sulfonate as a surrogate for the sulfate group present in natural proteoglycan structures, structures of which are shown below in figure 3.1.1, using two precursor monomers the sodium salt of 2-acrylamido 2 methylpropane sulfonic acid (NaAMPS) and the potassium salt of 3-sulfopropyl acrylate (KSPA).

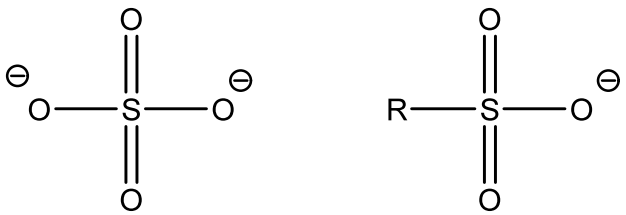


Fig 3.1.1 The sulfate and sulfonate functional groups, repsectively

The molecular structures of alkyl sulfate and alkyl sulfonate are very alike – both have very much larger hydration shells than either hydroxyl or carboxyl groups which

means that they are able more effectively to "shield" the hydrocarbon backbone. The hydration shell of the sulfonate group has been shown to extend over a larger portion of the hydrocarbon chain than that of the sulfate in simple alkyl derivatives. Various aspects of sulfate and sulfonate chemistry in compounds that come into bodily contact have been explored over many years. This is because of their use in the surfactant field. The ability of these groups to completely solubilise hydrocarbons in water beyond C10 contrasts with the fact that the hydroxyl group cannot completely solubilise more than a C3 chain. There is a long history of use of both sulfates and sulfonates without evidence of toxicity-related problems associated with either group. Since this is a commercially driven field greater versatility of synthesis and ease of production has led to a dominance of sulfonate-based over sulfate-based products. On the basis of the long history of surfactant use and performance it seems clear that there are huge practical advantages and no perceived disadvantages in using sulfonates as water binding groups in hydrogel-based proteoglycan mimics.

NaAMPs and KSPA are currently used in wound dressing applications. Due to the presence of the amide group within the structure and its subsequent interaction with the sulfonate group of an adjacent chain. KSPA is less cohesive due to the absence of the amide group. Further inclusion of monomers such as acrylic acid further enhance the cohesive forces within the hydrogel.

Monomers used in the preparation of generic and sulfonate based hydrogel materials are illustrated in figures 3.1.1 to 3.1.8.

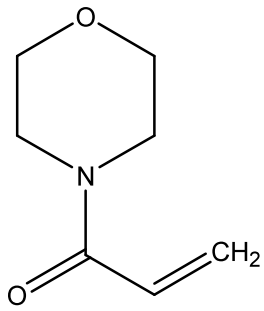


Fig 3.1.1 Acryloylmorpholine (ACMO)

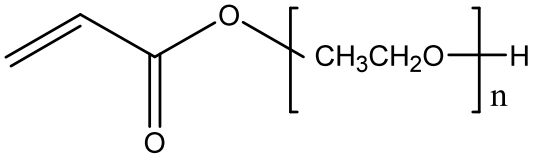


Fig 3.1.2 Poly(ethyleneglycol) acrylate (PEGA)

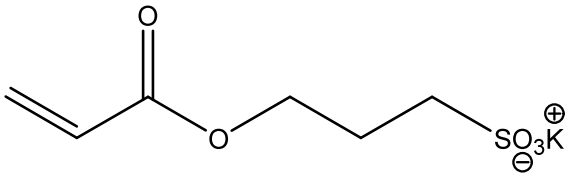


Fig 3.1.3 Potassium salt of 3-sulfpropyl acrylate (KSPA)

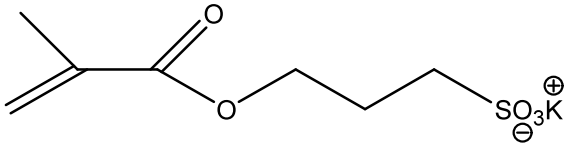


Fig 3.1.4 Potassium salt of 3-sulfopropyl methacrylate (KSPM)

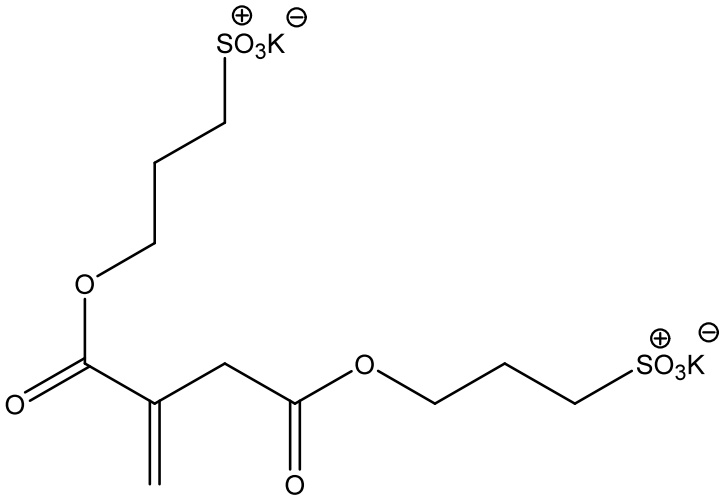


Fig3.1.5 Dipotassium salt of bis (3-sulfopropyl) itaconate (KSPI)

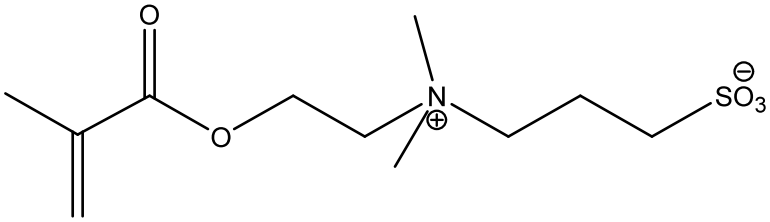


Fig3.1.6 N,N, Dimethyl N-(2-methacryloyloxyethyl)-N-(3-sulfopropyl) ammonium betaine (SPE)

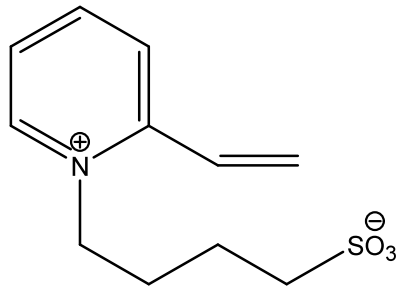


Fig 3.1.7 1-(3-Sulfopropyl)-2-vinylpyridinium betaine (SPV)

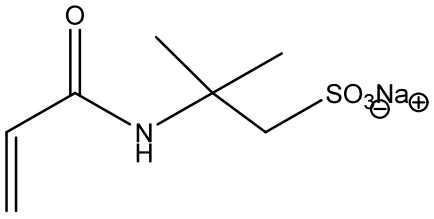


Fig 3.1.8 Sodium 2-acrylamido 2-methylpropane sulfonic acid (NaAMPs)

Alternative sulfonate monomers are illustrated in the previous figures, however NaAMPs and KSPA were deemed the most suitable monomers for the applications discussed within this thesis. This was due to the current use of both materials in a biological environment, such as wound dressings, amongst many others. Materials such as SPV and SPE, whilst sulfonate structures, possess toxicity characteristics that render them unsuitable for a biological application.

3.2 The investigation of suitable monomer systems, experimental considerations

Desirable properties as aforementioned are hydrophilicity (and thus swell behaviour) and mechanical strength, along with the obvious ability to polymerise under the required reaction conditions. The copolymer compositions which have been investigated in the scope of this research are represented in table 3.2.1. Initially homopolymerisation of just NaAMPs and KSPA was investigated followed by copolymer compositions.

Table 3.2.1 Possible homo and co-polymer compositions: combining column 1 and 2

Monomer 1	**Monomer 2**
NaAMPs	
KSPA	
NaAMPs	KSPA
KSPA	ACMO
ACMO	NaAMPs
NaAMPs	ACMO
NaAMPs	PEGA
KSPA	PEGA
NaAMPs	KSPI

3.2.1 Investigation of an NaAMPs polymer

Logically the first composition investigated was based on using NaAMPs on its own. The composition investigated is represented in table 3.2.1.1 below. The composition consisted of NaAMPs in a ratio of 1:2 with water, with 2% PEG575diacrylate

crosslinking agent. The sample was prepared using the method described in section 2.2.1, which consists of the mixing of the components: water, NaAMPs 58% solution, and crosslinking agent, within a glass sample *via*l in the presence of ambient oxygen. The redox initiators were then added to the *via*l, which was then inverted three times, and the contents transferred to a plastic petri dish at room temperature for polymerisation to occur. The petri dish is sealed with parafilm to prevent contact with excess oxygen during the polymerisation process.

Oxone and ascorbic acid were used as the redox pair, as these were known to produce relatively reproducible polymerisation.

Table 3.2.1.1. NaAMPs polymer composition

Gel	**NaAMPs (58%)**	**H_2O (g)**	**PEG575DA (g)**	**Oxone 0.1M (g)**	**AA 0.1M (g)**
A	6g 2.6×10^{-2} mol	3 0.16 mol	0.1 1.7×10^{-4} mol	0.65 6.5×10^{-5} mol	0.65 6.5×10^{-5} mol

Gelation was successful and reproducible in a timescale of circa. 2 minutes. This was as expected as acrylamide monomers are known to polymerise rapidly and efficiently even under mile conditions such as redox. Gelation occurred quickly suggesting that this composition would be rendered unsuitable for injection as the delivery may not occur in less than two minutes. The composition also has a very high water content that may not be mechanically suitable. Decreasing or removing the additional water would be advantageous but the time for gelation to occur would decrease due to the reduction in dilution of the system.

The swell behaviour of this partially hydrated material was investigated. This involved the cutting of 20mm discs of gel, and placing in water and 300 mOsm phosphate buffered saline respectively, at room temperature, the full procedure is detailed in section 2.2.1. The swell behaviour is illustrated in figure 3.2.1.1.

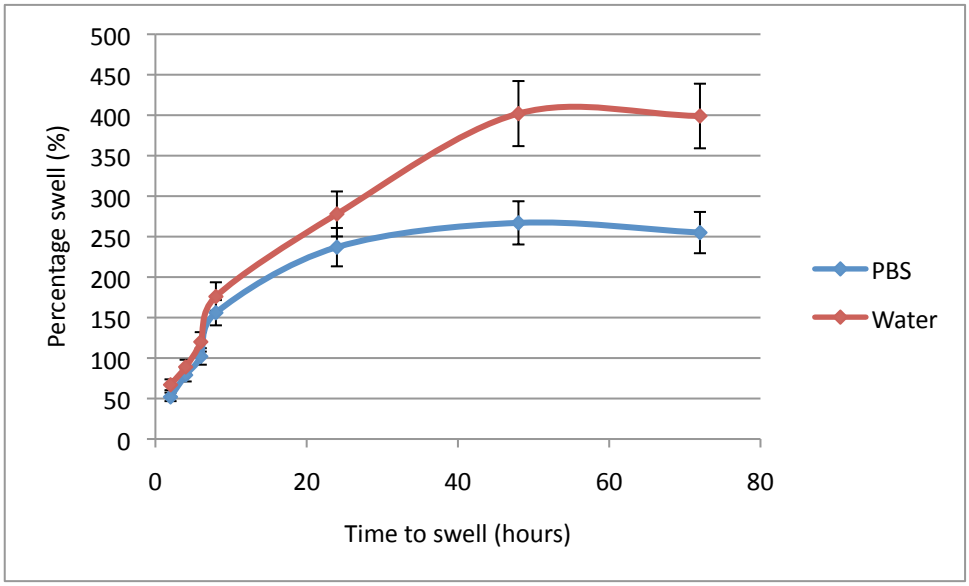


Fig 3.2.1.1 The swell behaviour of NaAMPs based gel A in water and PBS

The swell behaviour was relatively aggressive, often increasing in mass >4 fold in water and >2.5 in physiological saline, due to the presence of both Na^+ and the amide group (the gel was already partially hydrated due to the presence of water within the polymerisation system). The charge repulsion between both the sulfonate and the amide group provide this extra swelling capacity in comparison to a material containing simply one or the other.

When 20mm diameter discs were rheologically tested, the stiffness of the material also appeared to be greater than that required. The general procedure for rheology is detailed in section 2.2. Figure 3.2.1.2 illustrates the high stiffness of Gel A ~ 29000Pa whereas the stiffness of the natural nucleus pulposus is not expected to surpass 20000Pa.

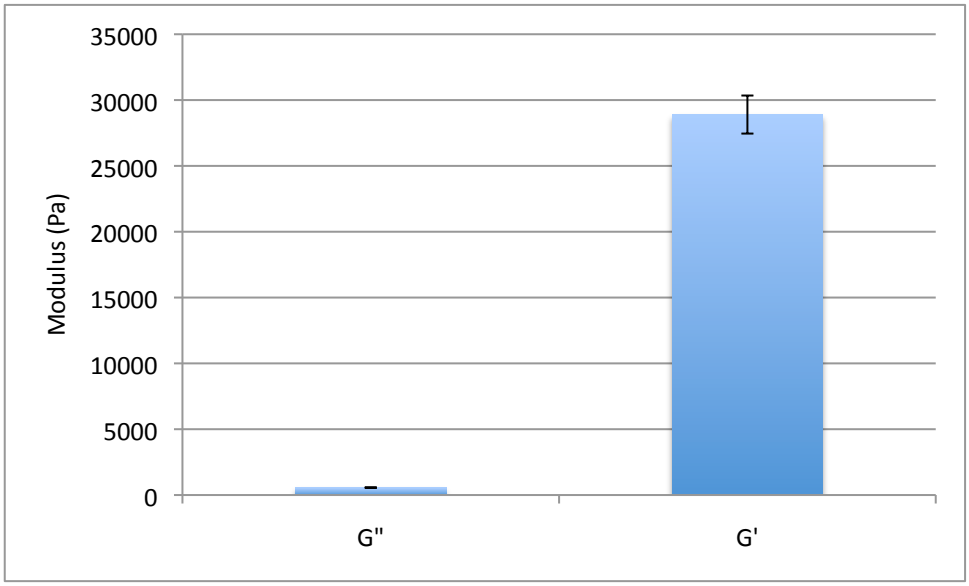


Figure 3.2.1.2 The viscous and elastic modulii of NaAMPs based Gel A

3.2.2 The homopolymerisation of KSPA

The polymerisation of KSPA was investigated as a 1:1 water to monomer mix, with a similar method to NaAMPs Gel A. No gel formed at room temperature or at 37°C, in the presence or absence of atmospheric oxygen.

3.2.3 The copolymerisation of NaAMPs/KSPA based systems

NaAMPs is available commercially as either a 58% or 50% solution due to its hygroscopic behaviour it is difficult to purify and supply as a solid material. The incorporation of a copolymer allows the percentage dry weight monomer in the final product to be increased. It also has the potential to reduce the fixed charge density of the polymer, thus reducing the aggressive swell behavior. Table 3.2.3.1 details the copolymerisation of NaAMPs and KSPA using ascorbic acid and Oxone as the initiation system, and crosslinking agent PEG575DA at 2%. Samples were prepared in a similar fashion to the previous experiment. Components were mixed in a sample *via*l prior to the addition of initiators, solutions 1 to 1b were then transferred to petri dishes for polymerisation to occur. Compositions 1c and 1d were left in the glass *via*ls to polymerise. Gels 1, 1a, and 1c were allowed to polymerise at room temperature while 1b and 1d were polymerised at 32°C

Table 3.2.3.1 The copolymerisation of NaAMPs with KSPA: The effect on polymerisation

Gel	NaAMPs (58%) (g)	KSPA (g)	H_2O (g)	PEG575DA (g)	Oxone 0.1M (g)	AA 0.1M (g)	Observations
1	3	1.4	4.3	0.2	0.65	0.65	20°C, O_2 no gel
1a	3	1.4	4.3	0.2	0.65	0.65	20°C, no O_2, complete gel
1b	3	1.4	4.3	0.2	0.65	0.65	37°C, O_2, partial gel
1c	3	1.4	4.3	0.2	0.65	0.65	20°C, O_2, partial gel, glass
1d	3	1.4	4.3	0.2	0.65	0.65	37°C, O_2, complete gel, glass

Where n= NaAMPs 1.3 $x10^{-2}$mol, KSPA 6 x 10^{-3}mol, H_2O 0.24 mol, PEG575DA 3.5 x 10^{-4} mol, Oxone 6.5 x 10^{-5} and AA 6.5 x 10^{-5}mol.

At 20°C in the presence of ambient oxygen the polymerisation was not reproducible, on occasion an incomplete gel was formed. In comparison when the system was purged with nitrogen prior to addition of the initiators, as was the case in 1b, a complete gel was formed. Gelation also appeared much more reproducible at 37°C. This set of experiments introduced the obstacles involved in polymerising acrylamide/acrylate copolymers using a redox method.

3.2.3.1 The effect of initiator concentration and volume on an NaAMPs/KSPA and NaAMPs based system

For the next set of experiments the concentration and volume of initiators were varied whilst the monomer composition of the gels remained similar. This was to investigate the effects of increasing the initiator concentration and/or adding a larger volume of a less concentrated initiator to assess the effect of dilution. The compositions were prepared in a similar way as previously described in section 2.2.1, each polymerisation was carried out in a plastic petri dish, at room temperature, in ambient oxygen. Compositions are detailed in table 3.2.3.1.1.

Table 3.2.3.1.1 The effect of initiator concentration and volume on the copolymerisation of NaAMPs and KSPA

Gel	**NaAMPs (58%)(g)**	**KSPA (g)**	**H_2O (g)**	**PEG575DA**	**Oxone**	**AA**	**Observations**
3p	3	1.4	4.3	0.2	0.1M 0.7g 0.42% 7×10^{-5} mol	0.1M 0.7g 0.42% 7×10^{-5} mol	partial gelation
3pa	3	1.4	4.3	0.2	0.1M 0.75g 0.46% 7.5×10^{-5} mol	0.1M 0.75g 0.46% 7.5×10^{-5} mol	partial gelation
3pb	3	1.4	4.3	0.2	0.1M 0.8g 0.48% 8×10^{-5} mol	0.1M 0.8g 0.48% 8×10^{-5} mol	partial gelation
3pc	3	1.4	4.3	0.2	0.15M 0.45g 0.414% 6.8×10^{-5} mol	0.15M 0.45g 0.414% 6.8×10^{-5} mol	partial gelation
3pd	3	1.4	4.3	0.2	0.15M 0.50g 0.460% 7.5×10^{-5} mol	0.15M 0.50g 0.460% 7.5×10^{-5} mol	complete gelation
3pe	3	1.4	4.3	0.2	0.15M 0.55g 0.506% 8.3×10^{-5} mol	0.15M 0.55g 0.506% 8.3×10^{-5} mol	complete gelation

Where n= NaAMPs 1.3 $x10^{-2}$mol, KSPA 6 x 10^{-3}mol, H_2O 0.24 mol, PEG575DA 3.5 x 10^{-4} mol,

This yields similar results when Oxone is replaced with potassium persulfate.

From this set of experiments it can be deduced that a smaller volume of initiator at a higher concentration produced more effective polymerisation in comparison to the larger volume of a weaker initiator that still provides radicals at a similar percentage. This is likely due to the probability of radicals coming into contact with monomer being greater in a less dilute solution.

As NaAMPs was previously deemed to be more reactive than with KSPA as a co-monomer, the following experiments represented in table 3.2.3.1.2 detail the effect of reducing the volume of initiator on the polymerisation time of an NaAMPs homopolymer. The polymerisation was carried out using the same method as

previous experiments at both room temperature and at 37°C to mimic body temperature.

Table 3.2.3.1.2 The polymerisation time of an NaAMPs system in response to initiator volume and temperature, time to complete gelation is recorded visually

Gel	NaAMPs (58%)(g)	H_2O (g)	PEG575DA (g)	AA 0.1M (g)	Oxone 0.1M (g)	Temperature and time to complete gelation
6	6	3	0.2	0.65	0.65	20°C, O_2, 2 min 0 sec
6a	6	3	0.2	0.5	0.5	20°C, O_2 2 min 23 sec
6b	6	3	0.2	0.5	0.5	37°C, O_2 1 min 30 sec
6c	6	3	0.2	0.4	0.4	20°C, O_2 2 min 35 sec
6d	6	3	0.2	0.4	0.4	37°C, O_2, 2 min 0 sec
6e	6	3	0.2	0.3	0.3	20°C, O_2, 3 min37 sec
6f	6	3	0.2	0.3	0.3	37°C, O_2, 4 min 30 sec

Where n= NaAMPs 2.6 $x10^{-2}$mol, H_2O 0.17 mol, PEG575DA 3.5 x 10^{-4} mol, AA and Oxone 6.5-3 x 10^{-5}mol respectively

All compositions were successful, the time taken to achieve complete gelation as observed visually, is given in the observation column. Experiments were carried out at both 20°C and 37°C to assess the effect of temperature on the time taken for complete gelation to occur. Typically due to the energy supplied to the system by increasing the temperature to 37°C, the experiments carried out at this composition demonstrated a quicker gelation time. As expected, decreasing the volume of initiators increased the time taken for complete gelation to occur.

3.2.3.2 The effect of varying of the ratio of NaAMPs:KSPA in a copolymer system

As the previous NaAMPs/KSPA copolymer systems were based on a mass ratio of 1.74NaAMPs to 1.4KSPA the next step was to vary the copolymer composition. The compositions investigated are detailed in table 3.2.3.2.1 The initiator concentrations were increased to 0.15M to increase reproducibility of polymerisations due to the production of more radicals, reactions were carried out at room temperature in the presence of ambient oxygen, prepared in a similar method to previous gels

Table 3.2.3.2.1 The effect of varying KSPA content in an NaAMPs copolymer system

Gel	NaAMPS (58%)	KSPA	H_2O	PEG575DA	AA 0.15M	Oxone 0.15M	Observations
3pd	3g 1.3×10^{-2} mol	1.4g 6×10^{-3} mol	4.3g 0.24 mol	0.2g 3.4×10^{-4} mol	0.5g 7.5×10^{-5} mol	0.5g 7.5×10^{-5} mol	complete gel
2a	2g 8.7×10^{-3} mol	2g 8.6×10^{-3} mol	4.7g 0.26 mol	0.2g 3.4×10^{-4} mol	0.5g 7.5×10^{-5} mol	0.5g 7.5×10^{-5} mol	complete gel, not reproducible
2b	1.5g 6.5×10^{-3} mol	2g 8.6×10^{-3} mol	5.2g 0.29 mol	0.2g 3.4×10^{-4} mol	0.5g 7.5×10^{-5} mol	0.5g 7.5×10^{-5} mol	partial gel
2c	1.5g 6.5×10^{-3} mol	3g 1.3×10^{-2} mol	4.2g 0.23 mol	0.2g 3.4×10^{-4} mol	0.5g 7.5×10^{-5} mol	0.5g 7.5×10^{-5} mol	no gel
2ca	1.5g 6.5×10^{-3} mol	3g 1.3×10^{-2} mol	3.2g 0.18 mol	0.2g 3.4×10^{-4} mol	1.0g 1.5×10^{-4} mol	1.0g 1.5×10^{-4} mol	no gel
2d	4g 1.7×10^{-2} mol	1g 4.3×10^{-3} mol	3.7g 0.21 mol	0.2g 3.4×10^{-4} mol	0.5g 7.5×10^{-5} mol	0.5g 7.5×10^{-5} mol	complete gel

The formation of a complete and reproducible gel was clearly influenced *via* the increase in KSPA content. As a rule, when the KSPA content was above a ratio of 3:2 with NaAMPs a successful gel was not formed. This may be due to the fact that KSPA is less reactive to polymerisation *via* a redox mechanism under these mild conditions. This is supported by the inability of KSPA to form a homopolymer.

3.3 Copolymerisation of NaAMPs with PEG acrylate

NaAMPs and poly-(ethylene glycol) acrylate copolymers were investigated at low levels of PEGA ~5-15% due to its low aqueous solubility. Compositions are represented below in table 3.3.1. Each reaction was carried out in a similar manner to previous experiments at room temperature with ambient oxygen. Initiator concentrations were kept high to enable efficient reproducible polymerisation.

Table 3.3.1 The effect of using PEGA as a co-monomer with NaAMPs

Gel	**NaAMPs (58%)**	**PEGA**	**H_2O**	**PEG575DA**	**AA 0.15M**	**Oxone 0.15M**	**Observations**
Peg1	6g 2.6×10^{-2} mol	0.5g 1.3×10^{-3} mol	2.3g 0.13 mol	0.2g 3.4×10^{-4} mol	0.5g	0.5g	gel formed
Peg2	6g 2.6×10^{-2} mol	1.0g 2.7×10^{-3} mol	1.8g 0.10 mol	0.2g 3.4×10^{-4} mol	0.5g	0.5g	gel formed
Peg3	6g 2.6×10^{-2} mol	1.5g 4×10^{-3} mol	1.3g 0.07 mol	0.2g 3.4×10^{-4} mol	0.5g	0.5g	gel formed, PEGA slightly miscible

AA and Oxone were 7.5×10^{-5} mol respectively

Polymerisation was successful in all cases.

The swell behaviour of the gels was investigated in a similar manner to previous gels. Results are illustrated in figure 3.3.1. Gels were produced which exhibited low swell ability in comparison to a fully ionic copolymer. This was due to the reduced hydrophilicity and fixed charged density of the material suggesting that the use of a non ionic co-monomer may not be desirable for this application.

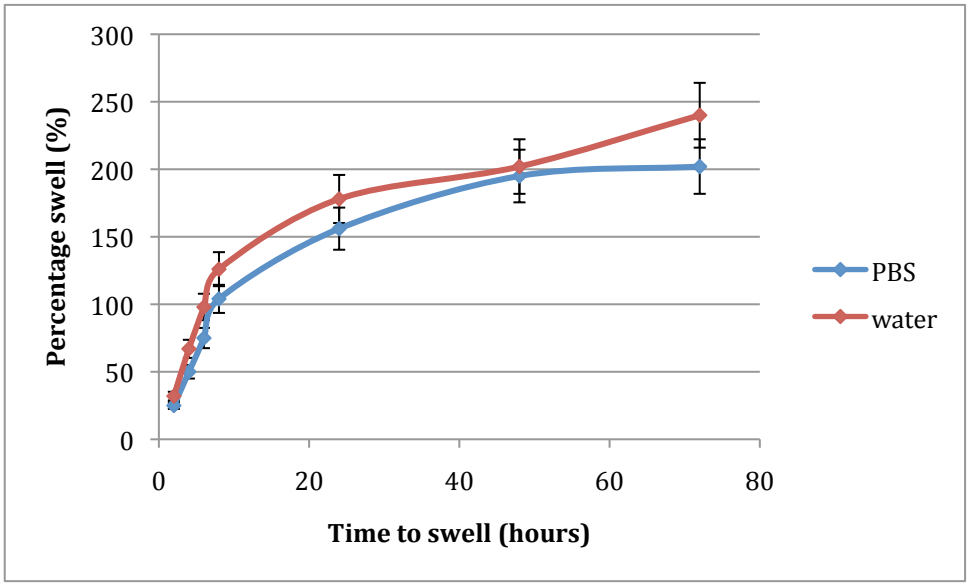


Fig 3.3.1 The swell behaviour of NaAMPs/PEGA copolymer gel Peg3 in water and PBS

The viscous and elastic behaviour of the NaAMPs/PEG acrylate copolymer was also investigated. This is illustrated in figure 3.3.2 .

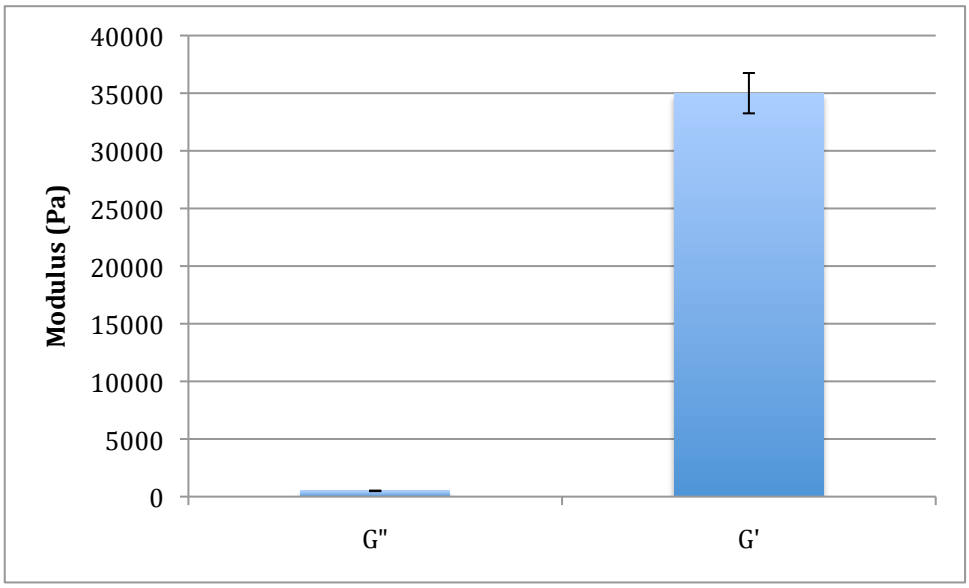


Figure 3.3.2 The viscous and elastic modulii of NaAMPs/PEGA based Peg1

The high mechanical strength is observed this could be due to reduced rotation of the structure due to the incorporation of a neutral monomer. As polymerisation is slow when incorporating PEG it is possible that the chains formed are quite long producing a material of higher mechanical strength in comparison to a NaAMPs/KSPA gel with

a similar w/w monomer content. Given that the human nucleus pulposus has a stiffness of less than 20000Pa this composition is likely to be unsuitable, particularly as this rheology trace represents the lowest percentage inclusion of PEGA investigated

Similar results were obtained with the incorporation of low levels of acryloyl morpholine (ACMO), also a neutral organic monomer (investigations were conducted by a previous MSc student and the results are not reported here). The incorporation of PEGA or ACMO with KSPA did not result in polymerisation[58].

3.4 The effect of incorporating KSPI as a co-monomer with NaAMPs

To improve the ability of the material to be biomimetic, incorporating the dipotassium salt of bis (3-sulfopropyl) itaconate (KSPI) in the proteoglycan analogue would increase the sulfonate content in comparison to a NaAMPs polymer or NaAMPs/KSPA copolymer. This has the potential to increase the responsiveness of the polymer in a biological environment, in particular its ability to imbibe and maintain water content, this is due to the water shielding behaviour of the sulfonate group. The charge repulsion of the two sulfonate groups enables the structure to exist in an extended form, however it does restrict rotation which in turn has the potential to affect the reactivity of the monomer.

Due to KSPA being discovered as less reactive it was assumed that KSPI would also have low reactivity. Therefore the inclusion of KSPA in the system to produce a tripolymer was not investigated, instead NaAMPs was the sole comonomer. Compositions are detailed in table 3.4.1 KSPI is commercially available as a 100% solid. Reactions were carried out in a similar manner to previous experiments, however due to the reported unreactivity of KSPI in oxygen rich environments, all reaction components were purged of oxygen using nitrogen, and polymerisation was carried out at 37^{o}C. Initiators were used at the higher concentration of 0.15M, and water content kept low to increase probability of radical-monomer interaction.

Table 3.4.1 The effect of incorporating KSPI as a copolymer with NaAMPs on gel formation

Gel	NaAMPs (58%)	KSPI	H_2O	PEG575DA	AA 0.15M	Oxone 0.15M	Observations
K1	6g 2.6×10^{-2} mol	1g 2×10^{-3} mol	1.8g 0.1 mol	0.2g 3.4×10^{-4} mol	0.5g 7.5×10^{-5} mol	0.5g 7.5×10^{-5} mol	viscous gel formed
K2	5.5g 2.4×10^{-2} mol	2g 4×10^{-3} mol	1.3g 0.07 mol	0.2g 3.4×10^{-4} mol	0.5g 7.5×10^{-5} mol	0.5g 7.5×10^{-5} mol	no gel
K3	5.5g 2.4×10^{-2} mol	2g 4×10^{-3} mol	0.3g 0.02 mol	0.2g 3.4×10^{-4} mol	1.0g 1.5×10^{-4} mol	1.0 1.5×10^{-4} mol	viscous gel formed
K4	5g 2.2×10^{-2} mol	2g 4×10^{-3} mol	0.3g 0.02 mol	0.2g 3.4×10^{-4} mol	1.25g 1.9×10^{-4} mol	1.25g 1.9×10^{-4} mol	partial viscous gel formed
K5	4g 1.7×10^{-2} mol	3g 7×10^{-3} mol	0	0.2g 3.4×10^{-4} mol	1.5g 2.3×10^{-4} mol	1.5g 2.3×10^{-4} mol	no gel

As reported in the table, despite increasing initiator volume and concentration to often threefold that used in NaAMPs/KSPA gels, a complete polymer was not formed. Whilst there was evidence of some polymerisation occurring due to a visible increase in viscosity of the component solution, this was not complete, confirming that the use if KSPI as a comonomer was not suitable.

Utilising the more reactive nelfilcon, the structure of which is shown in figure 3.4.1, as a crosslinking agent instead of PEGDA did improve gelation, however often a nucleus of gelation was formed instantaneously possibly preferentially incorporating the NaAMPs, and the remainder of the solution did not polymerise. This was consistent with bit UV and redox initiation. It was concluded that although KSPI incorporation has the potential to produce a gel with advantageous properties, the constraints of initiation render KSPI unsuitable.

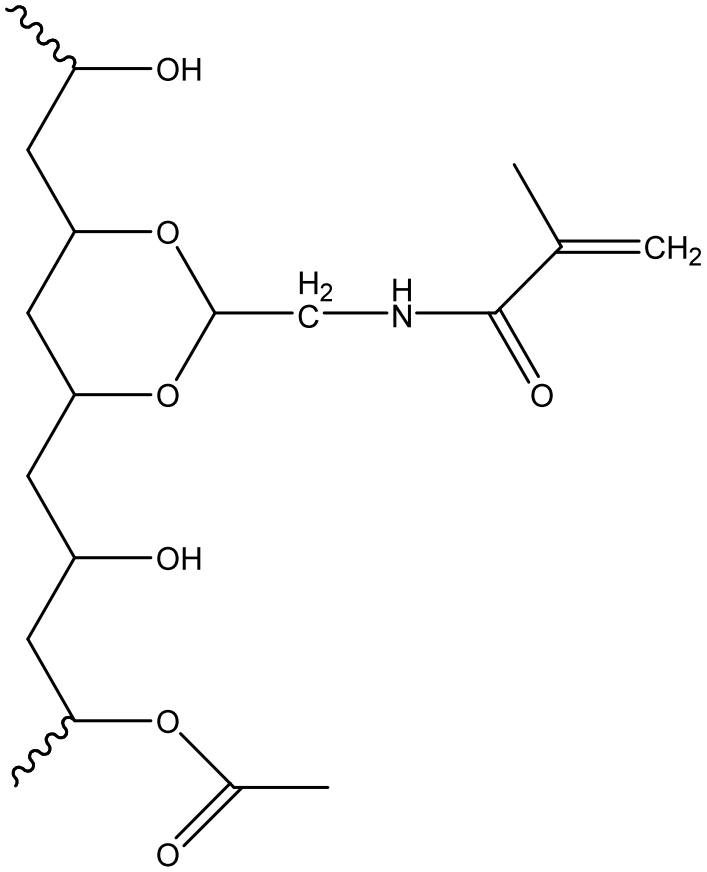


Fig 3.4.1 Nelfilcon A, modified PVA [50]

3.5 Initiator variation

3.5.1 The use of benzoyl peroxide and dimethyl toluidine as a redox pair

This set of experiments aimed to assess whether additional redox pairs would create a more efficient polymerisation system. The first pair investigated were benzoyl peroxide and dimethyl toluidine. Benzoyl peroxide is typically used in dental applications whereas dimethyl toluidine is a catalyst in epoxy resin formation. Gels were prepared as described previously. The volume and concentration of benzoyl peroxide was kept constant as this was found to be the maximum concentration compatible for use in an aqueous environment, the initiator was made up using poly (ethyleneglycol) acrylate as the solvent due to its poor aqueous solubility. Gels 4 and 4a were polymerised at room temperature in the presence of ambient oxygen, and 4b and 4c at 37^{o}C after degassing with nitrogen prior to addition of the initiators. The compositions are represented in table 3.5.1.1

Table 3.5.1.1 The effect of benzoyl peroxide and dimethyl toluidine as initiators in the polymerisation of an NaAMPs/KSPA copolymer

Gel	NaAMPS (58%) (g)	KSPA (g)	H_2O (g)	PEG400DA	DMT	BP 2M	Observations
4	3	1.4	4.3	0.2g 5×10^{-4} mol	0.05g 3.7×10^{-4} mol	0.65g 1.3×10^{-3} mol	20°C, O_2, partial gel
4a	3	1.4	4.3	0.2g 5×10^{-4} mol	0.1g 7.4×10^{-4} mol	0.65g 1.3×10^{-3} mol	20°C, no O_2, non uniform complete gel
4b	3	1.4	4.3	0.2g 5×10^{-4} mol	0.05g 3.7×10^{-4} mol	0.65g 1.3×10^{-3} mol	37°C, O_2, partial gel
4c	3	1.4	4.3	0.2g 5×10^{-4} mol	0.1g 7.4×10^{-4} mol	0.65g 1.3×10^{-3} mol	37°C no O_2, non uniform complete gel

Where n= NaAMPs 1.3×10^{-2}mol, KSPA $6 \times 10^{-}3$, H_2O 0.24 mol, PEG400DA 5×10^{-4} mol, AA and Oxone 6.5-3 $\times 10^{-5}$mol respectively

At 20°C in both the presence and absence of oxygen, a uniform complete gel was not formed, this was assessed visually. It is possible that this was due to the low level of initiator used, controlled by the organic nature of both the benzoyl peroxide and dimethyltoluidine. Increasing temperature did improve gelation but not sufficiently. Polymerisation in the absence of oxygen at 37°C produced a gel but with a brittle non-uniform structure. This can be attributed to both the poor solubility of initiators, when initiation did occur it tended to be rapid resulting in an erratic short chain polymer. This initiator was therefore deemed unsuitable for this application.

3.5.2 The use of hydrogen peroxide and dimethyl toluidine as a redox pair

In the next set of experiment BP was replaced with aqueous hydrogen peroxide and the volume of both DMT and hydrogen peroxide increased up to a point. All experiments were carried out in a similar manner to previous, and all components were purged with nitrogen prior to use, to minimise the interference of oxygen in the polymerisation. The compositions are detailed in table 3.5.2.1.

Table 3.5.2.1 Hydrogen peroxide and dimethyl toluidine in the polymerisation of an NaAMPs/KSPA copolymer

Gel	**NaAMPs (58%) (g)**	**KSPA (g)**	**H_2O**	**PEG400DA (g)**	**DMT**	**H_2O_2 8.8M**	**Observations**
5	3	1.4	4.3g 0.24 mol	0.2	0.02g 1.5 x 10^{-4} mol	0.046g 4 x 10^{-4} mol	20°C, no gel
5a	3	1.4	4g 0.22 mol	0.2	0.1g 7.4 x 10^{-4}mol	0.33g 2.9 x 10^{-3} mol	20°C, no gel
5b	3	1.4	3.9g 0.22 mol	0.2	0.2g 1.5 x 10^{-3} mol	0.66g 5.8 x 10^{-3} mol	37°C, no gel
5c	3	1.4	3.2g 0.18 mol	0.2	0.5g 3.7 x 10^{-3} mol	1.65g 1.5 x 10^{-2}	37°C, no gel

Where n= NaAMPs 1.3 x10^{-2}mol, KSPA 6 x 10^{-}3, PEG400DA 5 x 10^{-4} mol

Water content was adjusted accordingly to allow for water present in the H_2O_2 to allow the concentration of H_2O_2 used to reach 0.2%, 1%, 2% and 5% respectively.

Despite increasing initiator inclusion to high levels, ~10% w/v H_2O_2, no gel was formed. It would be detrimental to include a higher level of initiator so investigations were halted. This system was deemed unsuitable.

3.5.3 The use of tertiary butyl hydroperoxide with dimethyl toluidine as a redox pair

Tertiary butyl-hydroperoxide is another commercially available peroxide initiator, typically used in the oxidation of ketones. This was used as a pair with DMT. All reactions were carried out at 37°C in the presence of ambient oxygen, or after all components had been purged of nitrogen with oxygen. The compositions are represented in table 3.5.3.1.

Table 3.5.3.1 Tertiary butyl peroxide and dimethyl toluidine in the polymerisation of a NaAMPs/KSPA copolymer

Gel	**NaAMPs (58%) (g)**	**KSPA (g)**	**H_2O (g)**	**PEG575DA (g)**	**DMT**	**TbutH**	**Observations**
8	3	1.4	4.3	0.2	0.5g 3.7×10^{-3} mol	0.5g 5.5×10^{-3} mol	O_2, no gel
8a	3	1.4	4.3	0.2	0.5g 3.7×10^{-3} mol	0.5g 5.5×10^{-3} mol	no O_2, no gel
8b	3	1.4	4.3	0.2	1.0g 7.4×10^{-3} mol	1.0g 1.1×10^{-2} mol	O_2, no gel
8c	3	1.4	4.3	0.2	1.0g 7.4×10^{-3} mol	1.0g 1.1×10^{-2} mol	no O_2, no gel
8d	3	1.4	4.3	0.2	1.5g 1.1×10^{-2} mol	1.5g 1.7 x 10-3 mol	O_2, no gel
8e	3	1.4	4.3	0.2	1.5g 1.1×10^{-2} mol	1.5g 1.7 x 10-3 mol	no O_2, no gel
8f	3	1.4	4.3	0.2	2.0g 1.5×10^{-2} mol	2.0g 2.2×10^{-3} mol	O_2, no gel
8g	3	1.4	4.3	0.2	2.0g 1.5×10^{-2} mol	2.0g 2.2×10^{-3} mol	no O_2, no gel

Where n= NaAMPs 1.3 $x10^{-2}$mol, KSPA 6 x 10^{-}3, H_2O 0.24 mol, PEG575DA 3.4 x 10^{-4} mol

As demonstrated in the table, no gelation was successful and increasing initiator concentration further to over 5% of the end product would be detrimental to both the structure and surrounding tissues. This thus deemed an unsuitable redox pair.

3.5.4 The use of TEMED and potassium persulfate as a redox pair

A suitable redox pair for investigation was potassium persulfate (KPS) and *N'N'N'N* tetramethy ethylenediamine (TEMED). These initiators are used together as a pair to initiate gelation within electrophoresis SDS page gels. The procedure for preparation was similar to previous gel compositions, polymerisation was carried out at room temperature in the presence of ambient oxygen. The compositions are detailed in table 3.5.4.1

Table 3.5.4.1 The use of N'N'N'N tetramethyethylenediamine (TEMED) and potassium persulfate (KPS) in the polymerisation of a NaAMPs/KSPA copolymer

Gel	**NaAMPs (58%) (g)**	**KSPA (g)**	**H2O (g)**	**PEG575DA (g)**	**TEMED**	**KPS**	**Observations**
9a	3	1.4	4.3	0.2	0.1M 0.65g 6.5 x 10-5 mol	0.1M 0.65g 6.5 x 10-5 mol	Complete gel, slow polymerisation
9b	3	1.4	4.3	0.2	0.15M 0.65g 9.8 x 10-5 mol	0.15M 0.65g 9.8 x 10-5 mol	Complete gel, relatively slow polymerisation
9c	3	1.4	4.3	0.2	0.2M 0.65 1.3 x 10-4 mol	0.15M 0.65g 9.8 x 10-5 mol	Complete gel
9d	3	1.4	4.3	0.2	0.2M 0.65 1.3 x 10-4 mol	0.2M 0.65 1.3 x 10-4 mol	Complete gel, polymerised less than 10 minutes
9e	3	1.4	4.3	0.2	0.3M 0.65g 2 x 10-4 mol	0.2M 0.65g 1.3 x 10-4 mol	Complete gel polymerised ~ 3 minutes
9f	3	1.4	4.3	0.2	0.4M 0.65g 2.6 x 10-4 mol	0.18M 0.65g 1.2 x 10-4 mol	Complete gel polymerised ~ 5 minutes

Where n= NaAMPs 1.3 $x10^{-2}$mol, KSPA 6 x 10^{-}3, H_2O 0.24 mol, PEG575DA 3.4 x 10^{-4} mol

Although successful gels were produced for all initiator variations, the most effective and reproducible was the 0.4M TEMED and 0.18M KPS. The slightly slower polymerisation time for composition 9f suggests that the initiation depends more on the concentration of KPS than TEMED.

3.5.5 Additional initiation systems

A full list of redox pairs investigated is represented in table 3.5.5.1

Table 3.5.5.1 Redox pairs investigated

Redox A	**Redox B**
Ascorbic acid (AA)	Oxone
Ascorbic acid (AA)	Potassium persulfate (KPS)
Ascorbic acid (AA)	Ammonium persulfate
Iron (II) lactate hydrate	Oxone
Iron (II) lactate hydrate	T-but hydroperoxide
Iron (II) gluconate	Oxone
Iron (II) gluconate	tert-butyl hydroperoxide
Dimethyl- toluidine (DMT)	tert-butyl hydroperoxide
Dimethyl- toluidine (DMT)	Hydrogen peroxide
Dimethyl toluidine (DMT)	Oxone
Dimethyl toluidine (DMT)	Benzoyl peroxide
2,2 azo bis (2-methylpropionamidine) dihydrochloride	Oxone
N'N'N'N tetramethyethylenediamine (TEMED)	Potassium persulfate (KPS)

It seems unnecessary to discuss in detail all compositions that were investigated if they were immediately deemed to be unsuitable for the application. For instance the use of iron lactate and or gluconate with a peroxide pair resulted in a nucleus of gelation forming almost instantly even in the presence of atmospheric oxygen. However gelation was not always complete with water contained in the composition often left not included in the polymer structure due to the initial aggressive nature of radical formation. The production of a nucleus of gelation within any system that is designed for use as an injectable is undesirable, the material simply would not be able to pass through the needle. For this reason these compositions were unsuitable.

Further hydroperoxides were investigated such as cumene hydroperoxide, but were immiscible in aqueous solution and were therefore inhibited in becoming part of the polymerisation due to the monomers being highly aqueous.

2,2-Azo bis (2-methylpropionamidine) dihydrochloride and oxone redox pair did not produce a gel even at levels of initiator higher than 5% of the total composition. The same was true for the use of DMT with oxone.

The active part of oxone is $2KHSO_5$. Oxone is already used as an initiator in some applications[37][48] and is readily soluble in aqueous solution. This is due to its relative stability, easy handling and non toxic nature; one of the uses of Oxone in a biological environment is to clean dentures, this highlights its non toxic nature[49]. KPS is similar in its active component but is both less soluble and less stable to storage in aqueous environment than Oxone, when used with AA. Oxone, potassium persulfate and ammonium persulfate all yield similar results. Ammonium persulfate has a greater degree of aqueous solubility, however gels formed appear visibly to have a less uniform structure. Therefore it was discovered that the two most suitable redox pairs were oxone and AA and, TEMED and potassium persulfate.

3.6 Choice of crosslinking agent

Two main crosslinking agents were investigated PEG diacrylate, and the modified PVA –nelfilcon, The structure of PEGDA is shown below in figure 3.6.1, nelfilcon has been previously represented in figure 3.4.1. These crosslinking agents were chosen as they have been previously used in biomedical applications using similar monomer materials. Longer chain cross linking agents also allow for a greater incorporation of water in the composition and the potential for further swelling in aqueous media after gelation has occurred.

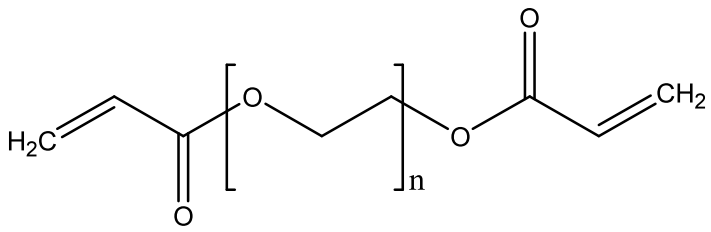


Fig 3.6.1 Poly (ethyleneglycol) diacrylate. Typically n~575

PEG diacrylate is an efficient di-functional crosslinking agent. Nelfilcon , or modified PVA is a multifunctional macromer, therefore it is capable of producing a greater number of crosslinks per unit weight. This has the advantage that an intricate network structure will be produced, but the disadvantage that due to its high proportion of reactive sites, polymerisation is often induced much more rapidly than without it. To assess the effect of varying both the percentage of initiator incorporated and the type of initiator had on the polymerisation a series of gels were made. The monomer and water composition and method of polymerisation remained similar to previous experiments, in the presence of ambient oxygen at room temperature. The results are represented in table 3.6.1.

Table 3.6.1 Crosslinking agent variations: effect on gel formation

Gel	NaAMPs (58%) (g)	KSPA (g)	H_2O (g)	Crosslinking agent	AA (0.1M) (g)	Oxone (0.1M) (g)	Polymerisation time
10a	3	1.4	4.3	PEG575DA 0.05g 8.7×10^{-5} mol	0.65	0.65	~ 7 minutes
10b	3	1.4	4.3	PEG575DA 0.1g 1.7×10^{-4} mol	0.65	0.65	~ 7 minutes
10c	3	1.4	4.3	PEG575DA 0.15g 2.6×10^{-4} mol	0.65	0.65	~ 7 minutes
10d	3	1.4	4.3	MPVA 0.02g 1.4×10^{-6} mol	0.65	0.65	No gel
10e	3	1.4	4.3	MPVA 0.05g 3.6×10^{-6} mol	0.65	0.65	Incomplete gel
10f	3	1.4	4.3	MPVA 0.1g $7.1 \times ^{10\text{-}6}$ mol	0.65	0.65	~ 2 minutes
10g	3	1.4	4.3	MPVA 0.2g 1.4×10^{-5} mol	0.65	0.65	~ 1minute

Where n= NaAMPs 1.3 $x10^{-2}$mol, KSPA 6 x $10^{-}3$, H_2O 0.24 mol, AA and Oxone 6.5 x 10^{-5} mol respectively

The results with 2% PEG diacrylate are not reported, this composition is represented in table 3.2.3.1 as gel 1.

It can be seen from the results above, the level of difunctional crosslinking agent included in the composition does not affect the polymerisation time, providing there is a high enough level to produce a crosslinked gel structure. However in the case of modified PVA, the high availability of reactive groups meant that initial gelation was almost immediate as it reacted with NaAMPs upon contact. This often resulted in a nucleus of gelation forming which would not allow the pregel to be injected. The highly crosslinked structure also results in a high stiffness and reduced swell capacity, as investigated by rheology, which is demonstrated in Figure 3.6.1.

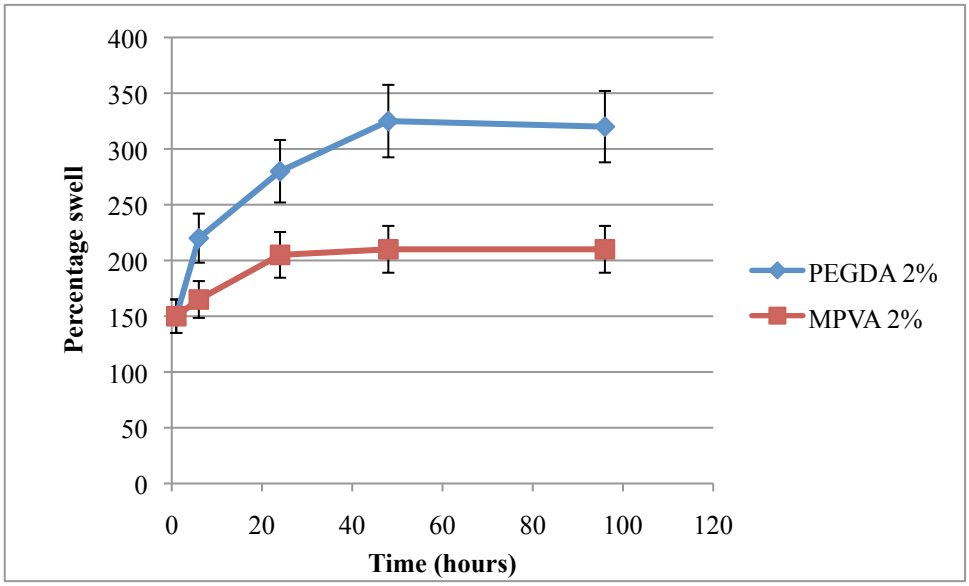


Fig. 3.6.1 The difference in swell behaviour of an NaAMPs/KSPA copolymer with 2% PEG diacrylate and 2% modified PVA

3.7 Discussion

The aim of the work conducted in this chapter was to find suitable monomer materials for the synthesis of the proteoglycan analogue. The sulfonate groups within NaAMPs and KSPA aim to mimic the natural sulfate groups found in proteoglycans. The natural structures are termed allyl sulfates and the synthetic alternatives alkyl sulfonates. It is not possible to provide a synthetic sulfate, and as an added advantage the carbon linked sulfur is much more stable to hydrolytic degradation than an oxygen linked sulphur atom. Sulfonates are advantageously the most hydrophilic monomers, the ability of the functional group to hydrophilically shield the carbon backbone is much greater than that of a hydroxyl or carbonyl group. Of the sulfonate monomers commercially available, NaAMPs and KSPA are the most cost effective and successfully synthesised, making them the ideal choice.

NaAMPs was initially investigated as a homopolymer, however the availability of the material as either a 50% or 58% solution warrants the addition of a comonomer to allow manipulation of the solid monomer content of the end gel. In addition the presence of both an amide group and the sulfonate produces an extremely large swell capacity. This was to such a degree to also suggest the use of a copolymer. Due to the requirements of the material to be osmotically responsive a neutral material would not be suitable as a comonomer.

Water is an important component of the polymer system. There is evidence to suggest that water in polymers can exist in more than one state. These states of water can affect the properties of the polymer. The water in hydrogel membranes can be characterised by the terms freezing and non-freezing. The analysis of the melting endotherms obtained by differential scanning calorimetery (DSC) have the ability to differentiate between freezing and non-freezing water. The water that is strongly associated with polar groups in the polymer matrix is bound and unable to freeze and would not, therefore, contribute to the melting endotherm. To further elaborate on this theory, the nature of water in a polymer can be considered as a combination of states which lies between the two extremes. The non-freezing water hydrogen bonds to the polar groups of the polymer matrix and has some interaction with the ionic residues. The freezing water lacks the interaction with the polymer matrix but

possesses characteristics similar to pure water due to its occupation of large pores and level of hydrogen bonding.

NaAMPs and KSPA are examples of acrylamides and acrylates, of which the reactivity of the monomers to each other is dependent on 3 factors: monomer reactivity, radical resonance stabilisation and steric hindrance[51]. Steric hindrance affects the ability of the monomer to react with the propagating radical, NaAMPs and KSPA – have similar steric bulk and would be expected to behave in a similar manner where reactivity is concerned. "The rate of reaction is proportional to the activated complex (propagating radical monomer) therefore it is not affected by mobility, viscosity or diffusion rate"[52]. However, despite these similarities their reactivity is in fact different and by experiment KSPA has been found to be preferentially incorporated into the polymer chain with the reactivity ratios KSPA to NaAMPs 1.7:0.55 respectively[53].

A range of initiators were investigated with two successful systems discovered as a result. These were ascorbic acid and oxone and TEMED and potassium persulfate. They produced the most reproducible systems. Initiators based on transition metals paired with peroxides are known as effective redox pairs. However for this application the formation of instant gels is detrimental to the ability of the gel to be injected. No other system investigated produced reliable results. An interesting observation was the effect of dilution of initiators on the efficacy of polymerisation.

The effect of dilution of the proteoglycan analogue must be discussed in two separate instances. There is both the dilution of the monomer containing system and the initiator containing system. Hydrogels are unique in their ability to swell with water yet not dissolve. Through the production of the ideal gel it was important to form a balance between the volume of water added to the gel to be included in the polymerisation, and the capability of the polymerised gel to swell when placed in water. It was clear that although a dehydrated gel was able to swell to great extent when placed in water, it was not possible to polymerise a gel with this volume of water. This is related to both dilution of free radical species and the constraints of forming a loosely cross-linked gel structure.

Dilution can also be related to the molarity of the initators. It was of little surprise to discover that the use of a larger volume of a weaker initiator will not produce gelation in comparison to the use of a smaller volume of a stronger initiator (even when the end calculated percentage of initiator is the same). This is due to the dilution of the free radicals, and the reduction in the probability of contact between monomer and radical. This arises due to the radical pairs undergoing oscillation while surrounded by water molecules before they diffuse apart. The greater the number of water molecules the larger the 'cage' around the pair of radicals and therefore contact between monomer and radicals is less likely.

Polymerisation was only investigated at physiological pH due to this being the environment in which the analogue was designed for use. Altering the pH of the system would alter the ionicity of the polymer and thus the biocompatibility. Differences in pH would also have an affect on the production and dissociation of radical species *via* redox, for instance lowering pH will give a reduction in the production of AH radicals[37].

Having discussed the effect that oxygen has on the initiating systems, it would seem logical to purge the system of oxygen *via* degassing with nitrogen. However this is also no advantageous, primarily due to the resulting instantaneous gelation that did not allow time for any form of injection of the pregel to occur. Instantaneous gelation also resulted in solution remaining on the surface of the formed gel due to polymerisation occurring so rapidly that water was excluded from the structure.

The final investigations reported were the effect of different crosslinking agents. Two types were assessed, multifunctional and difunctional, modified PVA and PEG diacrylate respectively. Whilst a multifunctional macromer crosslinker may produce a more intricate network structure, its ability to induce polymerisation immediately renders it unsuitable for an injectable application.

To build on the discoveries made within this chapter the following will aim to refine the system to produce a workable injectable system.

Chapter Four

The Design and Synthesis of a Proteoglycan Analogue: Refinement of the Injectable System

4.1. Introduction

The previous chapter dealt with variations in composition of the proteoglycan analogue. This chapter will focus on developing these compositions into successful injectable systems. The reproducible systems and their subsequent storage stability will be discussed, in addition to variables that affect the efficacy of the polymerisation process.

4.2.1. Refinement of the composition

Of all initiator systems investigated, the two redox pairs seen to have the greatest efficacy and reproducibility were ascorbic acid and oxone, gel 1 table 3.2.3.1 and TEMED potassium persulfate gel 9f table 3.5.4.1 with the respective concentrations being 0.1M:0.1M and 0.4M:0.18M respectively. Initiator solutions are used at a total of 13% per polymer and resultant levels of initiator in the polymerised product are 0.0065M for ascorbic acid oxone and 0.032M:0.014M for TEMED potassium persulfate respectively.

The first point of investigation was to refine the compositions, in particular as the NaAMPs used for these investigations was available at 50% only. The rationale behind refining the compositions was to decide on two variations which provided reproducible gelation after injection, whilst providing mechanical properties similar to those of the natural nucleus pulposus. Two variations were needed to present the idea commercially, in particular the ability of the system to be modified to produce a range of properties depending on commercial market preference. The compositions were modified as represented in table 4.2.1 below. They are now referred to as Rf1 and Rf2 which denoted refined formulation one and refined formulation two. It was decided at this point, due to commercial interest and discussions with collaborators at Oxford, Oswestry and Keele, also to introduce another cross-linking agent to enable a difference between the two compositions. A PEG dimethacrylate with a chain length of 1000 was chosen for use with the TEMED potassium persulfate system. The increased length of this crosslinking agent will mean less cross-links per volume used, and therefore will not be suitable for use at levels as low as the PEGE575 diacrylate.

This was another deciding factor in including it in the amine initiated system, which is a more efficient producer of radicals than ascorbic acid, so therefore will create more of a reproducible network with this type of cross-linking agent.

Table 4.2.1 Refinement of the compositions: effective formulations

Gel	NaAMPs (50%)	KSPA	Water	Crosslinking agent	Initiator 1	Initiator 2
Rf1	3.7g 1.6 x 10^{-2} mol	1.4g 6 x 10^{-3} mol	3.6g 0.2 mol	0.025 – 0.2g PEG575DA 4.3 x10^{-5} mol – 3.4 x 10^{-4} mol	AA 0.1M 0.65g 6.5 x 10^{-5} mol	Oxone 0.1M 0.65g 6.5 x 10^{-5} mol
Rf2	3.7g 1.6 x 10^{-2} mol	1.4g 6 x 10^{-3} mol	3.6g 0.2ml	0.05-0.2g PEG1000DMA 5 x 10^{-5} mol – 2 x 10^{-4} mol	TEMED 0.4M 0.65g 2.6 x 10^{-4} mol	KPS 0.18M 0.65g 1.2 x 10^{-4} mol

Both systems allow crosslinking agent to be used over a range of 0.5% to 2%, and polymerisation is reproducible in a time scale of 3-7 minutes.

In order to allow the analogue to be used in an injectable application, a method of delivery must be devised. As the polymerisation method is based upon redox, the primary requirement is the separation of the two initiating components prior to the time at which gelation is required. It follows from this then, that a two-pack system has the potential to offer the desired solution. To deliver this a dual syringe system may be used similar to that which delivers fibrin glue[(54)]. For this to be effective the compatibility of components which will form each pre-gel solution was thoroughly investigated, and will be discussed in the following sections. A picture of the fibrin glue delivery system is shown below in figure 4.2.1.

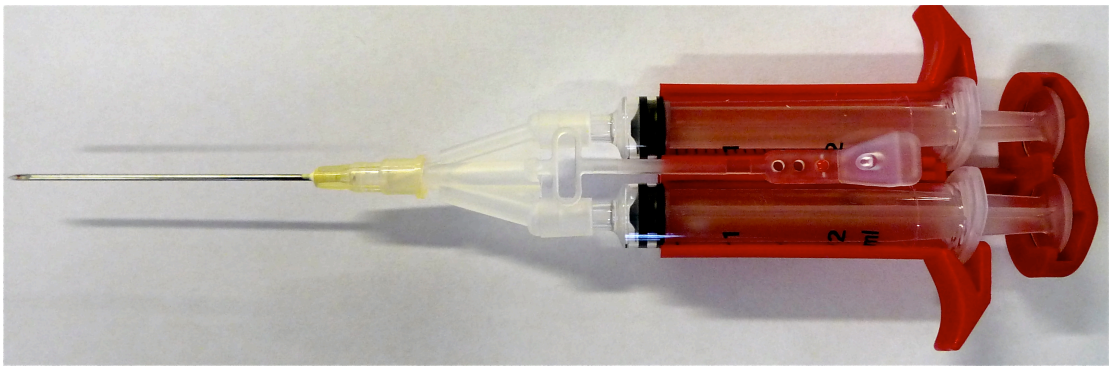


Fig 4.2.1 The dual syringe system

4.2.2 The storage of components: environmental effects

The factors which affect the storage of the components are represented in table 4.2.2.1 below

Table 4.2.2.1 Factors which affect the storage of components

Component	**Implications**
Redox initiators	Heat, light, oxygen
NaAMPs	Oxygen, presence of initiator
KSPA	Oxygen, presence of initiator
Water	N/A
Crosslinking agent	Light (if photoactive component is present) presence of monomer and/or initiator

The presence of oxygen is by far the most important factor when investigating storage stability. Oxygen is produced *via* the decomposition of peroxydisulfate ions, this in turn will be consumed by ascorbic acid which has the potential to decrease the induction period. Too much oxygen however will monopolise the radical species thus reducing the probability of contact with monomer and therefore polymerisation. Transition metals have also been shown to have a negative affect on the stability of Oxone[55]. This was an important factor to consider where the components may be stored in a container with a metal component, and also during the injection through a metal needle.

Although potassium persulfate has similar activity to oxone in relation to the active species $2KHO_5$, potassium peroxomonosulfate, oxone, upon investigation of storage of 0.1M, 0.15M, and 0.2M solutions, produced a more uniform gel after longer periods of storage. Potassium persulfate appeared to become degraded after 14 days (investigated each time with fresh redox B and monomer stock) polymerisation is visibly slower circa 30 seconds to 1 min, whereas there appears to be no degradation in the Oxone to 18 days. Both initiators do however produce a gel for up to 1 month providing they have been stored in optimal conditions, refrigerated at approximately 4^oC, in the dark, in a vessel where there is minimal air *i.e*. not a large gap between the solution and the lid of the vessel, with polymerisation carried out at 37^oC.

Similar experiments were carried out using ascorbic acid. Ascorbic acid was found to be highly light and heat sensitive with oxygen degradation being less of a problem, again the initiator produced a reproducible polymerised gel for up to one month. TEMED did not seem to degrade in a similar manner to the other initiators, continuing to produce a reproducible gel, the constraints of the other redox constituent are the limiting factor in the use of this intitiator. It is important to note that amongst other factors, due to potassium persulfate being more readily degraded than Oxone, a system of optimal reliability was formed using oxone as the redox agent not potassium persulfate as coupling the degradation of ascorbic acid and potassium persulfate produced a less reproducible system. A summary of the stabilities is shown in table 4.2.2.1 below.

Table 4.2.2.1 The stability of initiator stock solutions

Redox A	**Redox B**	***Via*bility time (days)**
AA	Fresh Oxone	19
AA	Oxone	17
AA	Fresh KPS	17
AA	KPS	12
Fresh AA	Oxone	18
Fresh AA	KPS	14
TEMED	Fresh KPS	30+
TEMED	KPS	14
Fresh TEMED	KPS	14

NB. Where fresh is not denoted, the solution had been stored for a number of days to assess *via*bility, *via*bility denoting when time taken to polymerise became noticeably altered.

With respect to monomer stability, NaAMPs and KSPA and also including in this section the crosslinking agent, which was either PEG575 diacrylate or PEG1000 dimethacrylate, which essentially, where stability is concerned, behave in a similar manner. Focusing on a dual-based system, stability was investigated in two ways. The first being to separate the monomers, incorporating the crosslinking agent with the least reactive monomer (KSPA as discussed in section 3.5), to make the two pre solutions of equal volume, the water component in each A and B was adjusted. Initial

stability tests discovered that on occasion the crosslinking agent and NaAMPS would form a gel over time if in solution. This resulted in solutions of the following composition:

Table 4.2.2.2 Suitable pregel compositions

Solution A	**Solution B**
NaAMPs	KSPA
Water	Water
Redox A	Crosslinking agent
	Redox B

This was a successful method when components were kept refrigerated ~4^{o}C, and in the dark.

Although it has already been discussed due to its unsuitability relating to the speed of polymerisation, the nelfilcon used had a photoactive agent attached to its modified structure. This meant that its stability under storage was only suitable in aqueous solution. It could not be stored for any length of time in presence of a monomer or an initiator pair.

The removal of oxygen from the polymerisation systems also had an effect on mechanical properties of the resultant gels. This is not surprising given that polymerisation often occurs much more rapidly which, would result in shorter chains, therefore a less elastic gel. This is demonstrated in the graph below which is an KSPA/NaAMPs gel 1:1:1 NaAMPs:KSPA:water with 1% PEGDA400 as crosslinking agent, polymerised with 0.1M AA and 0.1M Oxone. The monomer stock sample which was purged of oxygen had nitrogen bubbled through it for 10 minutes, as did the initiators separately prior to addition to the pregel. An increase in stiffness can clearly be observed which is demonstrated in figure 4.2.2

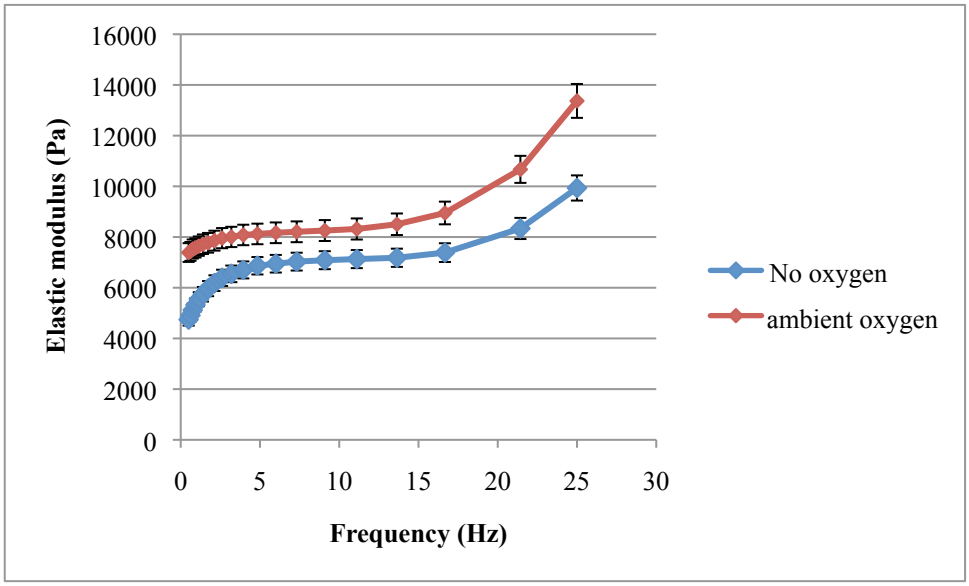


Fig 4.2.2.2 The effect of the removal of oxygen on the mechanical properties of the gels

A Mettler Toledo oxygen probe was used to measure the levels of oxygen in nitrogen purged, ambient, and oxygen saturated solutions, then the time taken to polymerise observed. Nitrogen purged solutions produced instantaneous gelation, ambient circa 5-10 minutes and oxygen saturated upwards of one hour, often resulting in incomplete gelation. This is represented below in table 4.2.2.3. The percentages detailed are relative percentages

Table 4.2.2.3 The effect of oxygen levels on polymerisation time

Conditions of solution	**Percentage O_2**	**Polymerisation time**
N_2 purged	0.0%	Instantaneous
O_2 saturated	28.8%	Incomplete after 1 hour
Ambient	8.5%	~ 5- 10 minutes

4.3 The use of dipicolinic acid as a stabiliser within the copolymer system

Ambient oxygen controls the rate of polymerisation in order to produce reproducible injectable gels in circa 3-7 minutes. The use of dipicolinic acid DPA as a stabiliser to delay polymerisation for Oxone and KPS to assess whether oxygen could be removed from stock solutions whilst still allowing sufficient time for injection prior to gelation was investigated. The structure of dipicolinic acid is shown in figure 4.3.1

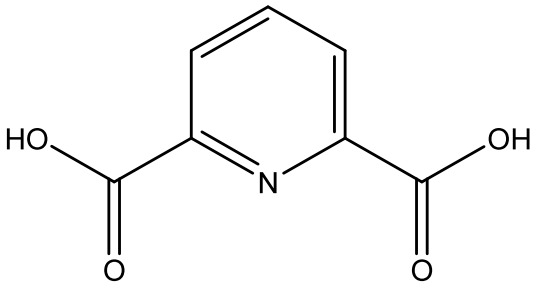


Fig 4.3.1 The structure of dipicolinic acid

Dipicolinic acid has been used to prevent the degradation of peracid solutions, reducing the loss of activity from 50% to 5% over a one month period, used at 50ppm[3]. Increasing levels to 1000ppm does not offer a significant increase in stabilisation, although in general the greater the inclusion of stabiliser, the greater the stabilising effect. As peracid has a strong oxidising potential, which is successfully stabilised by the dipicolinic acid, its affect on KPS and Oxone stability was investigated. Subsequent stabilisers which also may be suitable but were not investigated are ethylenediaminetetra(methylenephosphonic acid) (EDTPA) which acts as a chelating agent, and those based on stannates[56]. The proposed action of DPA within the Oxone and or KPS containing stock solutions was to sequester any peroxides formed prematurely *via* decomposition of the initiator, in particular Oxone which is widely reported to be unstable over storage in an aqueous environment[5]. Table 4.3.1 below illustrates the DPA containing compositions investigated

Table 4.3.1 A summary of gel compositions incorporating DPA

Percentage	**Redox solution A**	**Redox solution B**
Constant	NaAMPs	KSPA
Constant	Water	Water
Constant	AA/TEMED	Oxone/KPS
0.1-1%		DPA

Initially the stability of Oxone and potassium persulfate solutions where investigated prior to use in the two pack system. The results can be seen in table 4.3.2

Table 4.3.2 The effect of incorporating DPA on shelf life of initiator solutions

Redox A	**Redox B**	**Viability in days**
Fresh AA	Oxone 0.1% DPA	18
AA	Oxone 0.1% DPA	18
Fresh AA	Oxone 0.4% DPA	27
AA	Oxone 0.4% DPA	27
Fresh AA	Oxone 0.7% DPA	28
AA	Oxone 0.7% DPA	27
Fresh AA	Oxone 1.0% DPA	25
AA	Oxone 1.0% DPA	21

Each initiator solution was refrigerated at ~4^{o}C in a light insulated glass *via*l.

Initially, as demonstrated in the table above, DPA did improve the length of time which the solutions could be stored for and still produce a viable gel. However once incorporated as part of the pregel solution redox B, it was apparent that DPA will degrade on contact with oxygen therefore unless used immediately after receiving, these degraded products prevented subsequent polymerisation. This rendered the use of this particular stabiliser unsuitable for this application.

4.4 The injection process: effect of needle length and diameter

Because the injection process into the disc space will involve an analogue of the radiological process used to image potential flaws within the disc, the injection itself is a specific process. Therefore it was necessary to investigate the effects this process had on the polymerisation of the specific gel formulations, specifically the small diameter of needle required for a minimally invasive procedure, and any problems which may occur relating to back pressure created by injection of the pre-gel solution.

The pre-gel solutions Rf1 and Rf2, the most reproducible compositions chosen for investigation, were prepared as detailed in section 2.2.1 and placed in the dual syringe illustrated in figure 4.2.1. The primary reasons for this investigation was to explore the time taken to polymerise and assess whether or not this would allow for injection into the intervertebral disc cavity. The experiment was carried out at 37^{o}C in ambient oxygen, and the results are illustrated below in table 4.4.1.

Table 4.4.1 The effect of injection on polymerisation time

Syringe size	**Needle diameter vs length**	**Gelation time Rf1**	**Gelation time Rf2**
5ml	0.7 x 178mm (22)	3 mins	2.5 mins
5ml	1.1 x 50mm (19)	3 mins	2.5 mins
5ml	0.8 x 50mm	2.5 mins	2.25 mins
5ml	0.5 x 16mm	1.5 mins	1 min
1ml	1.1 x 50mm (19)	1 mins	1 min
1ml	0.8 x 50mm	30-40 secs	35 secs
1ml	0.5 x 16mm	30 secs	20- 30secs

It is apparent from the decrease in polymerisation time in relation to the diameter and length of the needle that shear forces are acting on the pre-gel inducing polymerisation. It is also feasible that metal mediated catalysis of the reaction is occurring.

4.5 Injection into a surrogate cavity

In order to successfully design an injectable proteoglycan analogue and assess its effectiveness, a suitable cavity is desirable. This was carried out in multiple stages. The first stage involved the injection of both Rf1 and Rf2 (compositions reported in table 4.2.1) into a balloon that had been flushed with nitrogen to represent the absence of atmospheric oxygen within the disc cavity. Gels formed reproducibly. As a larger volume was used, circa 5ml in total to inject into the balloon, it is feasible that this was not representative of the nucleus pulposus cavity. Therefore the balloon model was replaced by injecting into a finger cot, to represent the smaller volume. These are represented in figure 4.5.1

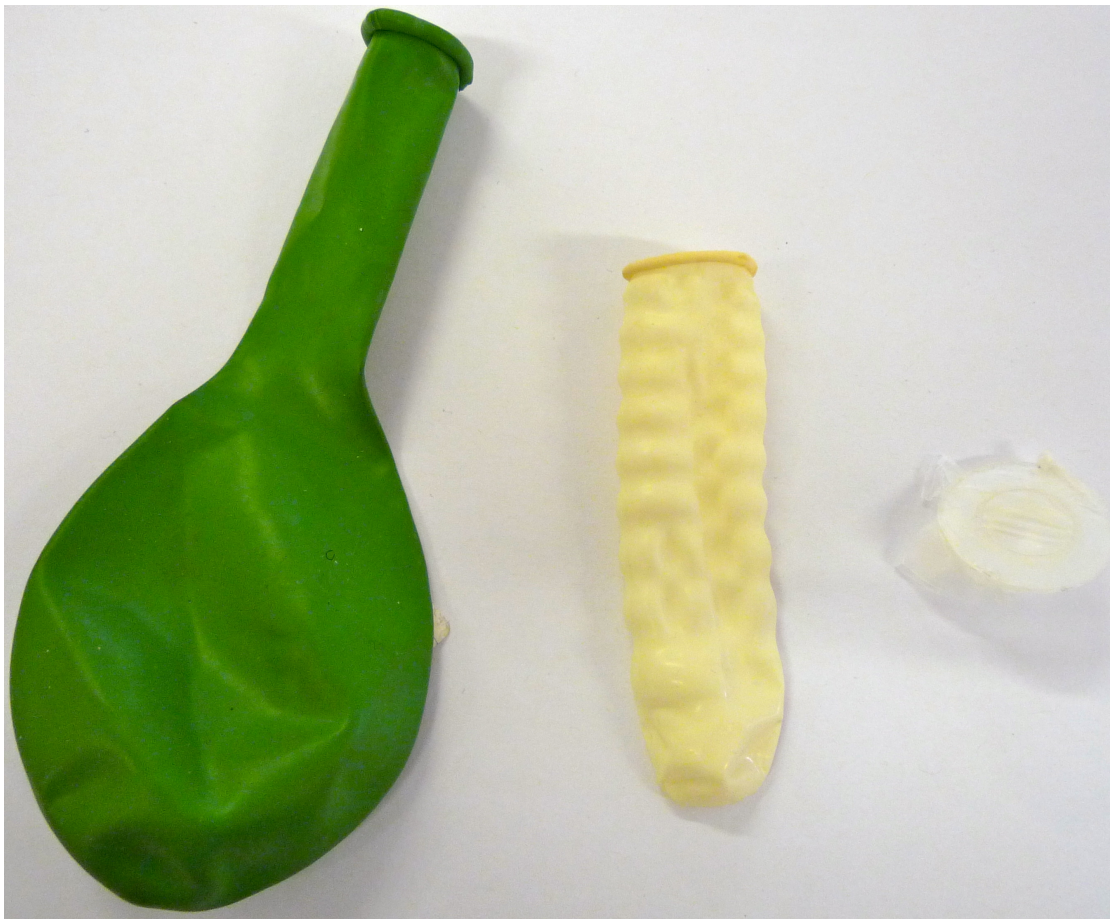


Fig 4.5.1 Balloon and finger cot models as surrogate cavities

Due to the possibility of residual liquid within the disc cavity, a small volume of water was injected into both the balloon and the finger cot ~0.5ml to represent this. The injection process was then repeated, with successful polymerisation.

The next logical step was to inject into the cavity of excised discs represented below in figure 4.5.2. This was carried out as part of dynamic mechanical testing to assess whether the proteoglycan analogue was capable of restoring compressive strength to the excised discs similar to their intact structure. The results are reported in the following chapter.

Fig 4.5.2 An excised sheep disc.

4.6 The incorporation of Histodenz into the gels and its subsequent effect on gelation time

In order for the gels to be visible throughout their lifetime it was deemed necessary to incorporate a radio-opaque material into the gel. The inclusion of a radio-opaque into the material was found to depend on both surgical and commercial preference. Histodenz was chosen as it is a readily available commercial radio-opaque marketed under the name Iohexol. Investigations were conducted preparing gels Rf1 and Rf2 with 2% crosslinking agent, to polymerise after injection into in a petri dish with varying amounts of radiopaque between 5 and 20% of the total gel volume, to see if the polymerisation was detrimentally effected. The results are represented in table 4.6.1 below.

Table 4.6.1 The effect of the addition of Histodenz to Rf1 and Rf2 gel compositions

Gel	0% Histodenz	5% Histodenz	10% Histodenz	15% Histodenz	20% Histodenz
Rf1	~5 mins	~5 mins	5 mins 15 secs	6 mins	10 mins
Rf2	~4 mins	~4 mins	4 mins	4 mins 40 secs	7 mins

The addition of histodenz did have an effect on the polymerisation time. This could be attributed to the radiopaque acting as a diluent within the polymerisation. At 20% there was visibly a slight difference in the gel structure, suggesting that use at such a high percentage in comparison to monomer content. On addition to the TEMED/KPS initiated gels there was a brown colour change. Initially this was suspected to be free iodine, however upon investigation with sodium thiosulfate titration this did not appear to be the case, and can be attributed to the presence of the aromatic amine initiator undergoing a form of oxidtive degradation.

4.6.1 The visibility of Histodenz within the polymerised gel

Discs of the samples above containing increasing amounts of Histodenz were X-rayed kindly by Birmingham Dental School using a Siemens Heliodent DS 7Ma 60kVp DC. The desitometry of the resultant X-rays was measured using a Densitometer X-rite 33. The results are represented in table 4.6.1

Table 4.6.1 The visibility of Histodenz within gel Rf1

Percentage Histodenz	Density of image
0	0.87
5	1.01
10	10.4
15	10.8

Figure 4.6.1 is an example of a large disc of gel x-rayed with smaller disc samples cut out. The density of the image of the gel in this case was 10.8.

4.6.1 An example of an x-rayed gel sample composition Rf1 with 5% histodenz w/w

It appears that whilst histodenz does show within an X-ray at the levels used, there is little differentiation between the percentage increases. A density of 4 is opaque, so it is debateable as to whether the gels would be visible when encased within endplates.

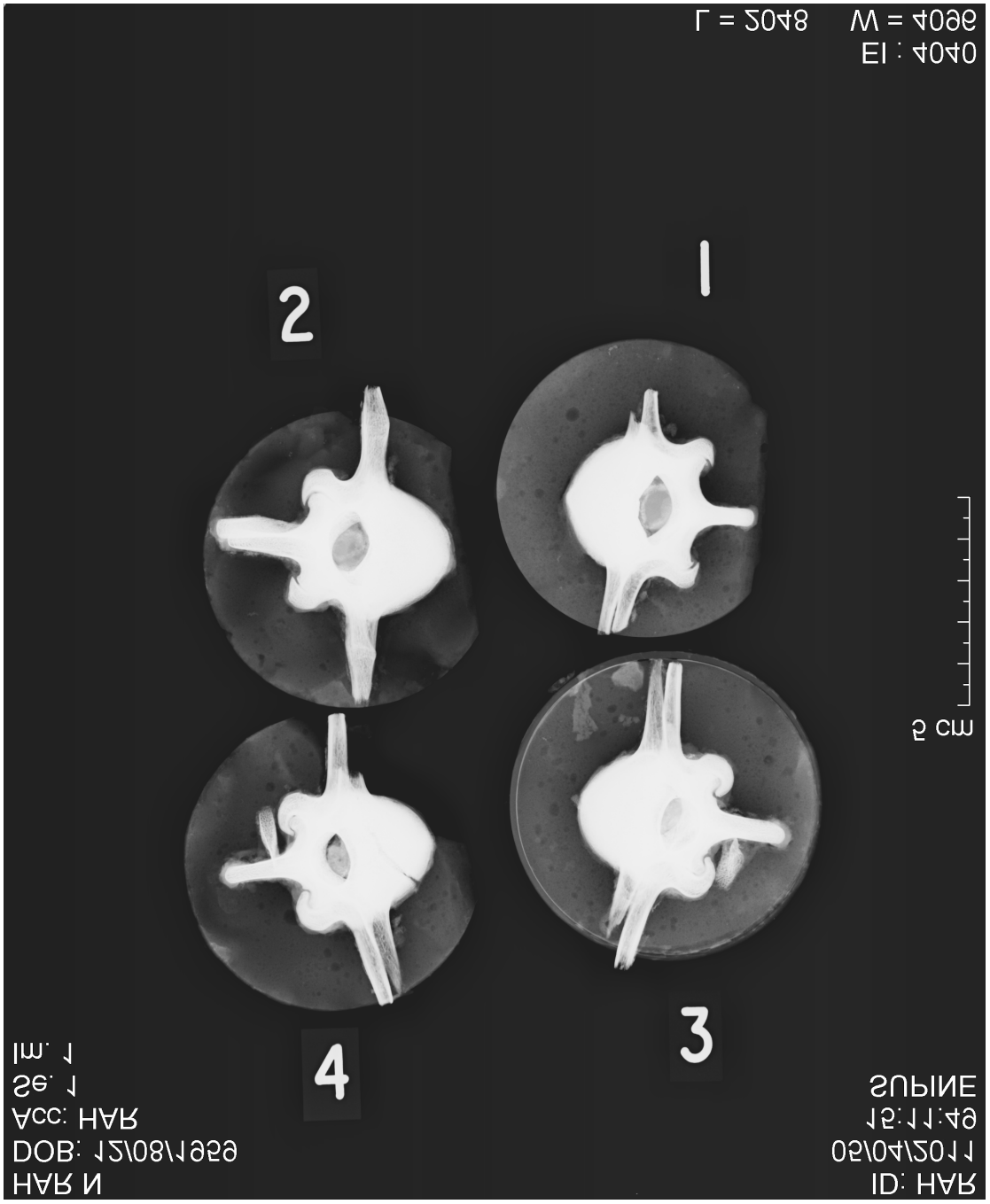


Fig. 4.6.2 The visibility of the gel within an excised disc segment, samples 1 and 2 contain no dye, samples 3 and 4 contain 5% histodenz.

Figure 4.6.2 represents excised sheep disc segments which were injected with pregel which was allowed to polymerise at room temperature prior to re-freezing in order to preserve the specimens. There appears to be very little visible difference between samples 1, 2 and 4, however sample 3 does appear to be slightly more dense. As the nucleus was removed prior to obtaining the samples, the efficiency of removal and size of the resultant cavity was not known. It could be that this cavity contained more

gel so thus resulted in a more dense image, rather than the presence of the histodenz improving visible clarity.

4.7 The residual monomer content

To assess the suitability of the two systems the residual monomer content must be analysed. This was conducted using discs of the two gel types Rf1 and Rf2 prepared with 2% crosslinking agent and polymerised within a petri dish. The full procedure is detailed in section 2.5. The calibration and subsequent results are detailed in figures and tables 4.7.1 through to 4.7.5

Table 4.7.1 Residual NaAMPs calibration by refractive index

NaAMPs conc	RI1	RI2	RI3	Avg RI
10%	1.34824	1.34827	1.34825	1.34825
5%	1.34030	1.34045	1.34035	1.34037
1%	1.33420	1.33421	1.33419	1.33420
0.50%	1.33333	1.33340	1.33335	1.33336
0.10%	1.33267	1.33272	1.33270	1.33270
0.05%	1.33258	1.33264	1.33260	1.33260
0.01%	1.33249	1.33258	1.33250	1.33252

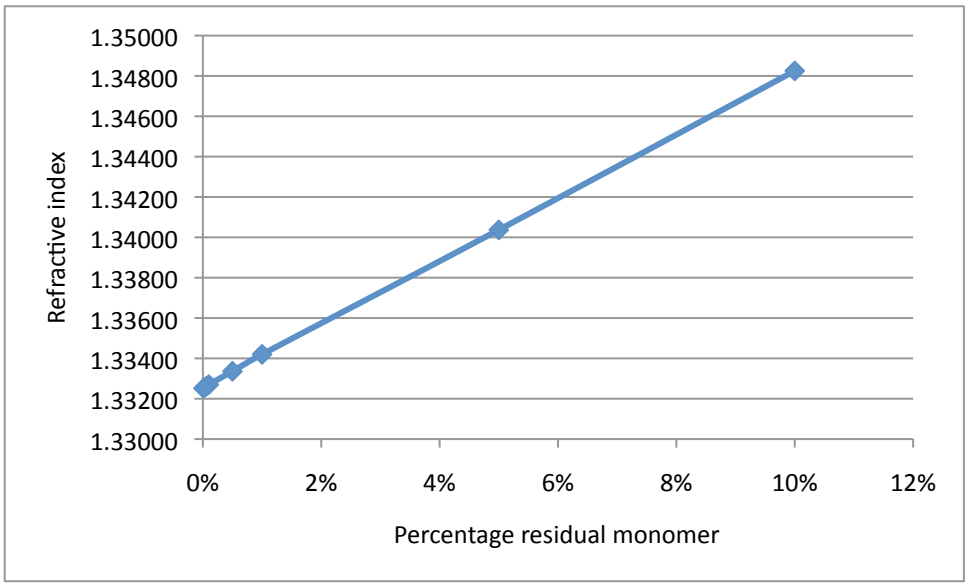


Fig 4.7.1 Residual NaAMPs calibration curve calculated by refractive index

Table 4.7.2 Residual KSPA calibration calculated by refractive index

KSPA conc	RI1	RI2	RI3	Avg
10%	1.34490	1.34490	1.34494	1.34491
5%	1.33890	1.33880	1.33871	1.33880
1%	1.33384	1.33393	1.33383	1.33387
0.50%	1.33316	1.33320	1.33315	1.33317
0.10%	1.33260	1.33267	1.33262	1.33263
0.05%	1.33254	1.33264	1.33259	1.33259
0.01%	1.33250	1.33256	1.33249	1.33252

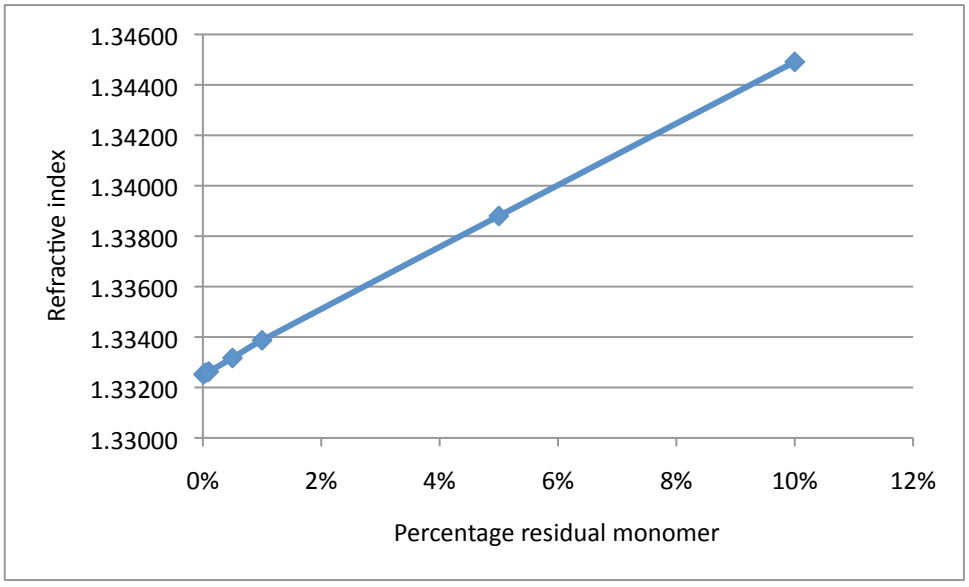


Fig 4.7.2 Residual KSPA calibration curve calculated by refractive index

Table 4.7.3 Mixture calibration for KSPA and NaAMPs calculated by refractive index

Conc	RI 1	RI 2	RI 3	avg
10% mix	1.34656	1.34666	1.34665	1.34662
5% mix	1.33953	1.33956	1.33954	1.33954
1% mix	1.33402	1.33401	1.33398	1.33400
0.5% mix	1.33327	1.33331	1.33328	1.33329
0.1% mix	1.33262	1.33265	1.33267	1.33264
0.05% mix	1.33266	1.33258	1.33256	1.33260
0.01% mix	1.33250	1.33249	1.33256	1.33252

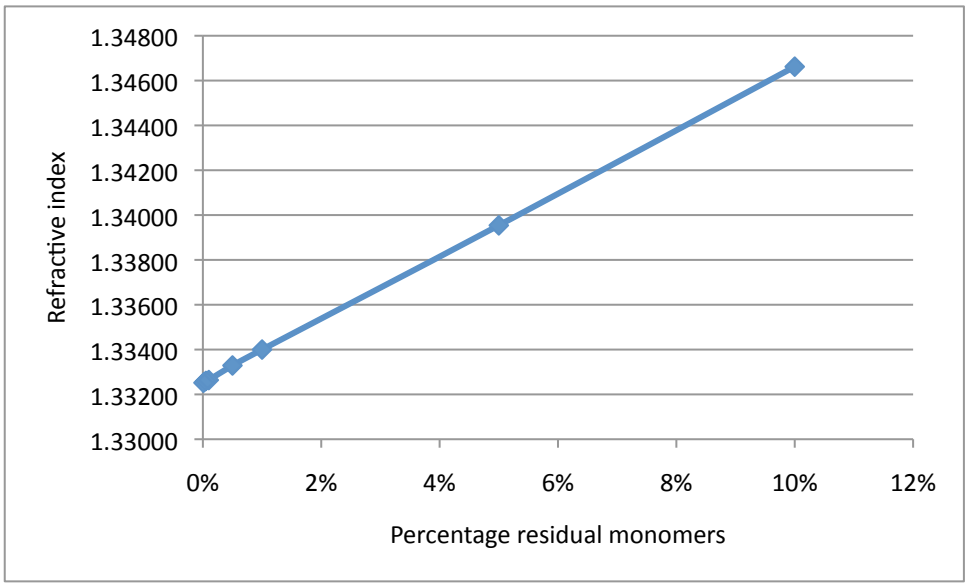


Fig 4.7.3 Residual NaAMPs/KSPA mixture analysed by refractive index

Table 4.7.4 The residual content of gel Rf1 calculated by refractive index

RI minus background	%R KSPA	%R NaAMPs	%R mix
0.00054	0.43	0.34	0.43
0.00055	0.44	0.35	0.35
0.00058	0.47	0.41	0.41

Average residual monomer content from a AA/Oxone initiated gel is 0.4%

Table 4.7.5 the residual content of gel Rf2 calculated by refractive index

RI minus background	**%R KSPA**	**%R NaAMPs**	**%R mix**
0.00039	0.30%	0.24%	0.28%
0.00038	0.30%	0.24%	0.28%
0.00038	0.30%	0.24%	0.28%

Average residual monomer content from a TEMED/KPS initiated gel is 0.27%
KSPA 0.001%

The results show very little residual content of the gels, especially if the structure of the ionic monomers are compared to sodium chloride of which typical physiological saline has a concentration of 0.9%. It is not surprising however that the gel initiated *via* TEMED and KPS demonstrates a lower level of residual monomer content due to the fact that it is perceived to be a much more efficient initiation system in comparison to AA and Oxone.

4.8 Discussion

The aim of the work described in this chapter was to refine the systems explored in the previous to produce systems that could be delivered *via* injection into the nucleus pulposus cavity. This resulted in two gels of similar composition, with different initiator systems and different crosslinking agents. These were denoted Rf1 and Rf2. Ascorbic acid and oxone with PEG575 diacrylate and TEMED and potassium persulfate with PEG1000 dimethacrylate. The incorporation of ascorbic acid and potassium persulfate may seem feasible, but in fact produced a less reliable system.

Methods of storage and stabilisation were explored with respect to monomers, initiators, and stock solutions as a whole. The factors that have the potential to affect storage stability were identified as heat, light and temperature. A cumulative affect of inclusion of certain components together was also discovered. For example, due to the high reactivity of NaAMPs it is not possible to store this component in the presence of a highly reactive initiator such as Oxone or potassium persulfate. Due to ascorbic acid possessing the ability to degrade when in direct light, it was deemed storage would only be suitable for an extended time period, in the dark.

The presence of oxygen within any polymerisation has the potential to cause detrimental effects. These could be either degradation of the initiator, which is particularly apparent in the case of ascorbic acid and potassium persulfate, which was discussed in the introduction. Oxygen also has the capacity to scavenge radicals during the reaction, preventing them from being available to initiate polymerisation. In solutions which were saturated with oxygen and measured *via* the use of an oxygen probe, this was shown to be an obvious detrimental effect. However the complete removal of oxygen from the system is also detrimental to the polymerisation reaction. This is particularly in this application which requires gelation to not commence until after at least 3 minutes. The inclusion of a level of oxygen that allows this is therefore advantageous. The desired result is that preparation and storage in ambient oxygen provide the most reliable reproducible systems.

Storage under refrigeration at approximately 4^{o}C hinders mobility of any radicals that are prematurely produced as a result of the presence of oxygen or just simply

degradation of the initiators. Both systems therefore were discovered to be stable under refrigeration for up to one month.

Injection into a suitable cavity was explored using both a balloon model and excised sheep disc and was deemed to be successful, and perceived not to alter the gelation time or efficacy. This was important especially in a tissue sample due to the presence of biological fluids and/or moisture on surfaces having the potential to dilute the polymerisation system and thus hinder polymerisation. The rationale for the selection of a sheep disc as a tissue model was due to the increasing reports in the literature of the behaviour of the disc being the closest analogue to that of human tissue.

It is important to mention the injection process. The use of different length and gauge of needle has a clear effect on the time taken to polymerise. Usually the greater the needle length and lesser the bore of the needle, less time is taken for a nucleus of gelation and thus complete polymerisation to occur. This could be an accumulative effect of surface area and shear forces.

Addition of a radio-opaque into the monomer system to allow the analogue to be visible throughout its lifetime was desirable. At levels up to 15% histodenz does not seem to detrimentally alter the properties of the gel. Analysis *via* X-ray also suggests that at these levels the implant should be visible. This was confirmed *via* both injection of free gel samples and x-ray of excised disc segments containing injected polymerised gel. However further investigation into the visibility within bone would be advantageous.

Finally the residual monomer content of the material was analysed *via* refractive index and perceived to be at an acceptable level. This can be equated to the concentration of physiological saline ~0.9%, NaAMPs and KSPA are analogous to the salt solution and the residual content of 0.4% or less is highly unlikely to produce a detrimental cell response.

Chapter Five

The Design and Synthesis of a Proteoglycan Analogue: Mechanical Properties

5.1 Introduction

This chapter will illustrate and discuss the rheological behaviour, swell behaviour and osmotic responsiveness of the material compositions discussed in chapters 3 and 4. This chapter in particular, like the previous, is focussed mainly on the properties of a material which will be suitable as an analogue for the natural nucleus pulposus of the intervertebral disc. The chapter follows the order of previous chapters, beginning with the effect of monomer variation, initiator variation, cross-linking agent variation, and water content variation before proceeding onto the swell behaviour and osmotic responsiveness of the compositions deemed the most suitable for the end application.

5.2 Rheological behaviour of different monomer compositions

As mentioned previously, the difficulty with using NaAMPs is its availability only as an aqueous solution and not in a solid form, therefore for this application KSPA is used as a co-monomer to increase the w/w solid content of the gel. The preparation of samples for rheology is discussed in section 2.2.4.

Figure 5.2.1 demonstrates a comparison between the viscous, elastic, and complex modulus of a NaAMPs polymer and an NaAMPs:KSPA copolymer. These are based on Gel 6 in table 3.2.3.1.2 for the NaAMPs gel and Gel Rf1 table 4.2.1 with 2% crosslinking agents. Gels are prepared within a Petri dish to allow a uniform structure and thickness of approximately 2mm. Discs of 20mm diameter are cut out before being tested on the rheometer through a frequency sweep of 5-25Hz at 37^{o}C to mimic body temperature. The results represented in the figure are taken at 10Hz. For all mechanical data reported in this and future chapters, where error bars are present a minimum of five samples were tested and the statistical average used to calculate error.

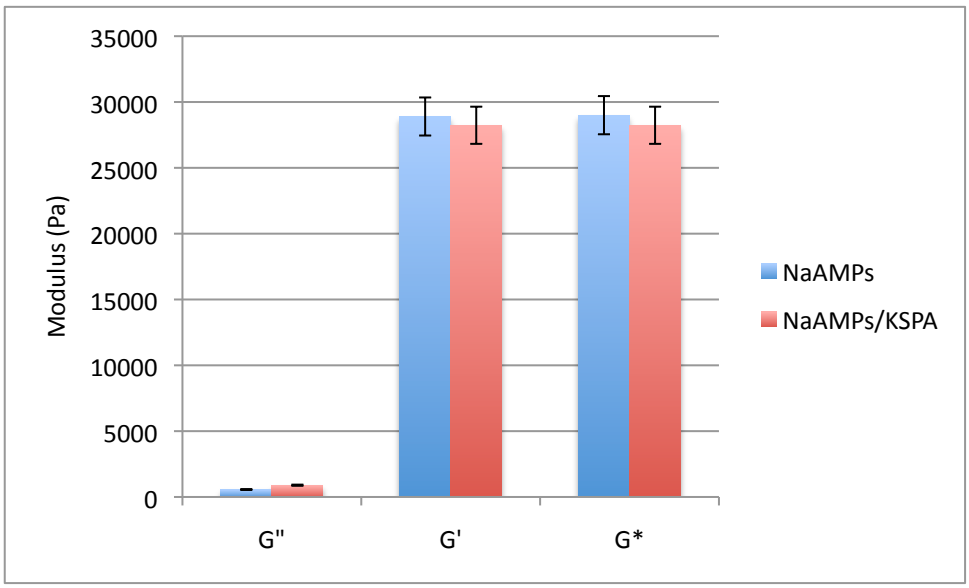


Fig 5.2.1 A comparison of viscous, elastic, and complex modulii recorded at ~10Hz for an NaAMPs based polymer vs an NaAMPs:KSPA copolymer of similar solid:water polymerisation ratios ~1:1, crosslinking agent PEG400DA at 2% w/w, initiated via AA and Oxone 0.1M

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It can be seen that there is very little difference in the mechanical properties of the material although NaAMPs tends polymerise quicker and produces a more reproducible network than its copolymer with KSPA.

Varying the monomer ratio has an effect on the resultant properties of the gels as is demonstrated in figure 5.2.2.1. These compositions are based on those detailed in table 3.2.3.2.1. The materials are prepared in similar manner as previously.

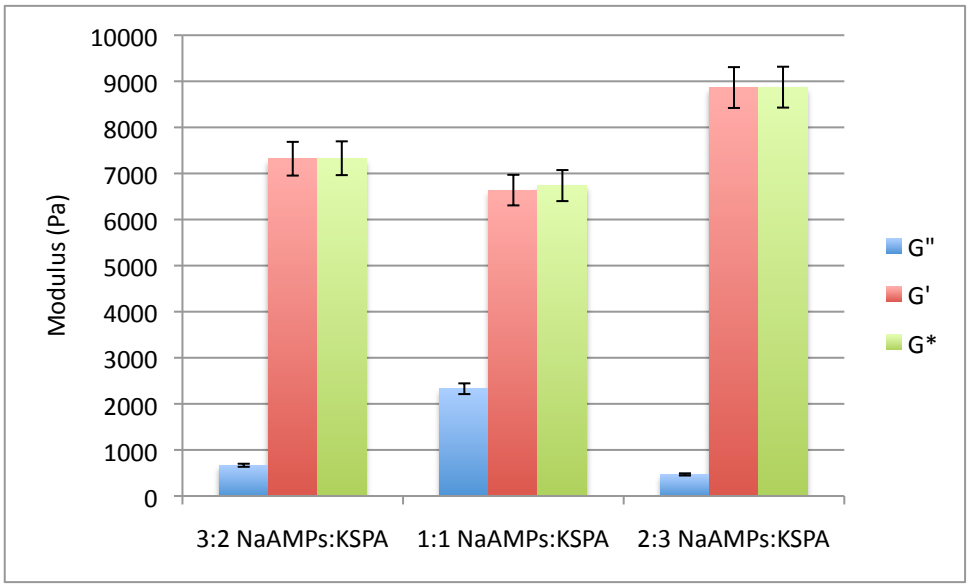


Fig 5.2.2.1 A comparison of the viscous, elastic, and complex modulii recorded at ~10Hz of three different ratios of NaAMPs:KSPA, 3:2, 1:1, and 2:3 respectively. Each composition consisted of similar water and crosslinking agent levels (1%), initators used were AA and Oxone 0.1M

The ration between the viscous and elastic modulus can be represented more clearly by the use of tan delta. This is represented in figure 5.2.2.2.

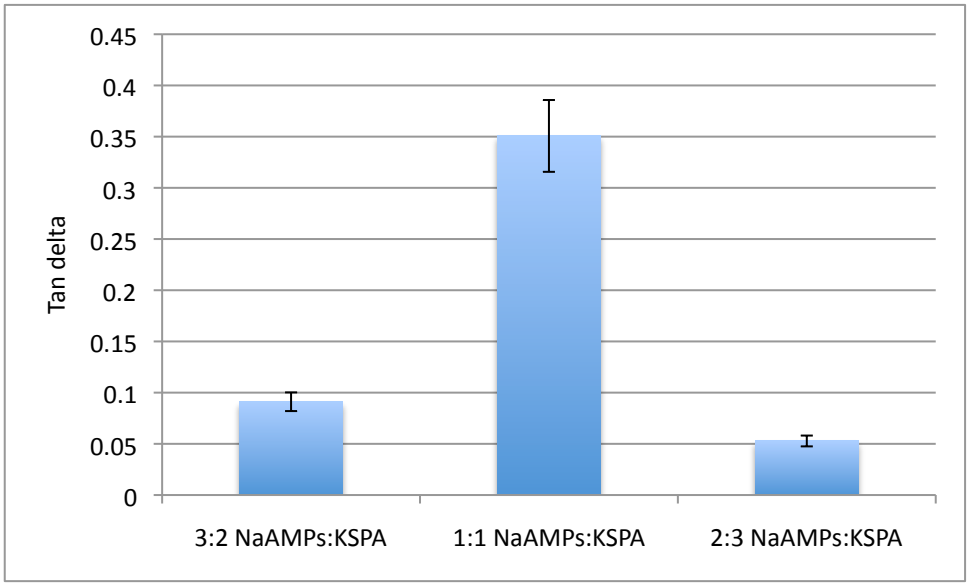


Fig 5.2.2.2 The effect of increasing KSPA content on Tan delta

The chart above demonstrates the effect of monomer variation, of particular interest is the increased mechanical strength of the gel containing more KSPA. Although KSPA is reluctant to polymerise, it appears that when it is used at a higher percentage it favours crosslinking with the PEG diacrylate. This can be explained by similar structures reacting more efficiently with each other, acrylate to diacrylate for instance. Gel samples were examined under a Dialux 20 Leitz microscope with darkfield illumination at x125 magnification, no particulates were visible suggesting that all the KSPA participated in the polymerisation therefore the increased mechanical stiffness was not due to particulates.

5.3 Initiator variation

5.3.1 The variation of ascorbic acid and oxone conentration

Whilst increasing the initiator concentration may increase the efficacy of polymerisation, it was important to assess the effect this has on the rheological properties of the gels, in particular the elastic modulus. Compositions were investigated based on those detailed in table 3.2.3.2.1, varying the ascorbic acid and oxone concentrations at 0.1M and 0.15M

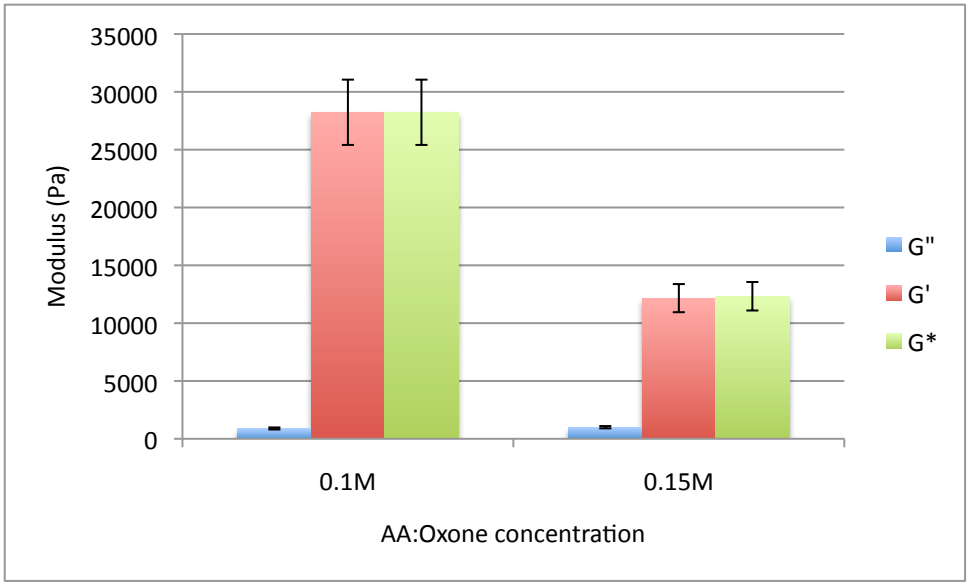


Fig 5.3.1 The effect of increasing the AA Oxone concentration on the rheological properties of an NaAMPs KSPA copolymer recorded at ~10Hz

It is clearly demonstrated that the use of a more concentrated initiator produces a gel with much less mechanical stiffness than a less concentrated initiator. This corresponds to both gelation and thus complete polymerisation occurring at a much greater rate. The chains that are formed are much shorter than those formed from the weaker initiator producing a weaker network structure and thus exhibiting a lesser degree of mechanical stiffness. It is important to note that although weaker, the structural response of the copolymer due to its monomer composition is similar, in that the ratio of viscous to elastic modulus changes minimally between the two systems. Therefore a 0.1M initiator system is not only more efficient due to the

degree of control it allows over the time taken for gelation to begin, but produces a stronger more intricate network.

5.3.2 The variation of TEMED and potassium persulfate concentration

The variation of the TEMED and potassium persulfate initiator systems requires a greater degree of investigation to fully understand the effect it has on the mechanical properties. As discussed in chapter 4, observation of polymerisation time and efficacy indicated that a compromise of 0.18M potassium persulfate and 0.4M TEMED provided the most efficient system, although polymerisation appeared to have greater dependency on the potassium persulfate concentration. Initially one concentration was kept constant, and then both were varied. The compositions were based on those detailed in table 3.5.4.1, however incorporating 1% of the crosslinking agent PEG 1000 dimethacrylate.

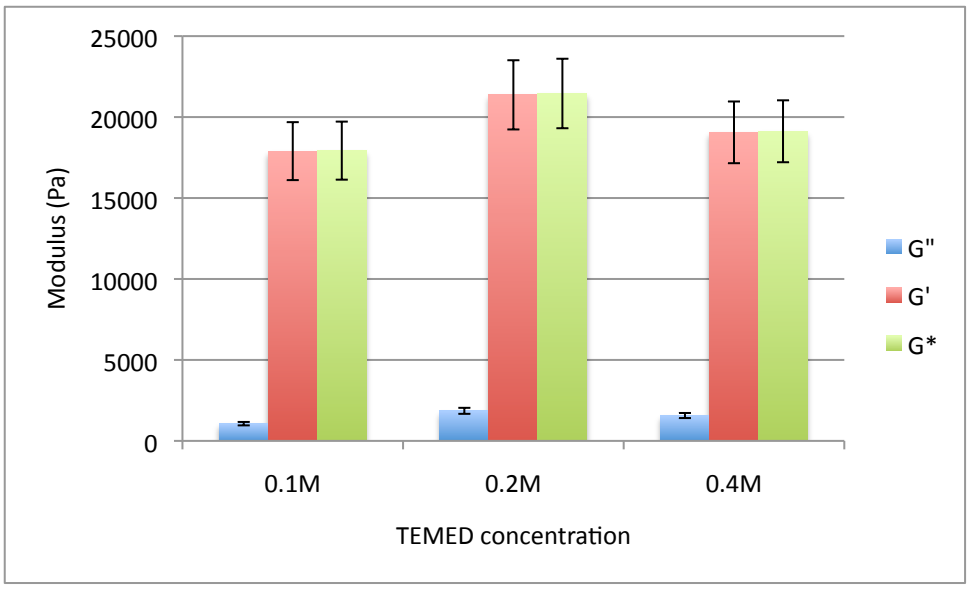


Fig 5.3.2.1 The effect of varying TEMED initiator concentration whilst keeping KPS constant at 0.18M, on viscous, elastic, and complex modulii recorded at ~10Hz

As with altering the concentration of the ascorbic acid and oxone redox pair, when altering the concentrations of TEMED and potassium persulfate, the ratio between elastic and viscous modulii differs very little. Essentially the gel properties with relation to their elastic behaviour remains similar, even if the gel produced is less stiff. This suggests complete gelation in all cases. Comparing figure 5.3.2.1 to figure

5.3.2.2 it becomes apparent that the initation and subsequent polymerisation has a greater dependence on the concentration of potassium persulfate as oppose to TEMED. As demonstrated above the alteration in elastic stiffness is small circa 2500Pa at most, in comparison to lowering the potassium persulfate concentration producing a gel nearly 10000Pa weaker.

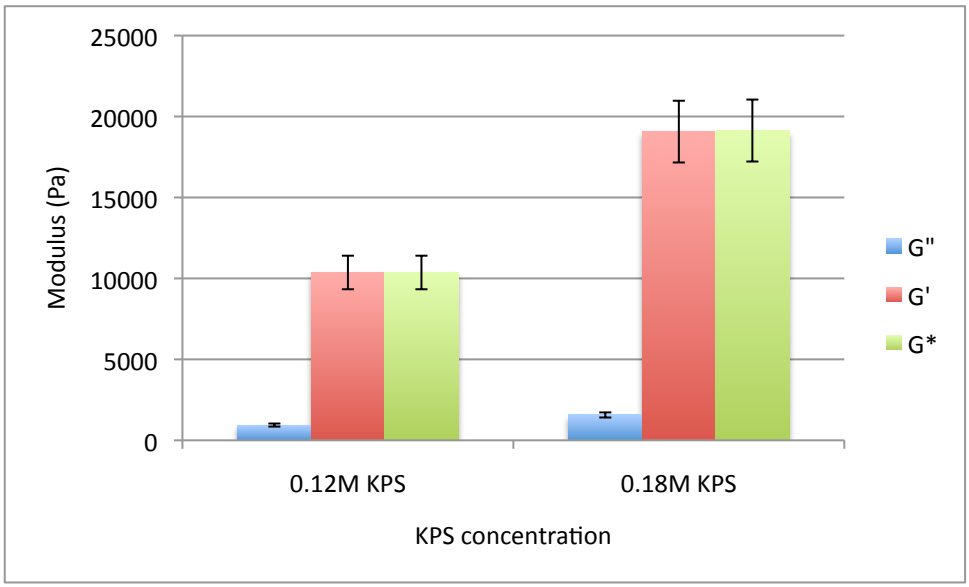


Fig 5.3.2.2 The effect of varying KPS initiator concentration whilst keeping TEMED constant at 0.4M, on viscous, elastic, and complex modulii recorded at ~10Hz

It was necessary to investigate the accumulative effects of altering the concentration of both of the initiators to discover the most effective combination that was both stable to the requirements discussed in the previous chapter, and still able to produce a mechanically suitable tissue analogue. Figure 5.3.2.3 below illustrates these investigations.

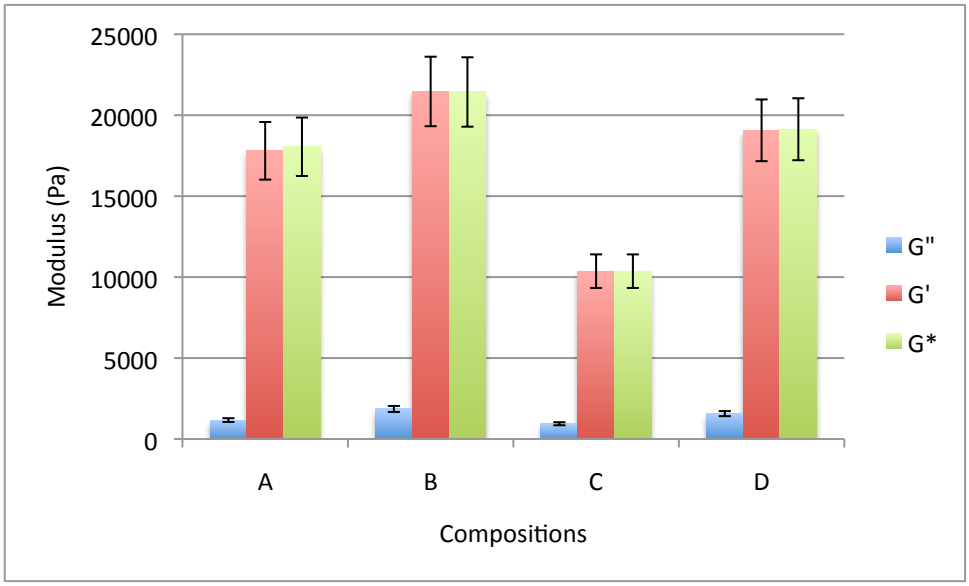


Fig 5.3.2.3 The effect of varying both initiator concentrations where A= 0.1M TEMED:0.18M KPS, B= 0.2M TEMED 0.18M KPS, C= 0.4M TEMED, 0.12M KPS, and D=0.4M TEMED and 0.18M KPS, recorded at ~ 10Hz

Potassium persulfate is again confirmed to be the dominating initiator, which is demonstrated by composition C, whereas lowering the TEMED concentration still produces a mechanically strong gel. These results support the decision that 0.4M TEMED and 0.18M potassium persulfate provide a suitable system for the tissue analogue.

To illustrate that the mechanical properties of each system are very similar fig 5.3.2.4 shows a comparison of the AA/Oxone initiated system based on the compositions Rf1 and Rf2 detailed in table 4.2.1 with 2% of their respective crosslinking agents PEG575 diacrylate and PEG1000 dimethacrylate.

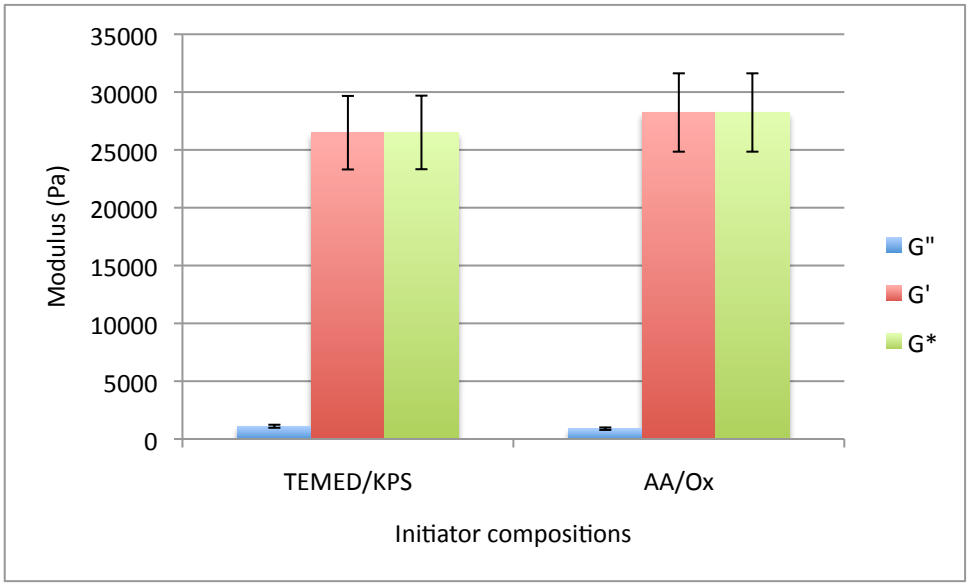


Fig 5.3.2.4 A comparison between TEMED/KPS initiated NaAMPS:KSPA copolymer system and an AA/Oxone initated system recorded at ~ 10Hz

5.4 Water content variation

The presence of water within a hydrogel is very important. This water may be structurally bound to the polymer or it may just behave in bulk. Figure 5.4.1 shows the effect on polymerising a similar monomer composition gel based on Rf2, the TEMED potassium persulfate initated gel with 1% crosslinking agent PEG 1000 dimethacrylate, with 40% and 60% water. The gel containing 60% water is noticeably less elastic, this may be due in part to water acting both as a spacing device, thus increasing the space between crosslinks, and as a plasticiser.

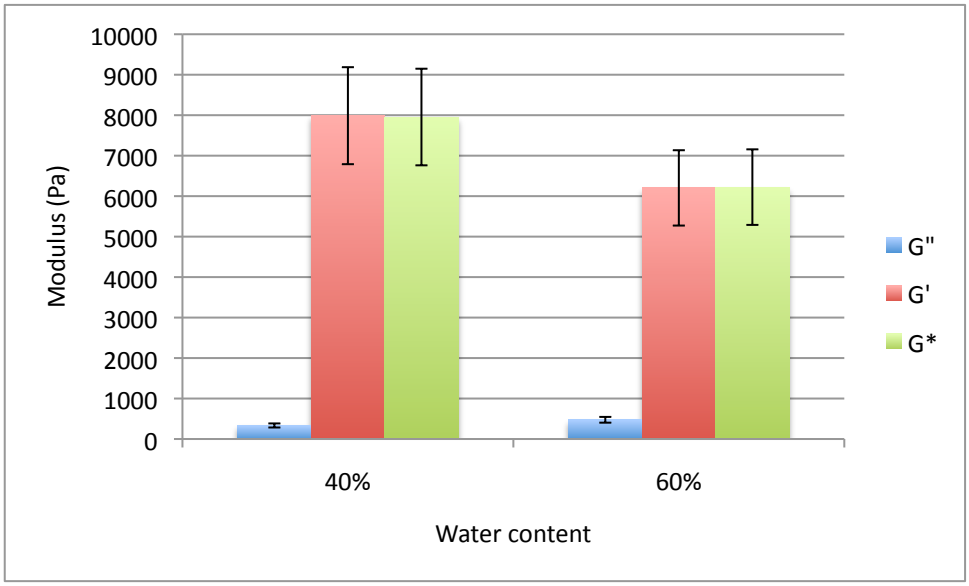


Fig 5.4.1 The effect of reducing the water component of the gel composition, recorded at ~ 10Hz

The chart above focuses on the water that is polymerised as part of the gel structure, however the gel is capable of imbibing large amounts of liquid (water or biological fluids), the procedure for preparing swollen discs of gels is detailed in section 2.3 Whilst the gel tested would never be expected to swell to a similar capacity in its application as a tissue analogue, it was necessary to test the maintenance of the mechanical properties once swollen. The gel featured in this chart was of the composition Rf2 with 1% crosslinking agent PEG1000 dimethacrylate. The swollen sample was prepared *via* swelling a whole petri dish size sample in phosphate buffered saline for 72 hours. A disc of diameter 20mm was cut out to test after this time, and tested in the same manner as a similar unswollen sample which was polymerised alongside the swollen sample. Also demonstrated in the chart is the very slightly increased mechanical strength of a gel, of the same unswollen composition, whose components were purged of oxygen prior to polymerisation. It can be seen that whilst oxygen clearly does have a role in hindering the production of a strong network, the difference is not of such significance ~500Pa to necessitate polymerisation permanently in the absence of oxygen.

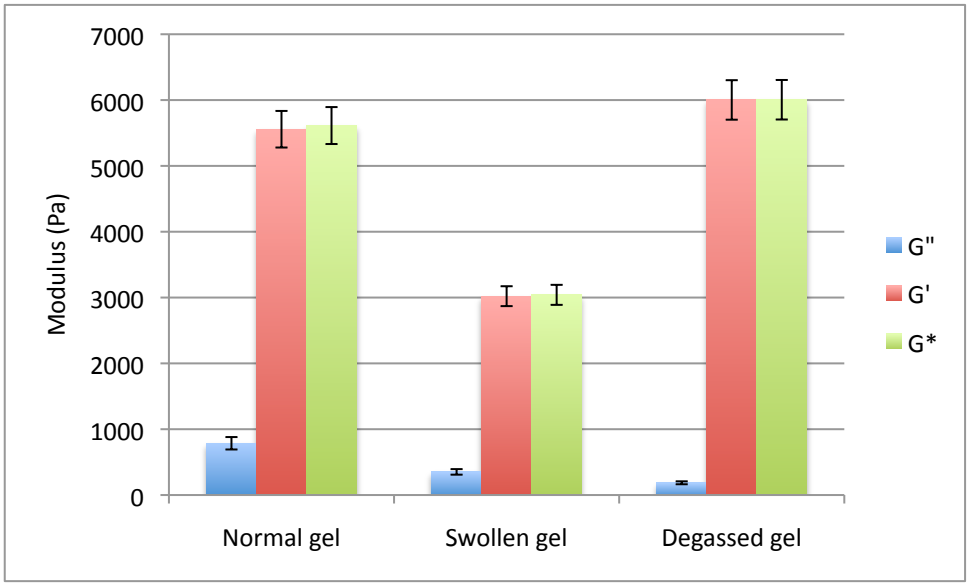


Fig 5.4.2 A comparison of the viscous, elastic and complex modulii of a normal unswollen gel, a similar swolled gel, and an unswollen gel which was polymerised in the absence of ambient oxygen. Recorded at ~ 10Hz

5.5 Crosslinker variations

The three crosslinking agents under investigation were Nelfilcon (functionalised PVA macromer, PEGDA at either n=400 or 575, and PEG1000DMA. As mentioned in section 3.3, the use of nelfilcon creates the problem of a rapid gelation point occurring but polymerisation can often be incomplete, this is particularly apparent when nelfilcon is used at a level higher than 0.5%, this is demonstrated in fig 5.5.1. The monomer and water composition of each gel was kept constant. Although 0.5% nelfilcon produces a less elastic gel than 0.5% PEG400DA, a complete network is formed in both cases and therefore the overall mechanical behaviour is similar, demonstrated by the similarity in the tan delta curves for both. However for the inclusion of 1% of nelfilcon, the network formed was obviously incomplete and as the frequency of oscillation is increased, the elastic behaviour of the gel diminishes as demonstrated by the plummeting tan delta curve. This reinforces the argument for not selecting Nelfilcon as a suitable macromer-based crosslinking agent for the tissue analogue application. The compositions in this analysis were based on those described in table 3.5.1.

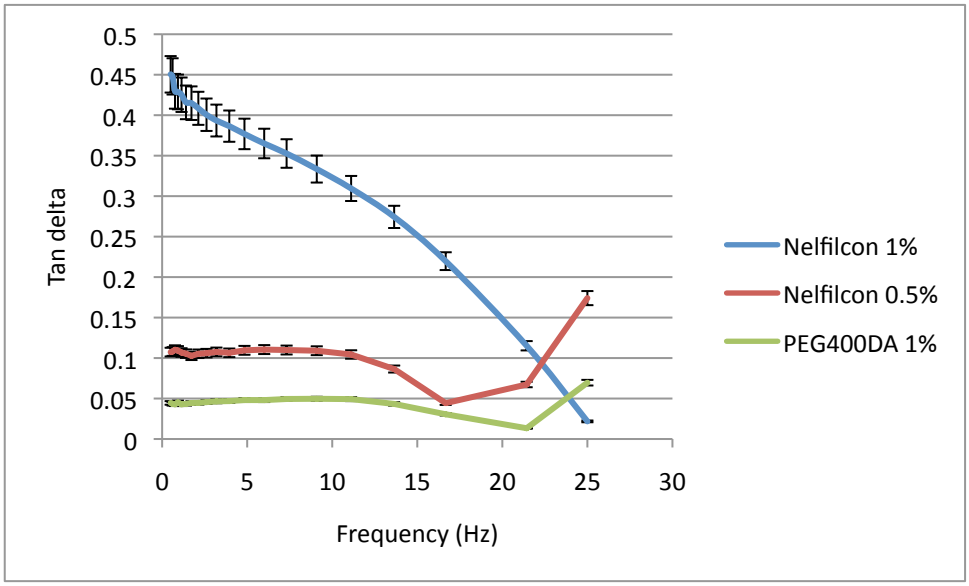


Fig 5.5.1 Tan delta demonstrating the effect of inclusion of an increased percentage of Nelfilcon macromer as a crosslinking agent.

The properties of both gel systems, the TEMED/KPS PEG1000 dimethacrylate, and AA/Oxone PEG575 diacrylate, can be manipulated less by altering the crosslink ratio. With respect to PEG575 diacrylate, this is represented in fig 5.5.2 below, and representative of the gels described in table 4.2.1.

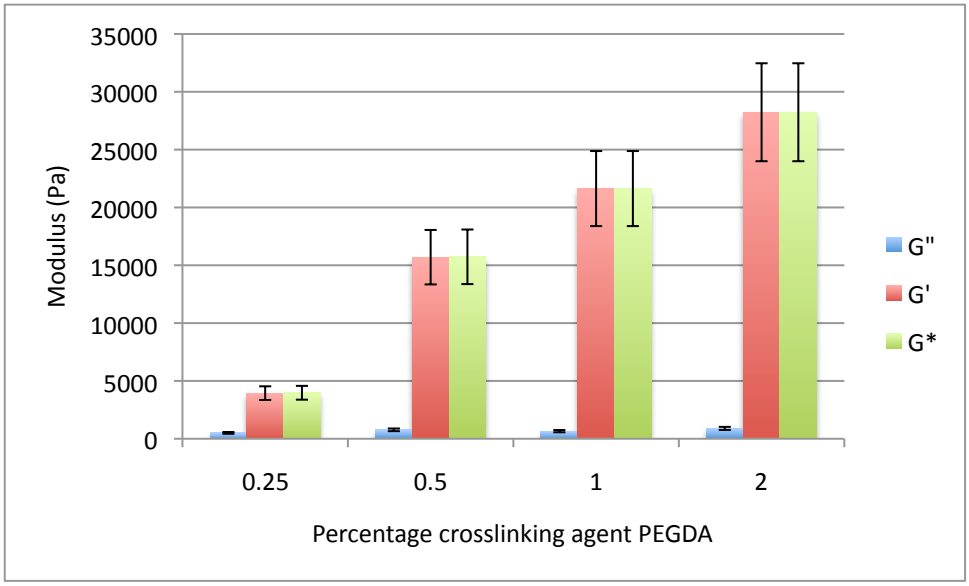


Fig 5.5.2 The viscous, elastic and complex modulii of an NaAMPs/KSPA, AA/Oxone initiated copolymer with increasing levels of PEG575DA crosslinking agent, recorded at ~10Hz

It is possible to incorporate quite a low level of crosslinking agent using PEG575 diacrylate and still produce an intact structure. In contrast to the use of PEG dimethacrylate, which has a molecular weight of 1000. Therefore it is assumed that per percentage of each, there would be more crosslinks formed when using PEG diacrylate. This is demonstrated when comparing fig 5.5.2 and fig 5.5.4 below. Each core gel composition is essentially very similar, so the differences in elastic modulus at 1% between each can be attributed to the crosslinking agent used with PEG diacrylate producing 21000Pa and PEG dimethacrylate 7500Pa respectively. Tan delta in fig 5.5.3 in comparison to Fig 5.5.5 illustrates the use of PEG diacrylate has less of an effect on gel properties between 0.5% and 1% crosslinking agent when using PEG diacrylate. Neither composition is more advantageous than the other, both can be structurally manipulated to give a desired end product.

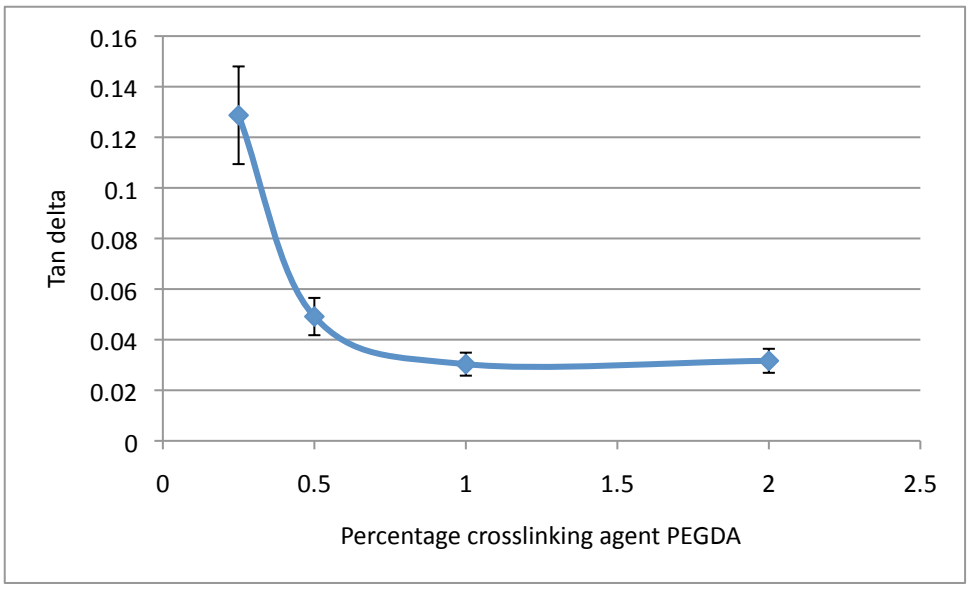


Fig.5.5.3 Tan delta, the ratio of viscous to elastic modulus for the increase of crosslinking agent PEGDA.

As more crosslinks are present within the gel structure, the material has a much greater elastic modulus, its viscous behaviour diminishes in comparison to lower levels and a considerably stiffer gel is the result. The ratio of viscous to elastic modulus is clearer to see in fig 5.5.5, with a rapid decrease in tan delta between 0.5% and 1% crosslinker.

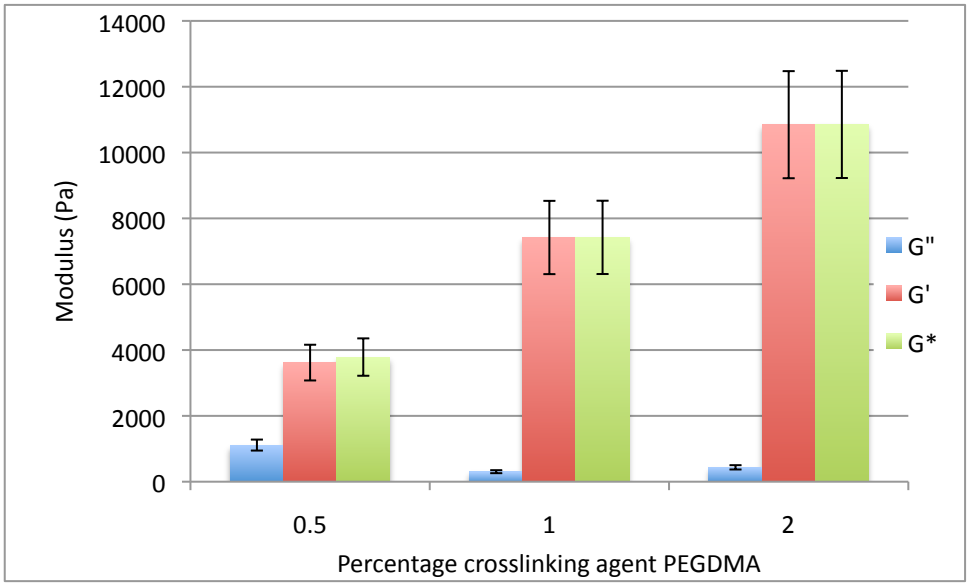


Fig 5.5.4 The viscous, elastic and complex modulii of an NaAMPs/KSPA, TEMED/KPS initiated copolymer with increasing levels of PEG1000DMA crosslinking agent, recorded at ~10Hz

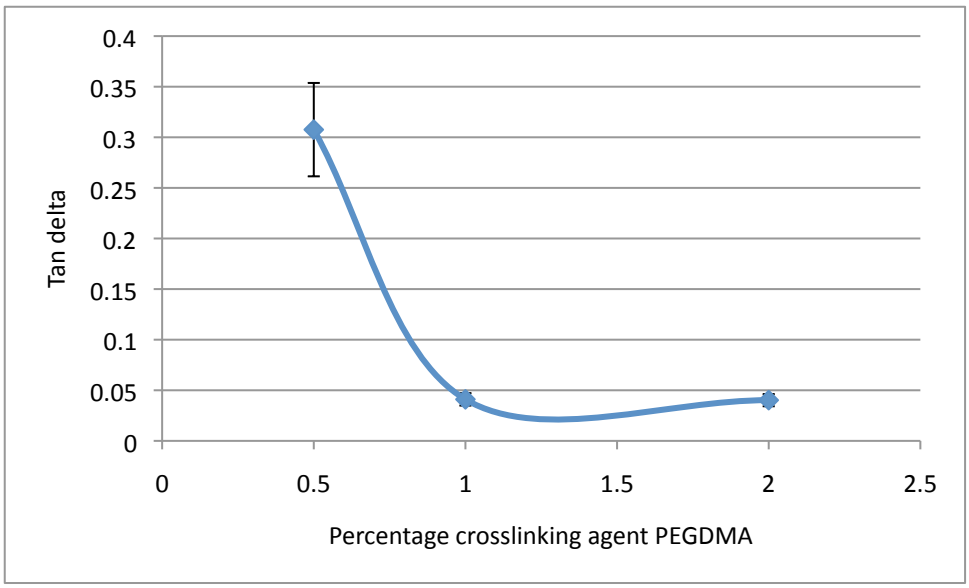


Fig.5.5.5 Tan delta, the ratio of viscous to elastic modulus for the increase of crosslinking agent PEGDA.

5.6 The incorporation of DPA as a stabiliser

The use of dipicolinic acid to stabilise KPS was discussed in section 4.3, the compositions are represented in table 4.3.1. Although it was deemed to have a detrimental effect on the polymerisation, the effect on the mechanical properties was investigated as demonstrated in fig 5.6.1 below.

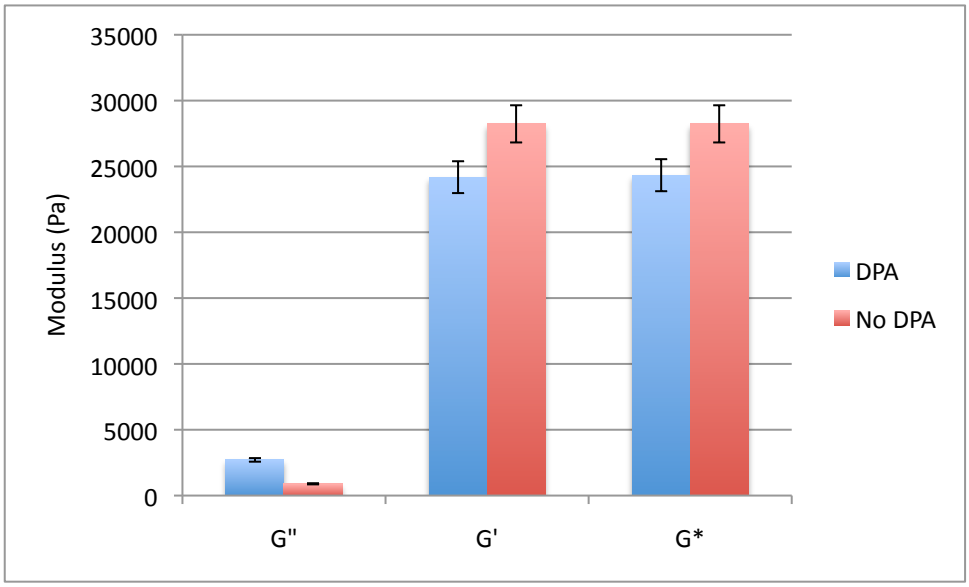


Fig.5.6.1 The incorporation of DPA into the TEMED/KPS system as a stabiliser, recorded at ~10Hz

The incorporation of DPA produces a gel with increased viscous and decreased elastic strength, this is probably due to the DPA interfering with the process of polymerisation. This was reported *via* the cessation of any gelation once DPA had been stored within a stock solution for any amount of time.

5.7 Incorporation of histodenz

The procedure for incorporating Histodenz into gels was introduced in section 4.6 along with the subsequent x –ray visibility of the gels. This section will focus on the effect of the inclusion of Histodenz on the mechanical properties of the two gels whose components are illustrated in table 4.6.1.

Adding Histodenz to the gel compositions does have a minor affect on their mechanical behaviour. This is represented in Figure 5.7.1 and 5.7.2, below. As Histodenz concentration increases, as a general trend, so does the elasticity of the material in contrast to the gel that contained no histodenz. This is due to the increase in solid content of the gel but is not detrimental to the resultant properties. Tan delta in Fig 5.7.3 does show an increase due to the slight increase in viscous modulus in comparison to the smaller increase in elastic modulus. In a gel of similar composition in the absence of histodenz, for instance Rf1, recording at a similar frequency tan delta would be expected to remain constant.

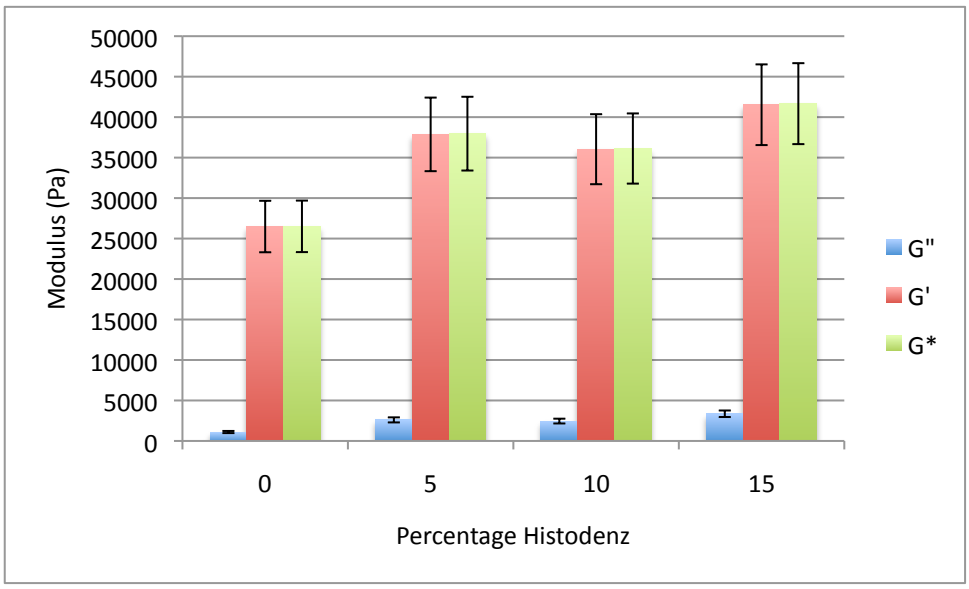


Fig 5.7.1 The effect of the inclusion on increasing levels of Histodenz on the viscous, elastic, and complex modulii of an NaAMPs:KSPA, AA/Oxone initiated gel with 2% PEG575DA crosslinking agent

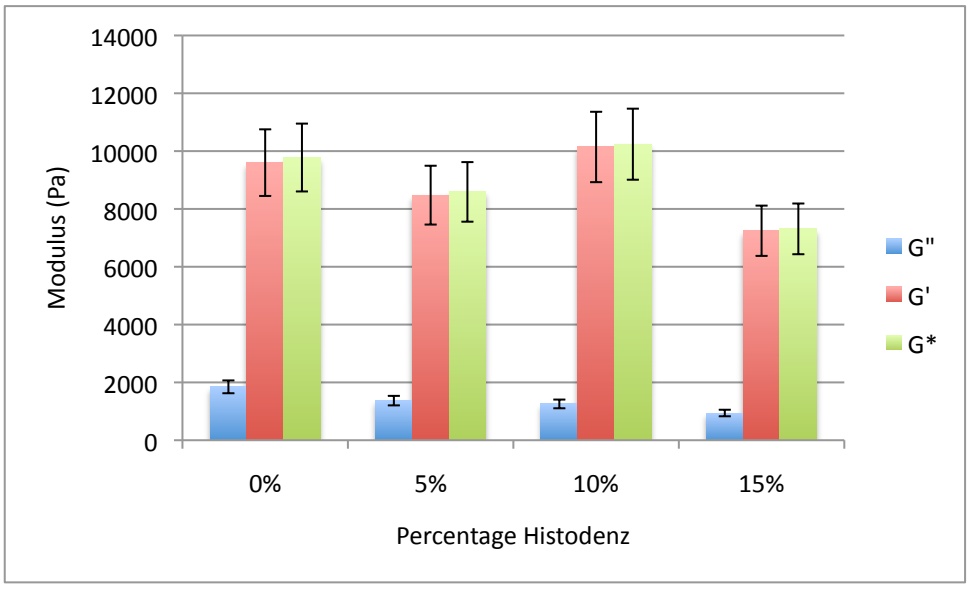


Fig 5.7.2 The effect of the inclusion on increasing levels of Histodenz on the viscous, elastic, and complex modulii of an NaAMPs:KSPA, TEMED/KPS initiated gel with 2% PEG1000DMA crosslinking agent

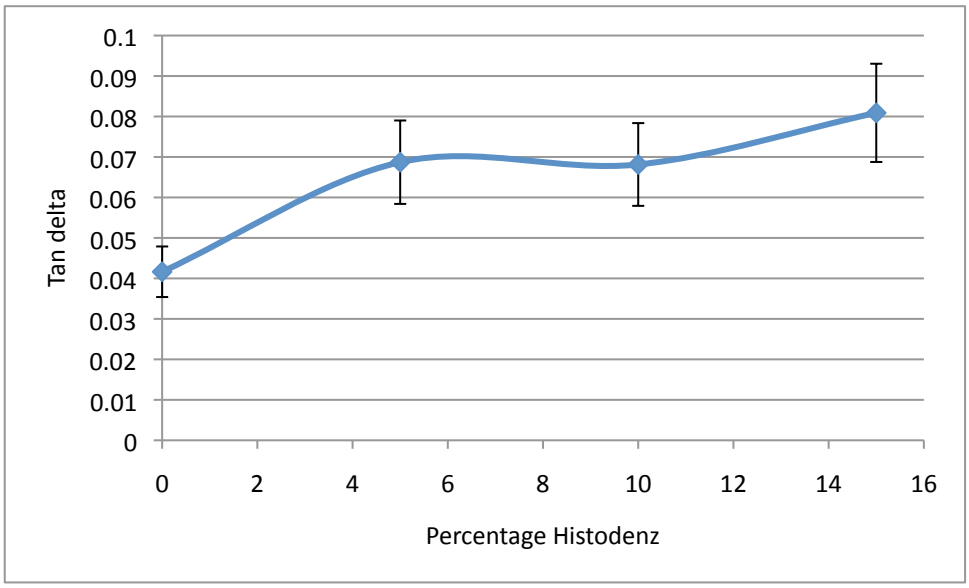


Fig 5.7.3 Tan delta for the increase in Histodenz on NaAMPs/KSPA, TEMED/KPS initiated gel with 2% PEG1000DMA

In the figure above it can clearly be seen that histodenz has had a slight interference in the polymer network formation. This could also be attributed to some degree to the increase in solid content of the gel.

5.8 The swell and subsequent compression behaviour in varying osmotic media

To assess the mechanical behavior of typical GAG-analogue hydrogels under different osmotic pressures various formulations were synthesized and prepared for compression monitoring as previously described in section 2.2.3. The two hydrogels were compressed to 50% and all remained intact after testing. The samples were swollen to different extents in response to the osmolarity of the solution displaying the osmotic driving potential of the sulfonated hydrogels. The samples displayed differing compression ratios, which is a function of their swell in response to the osmolarity of the solution.

This differential swelling property indicates that the gels can respond to osmotic gradients, in a similar manner to the natural tissues. The osmotic driving potential of the synthesised hydrogels can be manipulated *via* alteration of its fixed charge density, this is affected by controlling the concentration of the sulfonate-containing monomer. Figure 5.8.1 is based on gel Rf1 (table 4.2.1), and shows the development of compression modulus as a function of time swelled in a 300mOsm PEG solution. Figure 5.8.2 is based on gel Rf2 (table 4.2.1) and illustrates the osmotic responsiveness to solutions of different osmolarities, after 3 days.

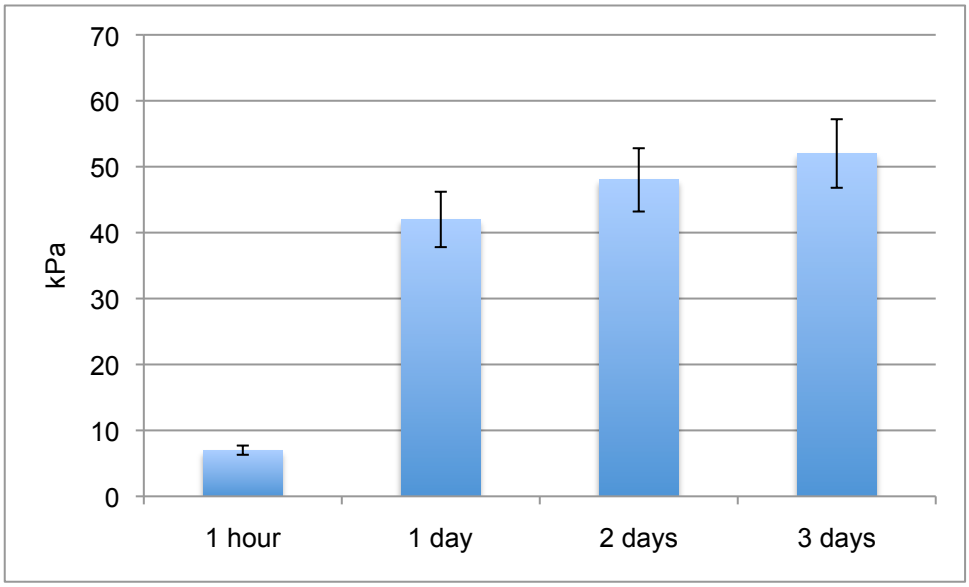


Fig 5.8.1 The compression modulus of an NaAMPs/KSPA AA/Oxone initiated gel with 1% PEG575DA, tested over a range of time

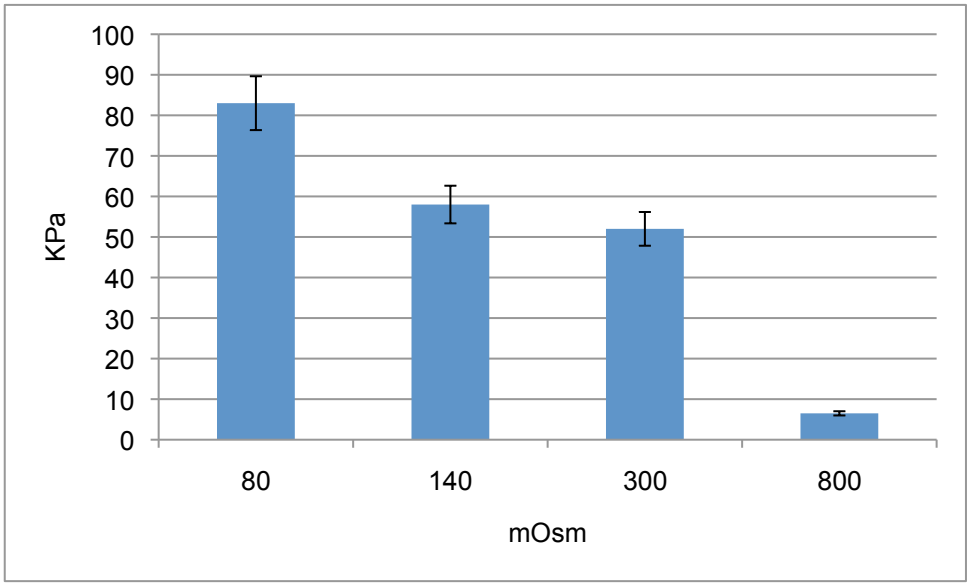


Fig 5.8.2 The compression behaviour of an NaAMPS/KSPA TEMED/KPS gel with 1% crosslinking agent PEG1000DMA swollen in varying osmotic media

These results are pleasing as they demonstrate the ability of the gels to respond osmotically in different media without any detrimental effects. In particular dehydration and/or sample collapse. This is advantageous in suggesting that the material will be able to restore a degree of disc height when *in situ.*

5.9 Dynamic mechanical analysis of excised sheep disc in three different states: intact nucleus, nucleus removed, and containing injected gel

The storage stiffness of excised sheep disc segments was measured *via* DMA at the University of Birmingham at the permission of Dr Duncan Shephard. Tests were conducted with the disc still intact, the nucleus removed, and after the nucleus has been replaced with a gel comprising of gel Rf1 with 1% crosslinking agend PEG 575 diacrylate. Samples were prepared as dual pre-gel solutions as detailed in section 2.2, and upon injection of the gel disc segments were left for 30 minutes to ensure complete gelation had occurred.

Dynamic mechanical analysis determines the dynamic viscoelastic properties of materials or components as a function of a wide range of test conditions. DMA application applies a sinusoidal excitation to the test specimen, collects timed data of force and displacement and provides viscoelastic analysis of the data based on Fourier

analysis techniques. To begin the test, the system ramps to a specified load hold level (-0.5) and records the current displacement feedback. This reading is now used as a relative zero position for all subsequent commands and displacement feedback. Once the dynamic cycling has begun the specimen is precycled for a user specified time (in secs) (for this test 3 precycles were conducted). This allows for amplitude control and specimen stabilisation to occur prior to data being taken. The WinTest DMA software uses a Fourier analysis technique to analyze the timed data of a reference and measured channel (in this case the reference is loading and measured displacement). The Fourier analysis technique models a measured signal as a series of sine functions of varying amplitude and phase.

The following parameters are calculated

- Dynamic stiffness K*
- Phase between the reference channel and feedback- Delta
- Storage stiffness K'
- Loss stiffness K"
- Damping C
- Hysteresis energy
- Transmissibility- Tr

The DMA testing machine used was BOSE Endura TEC ELF 3200. It applies a mean compressive load of 40N with a dynamic amplitude of 4 and the dwell time set as 1 minute. Frequency is oscillated from 0.1Hz to 35Hz. Eight disc segments were mounted in acrylic cold cure dental cement and surrounded by Ringers solution to maintain hydration. Figure 5.9.1. shows a picture of the testing rig with a disc under load.

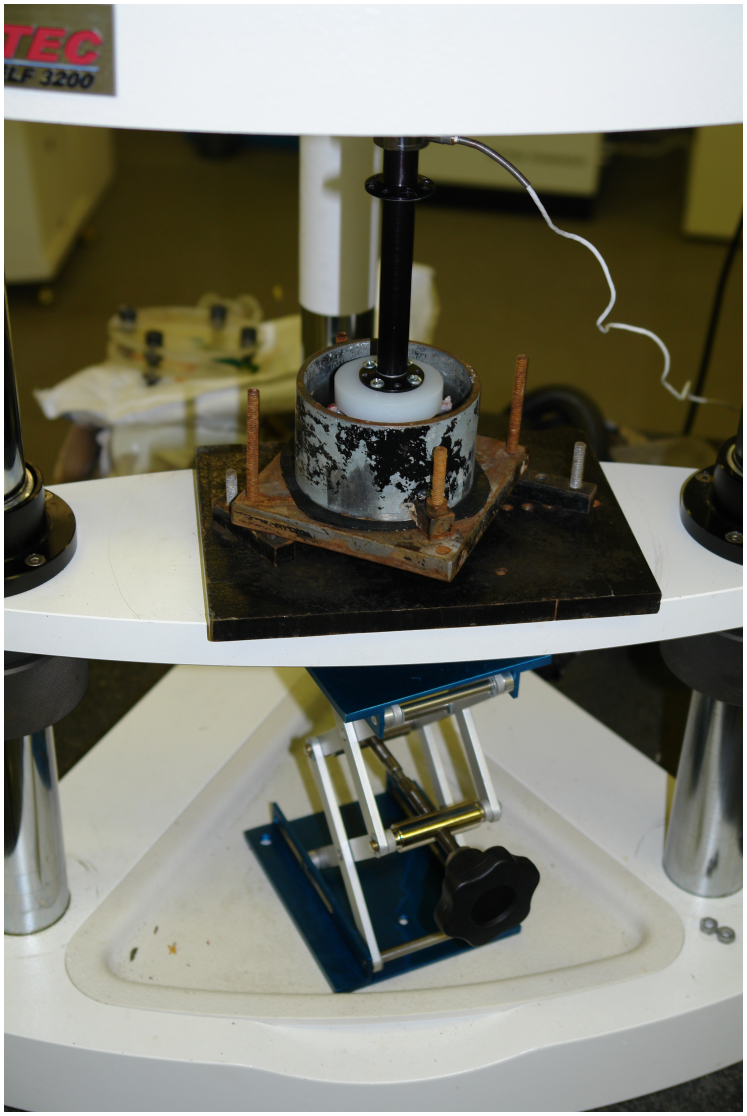


Fig 5.9.1 DMA testing kit with disc under load

The results are demonstrated in fig 5.9.2 below using an average from the eight results.

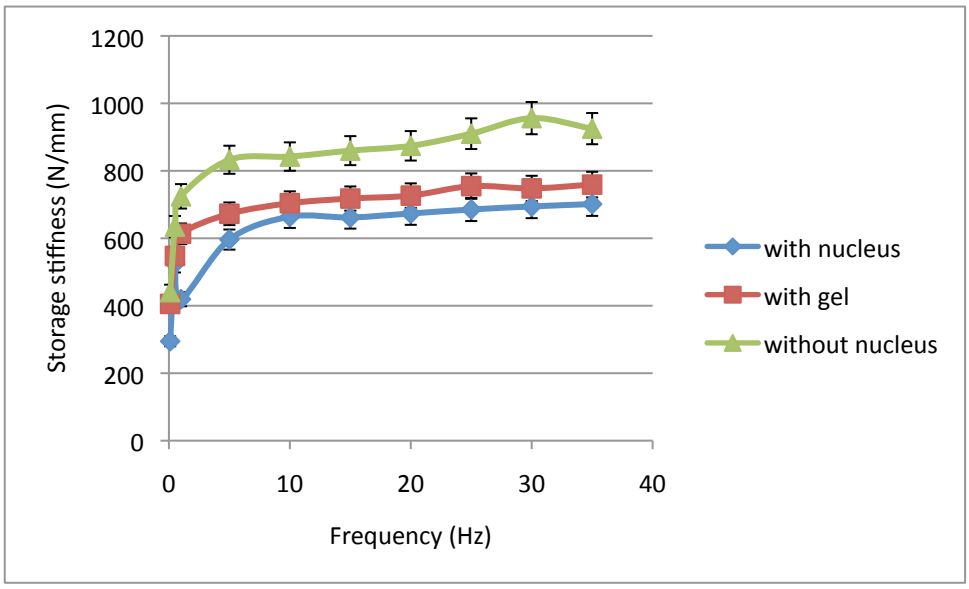


Fig 5.9.2. A comparison of the storage stiffness with and without the nucleus pulposus and with the gel to demonstrate the restoration of a degree of elasticity

As expected removal of the nucleus pulposus results in a stiffer disc. This can be likened to the removal of a cushioning sponge between two plates. When this is removed there is nothing to absorb and shock or spread the load, which is essentially the role of the nucleus pulposus. Upon refilling of the cavity with the analogue gel at least two thirds of the properties of the natural nucleus pulposus were restored. This proves that in theory, that as the gel has exhibited this response in an in-house testing model, it does have the ability to restore disc height.

5.10 Discussion

The aim of this chapter was to illustrate the effects that the composition manipulations carried out in the previous two chapters have on mechanical properties. It has been demonstrated that monomer composition has the ability to alter the mechanical strength, in particular with the use of similar reagents for example PEG diacrylate and KSPA. Here the combination of like for like acrylate has the potential to produce a much more efficiently crosslinked network structure as is illustrated by the increase in stiffness. This response to manipulation of monomer composition, is however negligible in comparison to the effect observed later on, on the variation of crosslinking agent content.

The concentration of initiators has a considerable effect on the structure of the gel. It has been reported that a higher concentration of ascorbic acid and Oxone produces a gel with much shorter chain lengths due to the rapid polymerisation which occurs. This suggests that initiators at a concentration of 0.1M are the most suited for this application, in comparison to a composition of 0.15M which coupled with its faster polymerisation times, produced a brittle gel structure. With respect to TEMED and potassium persulfate, the initiators behave in a more synergistic manner, however appearing considerably more dependant on the potassium persulfate concentration. The best match was found to be TEMED at 0.4 M and potassium persulfate at 0.18M.

The water content of resultant gels also has a clearly defined effect on their mechanical properties, with an increase in water content of 20% decreasing mechanical strength by almost a third. This reiterates the behaviour of water both as a spacing device and a plasticiser. Clearly here reduction in stiffness in response to the increase in water content suggests a behaviour in bulk, as oppose to participation in the structure of the gel. Gels which were swollen in water still maintain their structure during mechanical testing, however exhibit a much lower modulus of elasticity, as would be expected.

Varying the crosslink ratio between 0.25% and 2% for PEG diacrylate and 0.5 and 2% for PEG dimethacrylate in compositions Rf1 and Rf2 respectively achieved gels

with a modulus of elasticity ranging between 3kPa and 28kPa. This dictates that achieving properties similar to that of the natural nucleus pulposus which is reported to have values between 7kPa and 20kPa[26] perfectly achievable. The manipulation of crosslink ratio was by far the most influential method of altering the resultant properties of the gels.

The effect of stabiliser, although deemed unsuitable prior to mechanical testing, reinforces the decision that they are unsuitable. This is demonstrated by the increase in viscous modulii of these gels in response to ineffective gelation. Polymerisation was unable to produce an effective elastic gel due to the presence of the additive.

The incorporation of Histodenz was demonstrated to largely have no detrimental effect on the mechanical properties of both gel compositions when used at levels below 15%.

Compression testing of the two compositions Rf1 and Rf2, swollen within dialysis membrane in solutions of varying osmotic media clearly demonstrates the responsiveness of the gels. This resulted in compressive strengths of 800kPa in 800mOsm media and 8kPa in 80mOsm media.

Injection of the gel into excised sheep disc samples appeared to suggest the restoration of a degree of stiffness closer to that of the intact disc segment. It is feasible to assume that the gel has demonstrated the ability to restore a degree of advantageous properties to the natural disc. This supports the development of the injectable gel for nucleus pulposus repair.

Chapter Six

Towards Development of an Injectable Accommodating IOL

6.1 Introduction

The problem that exists with current IOL materials is their inherent stiffness due to their need to withstand manufacturing constraints. For example, typical IOLs are based on three main types of material: rigid PMMA, foldable acrylics, and silicone. The first two are lathe cut, with the latter being cast moulded. The reason for the difference in manufacturing methods is due to the glass transition temperature of the particular material. Typically PMMA and acrylic based materials are harder materials with a relatively high Tg, whereas silicone materials are much more rubber like with a lower Tg. These materials once implanted within the eye are too stiff to allow for natural accommodation. This usually results in the patient being completely presbyopic. The development of an injectable IOL, which does not have the rigid manufacturing constraints of current materials would allow for a softer material circa 3-8kPa which would allow for some natural accommodation. The use of injection would also further minimise required incision size thus reducing prospective complications during surgery.

The material discussed in the previous chapters is already deliverable *via* injection as a low viscosity pregel, capable of polymerising *in situ* in an acceptable timeframe. The ability of the gel mechanical properties have clearly been demonstrated to be alterable depending on the crosslink ratio of the gels. This chapter will focus on adapting that material to be suitable as an injectable intraocular lens.

The chapter will begin with a short focus on current available materials before introducing the novel injectable analogue material.

I must express thanks to Umair Hussain, a MSc student who conducted his project within the Biomaterials Research Unit, who is responsible for testing some of the compositions detailed in this chapter.

6.2 Current Materials

The crystalline lens of the eye has an elastic modulus ranging between 70Pa at birth and 2700Pa in later years[17]. To understand the properties and mechanical behaviour of current materials it is necessary to refer to both the material and the glass transition temperature of these materials. There are three types of typical IOL material, rigid poly (methylmethacrylate) PMMA, foldable acrylic and silicone based. PMMA and foldable acrylic materials typically have a high glass transition temperature. PMMA is usually in the range of 118-113.5°C, acrylic materials 15.5°C and 14°C and silicone materials -91.7°C to -119.6°C[57]. It is for this reason that PMMA and acrylic materials tend to be lathe cut whilst due to their low Tg silicone materials must be moulded.

Samples of Bausch and Lomb acrylic and silicone based IOLs were received and testing on the Bohlin CVO rheomoeter using a 10mm parallel plate with a roughened surface to help grip the lens. Samples were oscillated between 5 and 20Hz at three temperatures to ascertain the effect of temperature on properties. The results displayed in the figures below were recorded at 10Hz. Figure 6.2.1 reports the elastic modulus of the two types of lens.

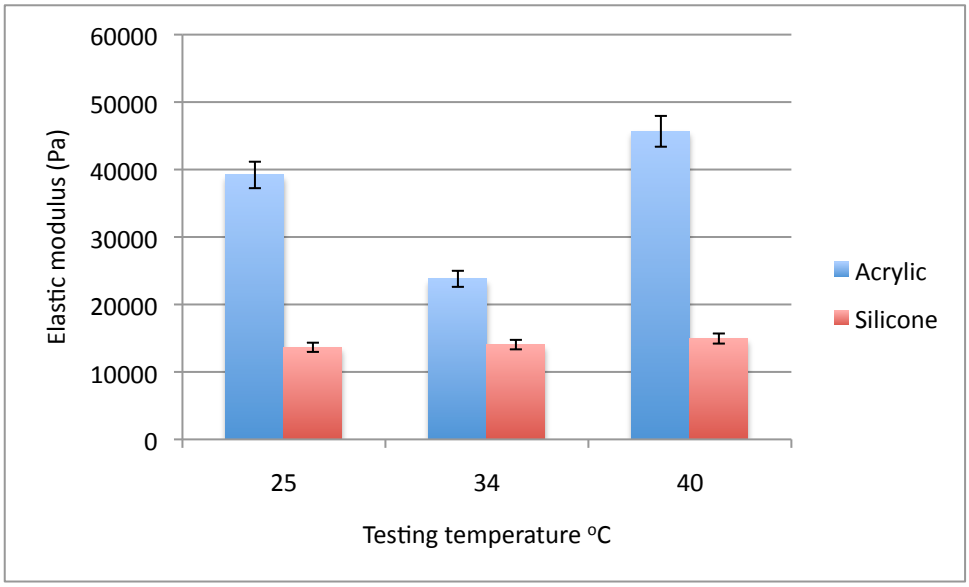


Fig 6.2.1 The elastic modulus of Bausch and Lomb acrylic and silicone based IOLs

As demonstrated in figure 6.2.1, the temperature range studied does not seem to affect the elastic behaviour of the silicone lens, this is due to the temperature of testing being significantly higher than that of the materials glass transition temperature. However with regards the acrylic material, which is essentially still within range of its Tg, the properties vary with temperature. It is important to state here that the glass transition temperature whilst a specific value, changes within the material occur over a 30°C temperature range This illustrates problems that may occur on insertion of the lens and its subsequent unfolding in relation to the temperatures at which this occurs. For example, lenses may be folded at approximately 20°C which is significantly close enough to the Tg of the material to induce creases and prove detrimental to the lenses ability to unfold correctly once implanted in the eye.

Figure 6.2.2 below demonstrates the viscous modulus of the same lenses which again display a similar pattern to that of the elastic modulus in response to temperature.

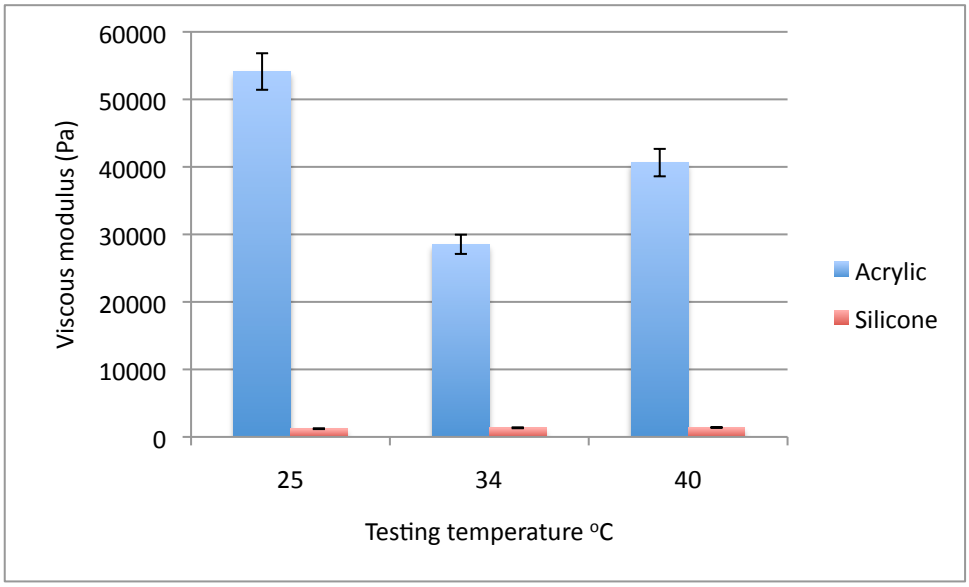


Fig. 6.2.2 The viscous modulus of Bausch and Lomb acrylic and silicone IOLs

Figure 6.2.3 below shows a comparison of the two, clearly demonstrating a more flexible structure at 40°C, attributable to the material being clear of its glass transition temperature.

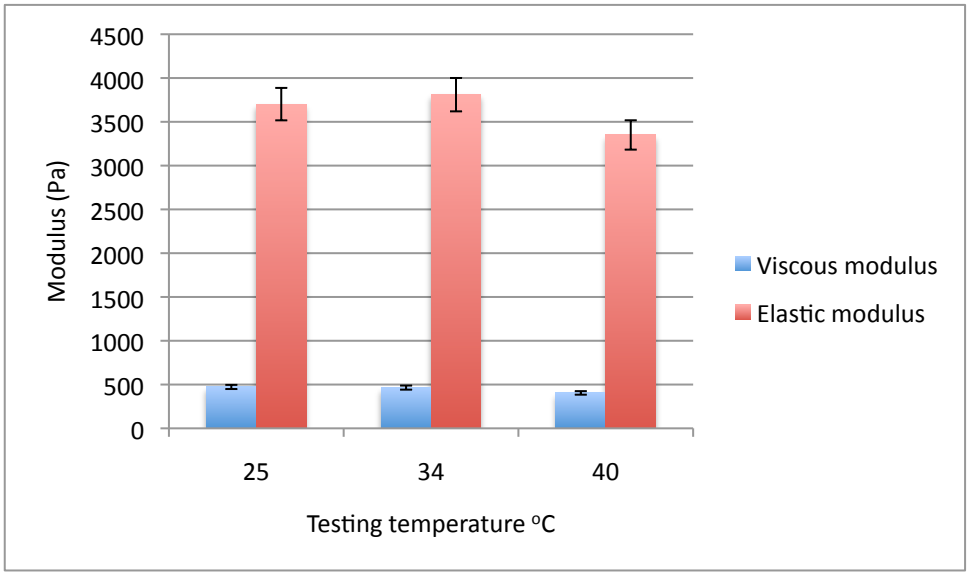


Fig 6.2.3 A comparison of the elastic and viscous modulii of Bausch and Lomb acrylic and silicone IOLs

In comparison, analysis of an acrylic based material which has a Tg within the range of use exhibits completely different properties. This is demonstrated in figure 6.2.4

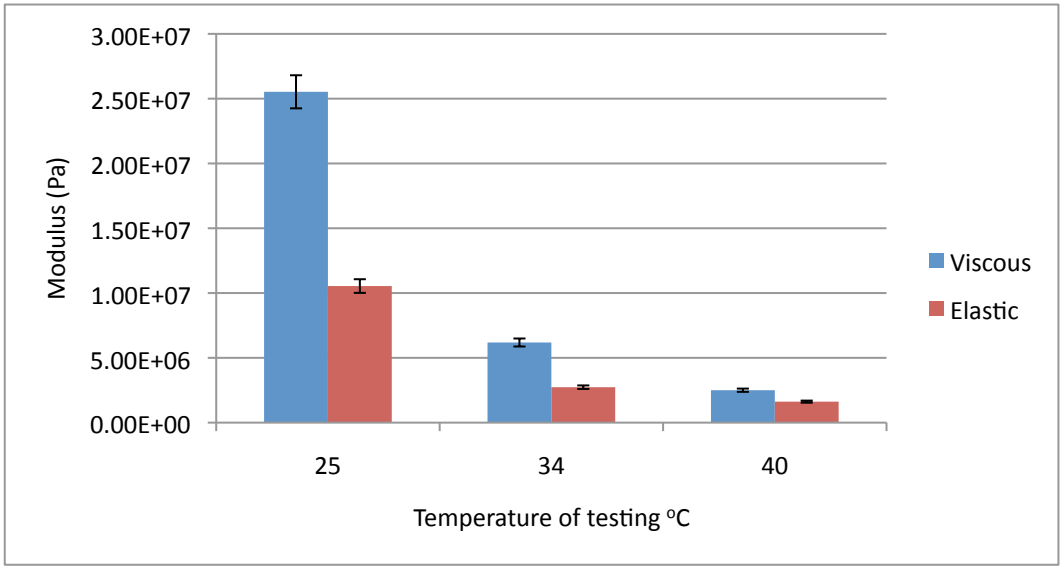


Fig 6.2.4 The rheological behaviour of an acrylic IOL material with a Tg in the range of use

Here it can clearly be seen that around its temperature of folding, the material has a much higher stiffness, which will affect both folding and unfolding. However at 'in

eye' temperature the stiffness bears almost no comparison to that of its folding temperature. A DSC trace for this material is shown in figure 6.2.5.

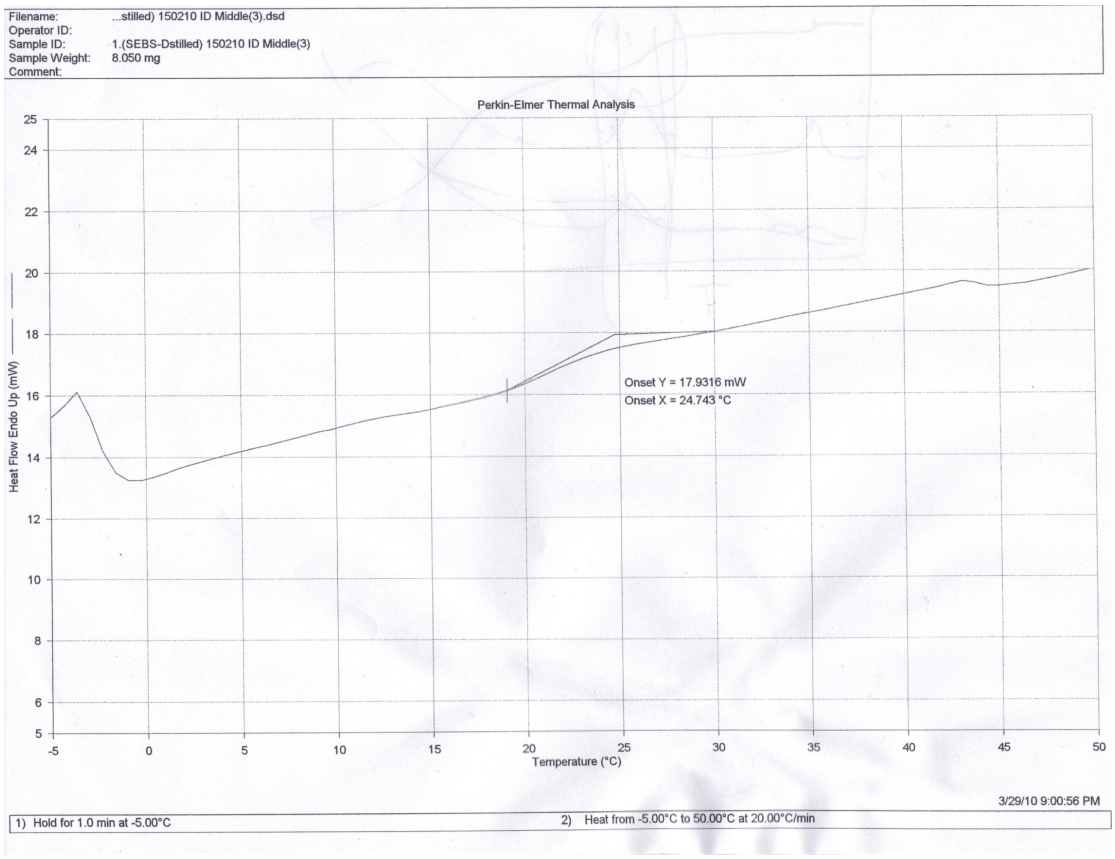


Fig 6.2.5 A DSC trace for a acrylic IOL material with Tg ~ 24°C

The trace illustrates the Tg as 24°C, however the shape of the trace also demonstrates that the range is between 18°C and 30°C. This explains the reduction in stiffness of the material as temperature increases. The chains have more energy to rotate and thus occupy a much lower energy configuration.

Introducing the current materials has given an insight into the problems which are incurred during manufacture and use of an IOL. To develop a successful injectable alternative there are a set of requirements which must be met:

- Material must be injectable into the cavity
- Must be retained within the cavity until complete polymerisation has occurred
- Must completely fill the cavity so as to reduce the possibility of posterior capsular opacification

It is logical now to discuss the proposed injectable alternative.

6.3 An injectable IOL

One of the primary steps within cataract surgery is the use of a viscoelastic to maintain the *via*bility of the cavity before and during surgery. This protects the epithelium from damage and prevents the front and back surfaces of the capsular bag from coming into contact with each other. Discussions with surgeons have indicated that it is not feasible to dry the cavity before injection of the proteoglycan analogue. Therefore displacement or incorporation of the viscoelastic into the pregel was agreed as a logical and workable progression. Initial work on an injectable IOL based solely on the compositions used for the intervertebral disc application resulted in a composition which was not viscous enough to be maintained within the cavity of the eye. This was discovered upon injection of the material into an excised sheep eye which had its lens surgically removed. The pregel solution did not remain within the cavity due to its low viscosityTherefore it was proposed to incorporate the viscoelastic – hyaluronic acid (HA) into the previous developed proteoglycan analogue.

6.3.1 Incorporation of hyaluronic acid into the "disc" gel

Hyaluronic acid is an anionic non-sulfated glycosaminoglycan distributed widely throughout connective, epithelial and neural tissues. It is unique amongst the glycosaminoglycans due to its un-sulfated form, and also to the high molecular weight it reaches which is often in the millions. Present in the extracellular matrix hyaluronan contributes significantly to cell proliferation and migration. Its structure is illustrated below in figure 6.3.1

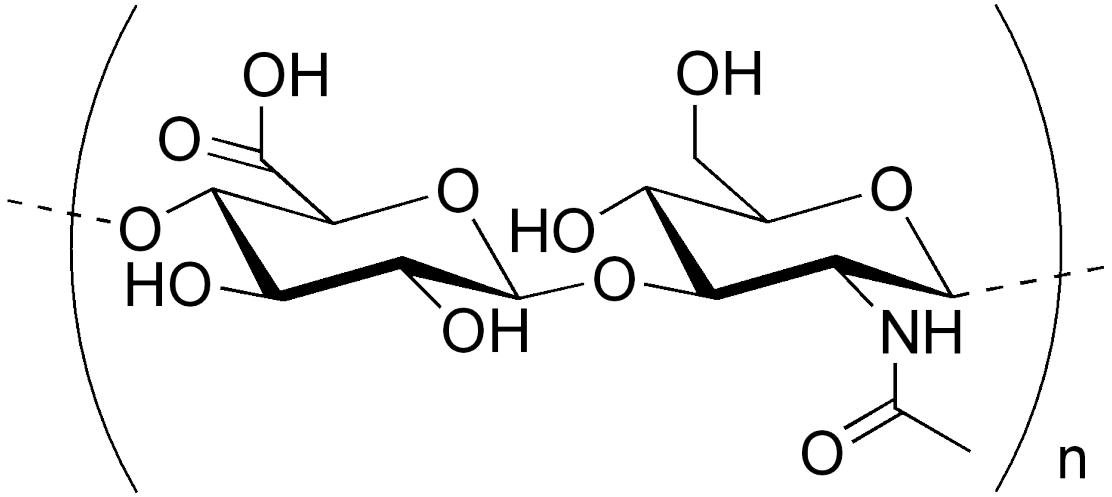


Fig 6.3.1 The structure of hyaluronic acid

Due to the high molecular weight of the material it is highly viscous in nature. For example the commercial Healon GV ocular viscoelastic device used in this research has a viscosity of 3,000,000 mPa. The incorporation of hyaluronic acid into the material will help increase the viscosity and thus improve the retention in the capsular bag prior to polymerisation.

Based on the general composition represented in table 6.3.1, attempts were made to incorporate HA into the composition.

Table 6.3.1 The generic analogue composition

Gel	**NaAMPs (50%)**	**KSPA**	**H_2O**	**Crosslinking agent**	**Initiator**	**Initiator (g)**
I1	3.7g 1.6 x 10^{-2} mol	1.4g 6 x 10^{-3} mol	3.6g 0.2 mol	PEG 575DA 0.1g 1.7 x10^{-4} mol	AA 0.1M 0.65g 6.5 x 10^{-5} mol	Oxone 0.1M 0.65g 6.5 x 10^{-5} mol
I2	3.7g 1.6 x 10^{-2} mol	1.4g 6 x 10^{-3} mol	3.6g 0.2 mol	PEG1000DMA 0.1g 1 x 10^{-4} mol	TEMED 0.4M 0.65g 2.6 x 10^{-4} mol	KPS 0.18M 0.65 1.2 x 10^{-4} mol

Hyaluronic acid was supplied as a 100% solid from Sigma Aldrich. The aqueous solubility was 0.005g/ml. Three different levels of HA were added to each composition as detailed in table 6.3.2 below, the composition of the gels is as table 6.3.1

Table 6.3.2 The incorporation of HA

Gel	HA (g)
I1a	0.034g
I1b	0.027g
I1c	0.02g
I2a	0.034g
I2b	0.027g
I2c	0.02g

Each gel was prepared in a similar manner as described in section 2.2.1, except for the additional component of HA. This was dissolved between the aqueous component and the 50% aqueous NaAMPs component before transferring into the respective Redox A and Redox B solutions. All reactions were carried out *via* injection into a petri dish to allow immediate de-selection of unsuitable systems. Each composition formed successfully. Molar concentrations of hyaluronic acid are not reported due to its extremely high molecular weight.

Once polymerised, 20mm discs of each sample were cut out for rhelogical testing. Figure 6.3.1. shows effect on the elastic and viscous modulii of decreasing amounts of HA in system I1.

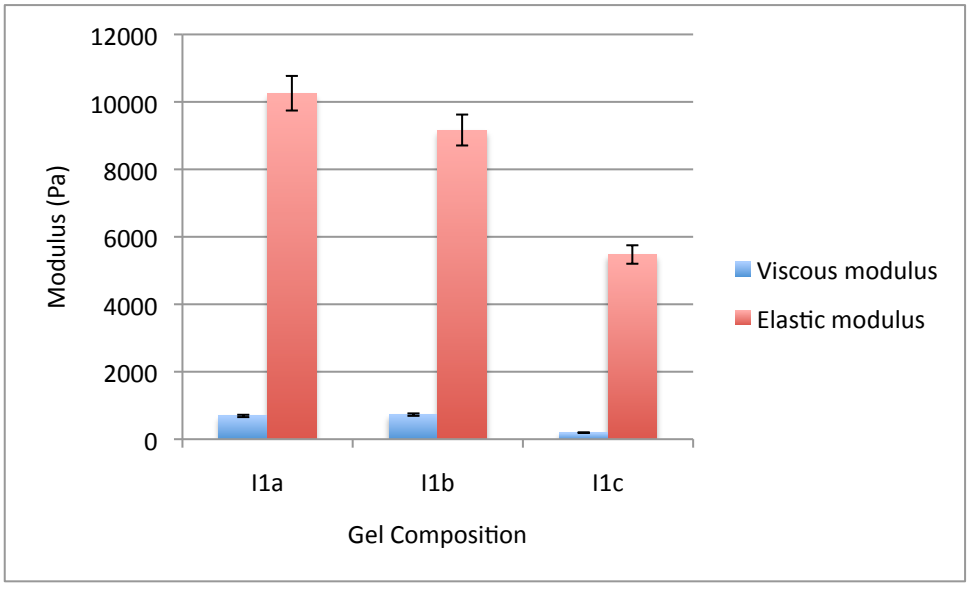


Fig 6.3.1 The elastic and viscous modulii of gel I1 containing decreasing amounts of HA

The results displayed in figure 6.3.1 suggest that at higher levels of HA, the material is behaving as a reinforcing agent, for instance as an interpenetrant. This effect decreases as HA content is reduced. It could also be proposed that the presence of HA is improving the ability of the material to polymerise, therefore a stronger network is formed in composition B.

Figure 6.3.2 below demonstrates the rheological behaviour of system two with decreasing amounts of HA

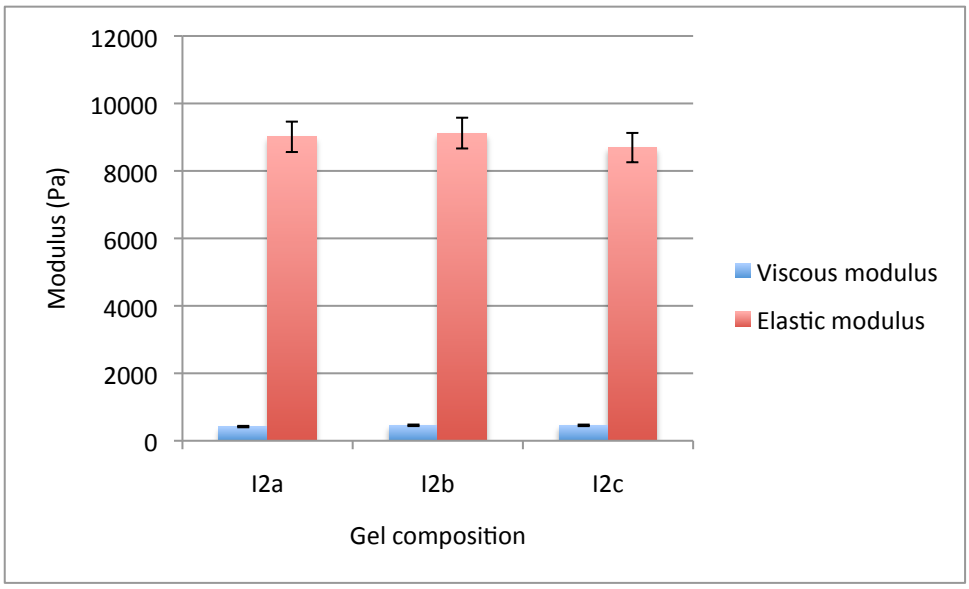


Fig 6.3.2 The elastic and viscous modulii of gel I2 containing decreasing amounts of HA

The results dictated in figure 6.3.2 for the TEMED and KPS initiated system suggests different results to that of the AA/Oxone. The amount of HA added does not appear to have such a drastic effect on the mechanical strength, which supports the theory that in the previous gel, HA was behaving as an interpentrant thus improving the effectiveness of the polymerisation. Here with the TEMED/KPS system it appears that HA is just behaving as bulk.

This evidence is further supported by the swelling behaviour of the two different gels in phosphate buffered saline and 10% PEG 35000 solution. Figure 6.3.3 below represents the AA/Oxone initiated gels I1a,b,c. Discs of 20mm diameter were cut from the petri dish and swollen in the required media until they appeared to reach equilibrium.

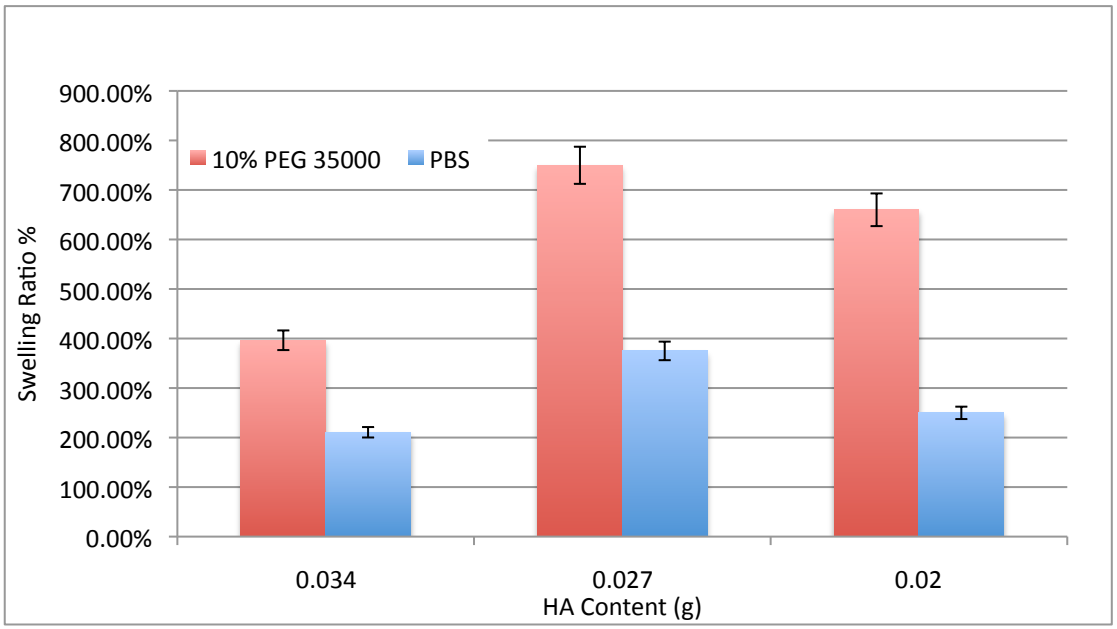


Fig 6.3.3 The swell capacity of gels I1a,b,c, in PBS and 10% PEG 35000

The results appear to suggest that there is an optimal inclusion level of HA which facilitates a greater degree of swell behaviour, illustrated by the greatest swell achieved at 0.027g inclusion. This may correspond with the HA acting as an interpenetrant and becoming part of the actual gel structure. Figure 6.3.4. below also supports this theory as the swell behaviour of the TEMED/KPS system does not seem so dramatically different between concentrations of HA including. This reiterates the fact that the HA must be behaving in bulk within the system as opposed to part of the structure.

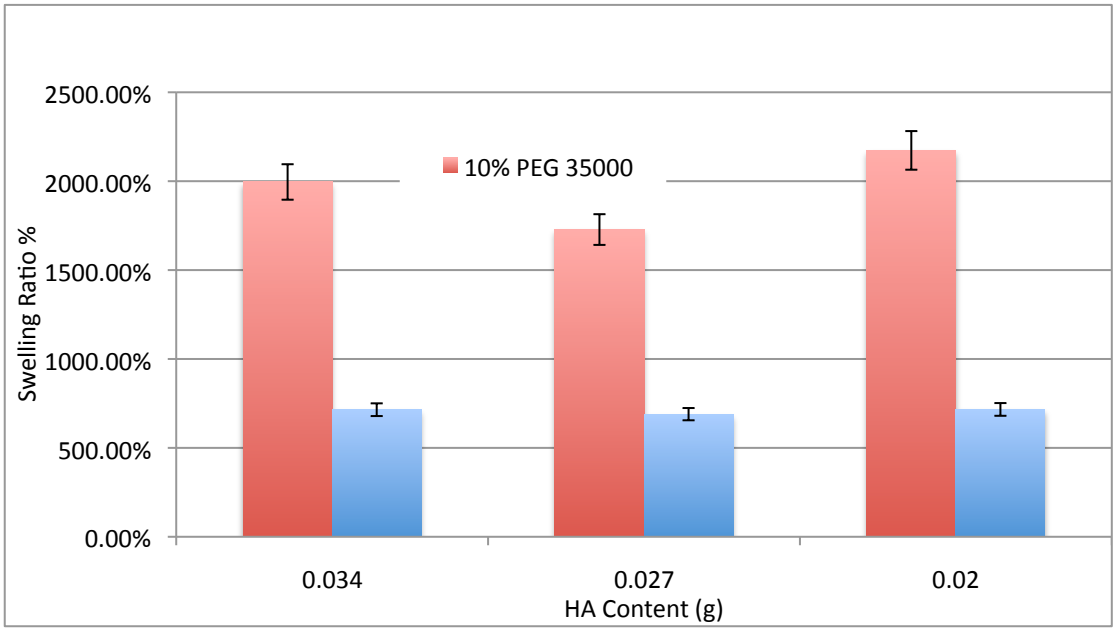


Fig 6.3.4 The swell behaviour of I2 a,b,c gels in PBS and 10% PEG35000

Sample I1a was tested for compressive strength after swelling of 3ml aliquots of the gel within dialysis bags immersed in three solutions of varying osmolarities for 72 hours. The procedure is detailed in section 2.3 and the results shown below in figure 6.3.5

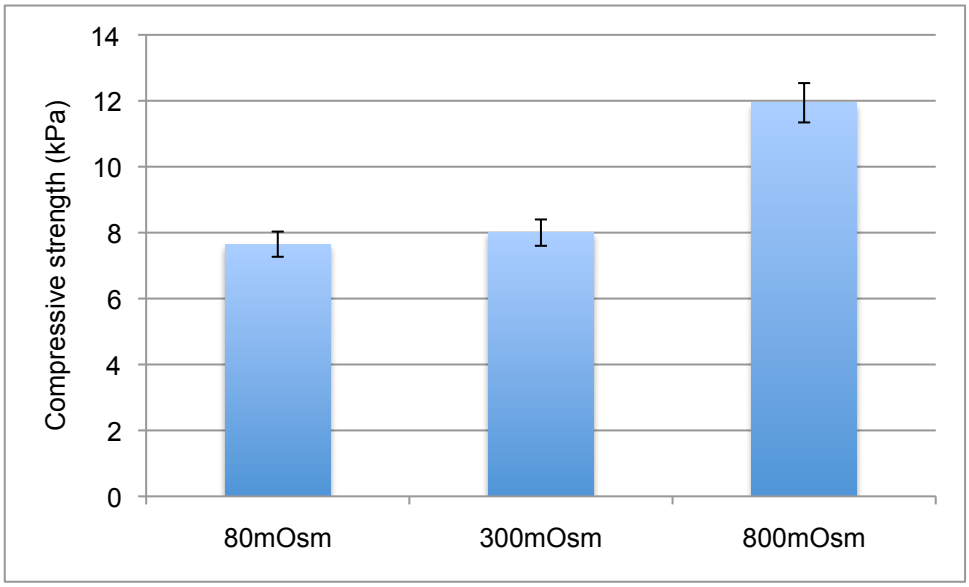


Fig 3.6.5 The response under compression of gel I1a after swelling in varying osmotic media

The responsiveness of the material is clearly demonstrated, although in comparison to similar material without HA, shown in figure 3.6.6, the response and compressive strength is slightly less. This could be due to the inability of the polymer chains to respond with HA as part of the structure, being almost restrained by its inclusion.

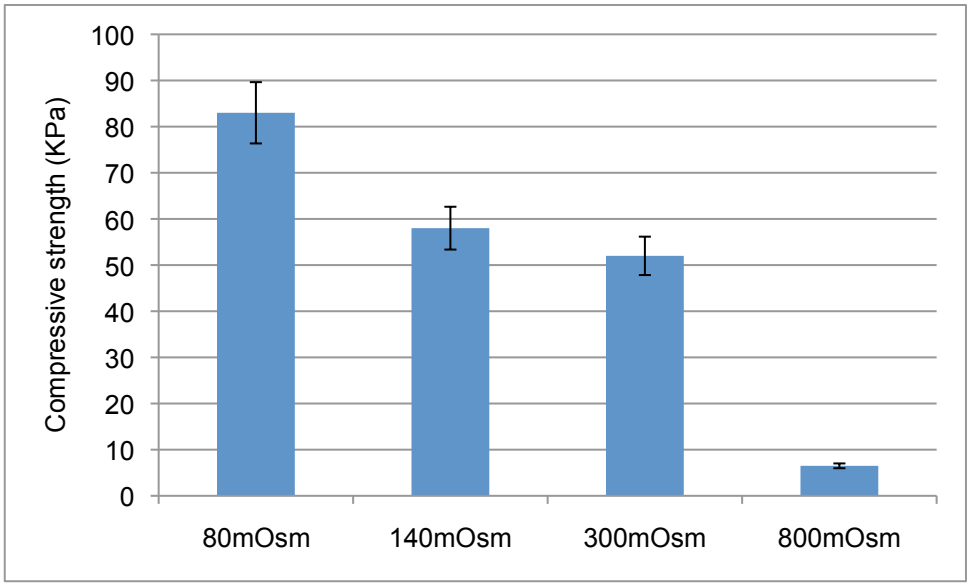


Fig 3.6.6 The compression behaviour of an NaAMPS/KSPA TEMED/KPS gel with 1% crosslinking agent PEG1000DMA swollen in varying osmotic media

6.4 The incorporation of a commercially available viscoelastic into the gel composition

During cataract surgery a viscoelastic consisting of hyaluronic acid is used to maintain *via*bility of the lens cavity. This is injected into the lens capsule during removal *via* phacoemulsification and acts to lubricate the surfaces and prevent them coming into contact. Previous experiments used solid hyaluronic acid to assess whether this could be *via*bly incorporated into the gel composition. The next logical step was to investigate the incorporation of a commercially available viscoelastic – Healon which is readily used in cataract surgey, into the gel in place of the solid HA previously used. For this the gel composition remained similar to previous, however the water content was replaced with commercial Healon, and is represented in table 6.4.1. 3.6g of Healon was used to replace the water component of the gel composition. The molar component of Healon is not reported due to its extremely high molecular weight ~ 40 million.

Table 6.4.1 Replacement of water with Healon

Gel	**NaAMPs (50%) (g)**	**KSPA (g)**	**Healon (g)**	**PEG575DA (g)**	**AA 0.1M (g)**	**Oxone 0.1M (g)**
I3	3.7g 1.2×10^{-2} mol	1.4g 6×10^{-3} mol	3.6g	0.05g 8.7×10^{-5} mol	0.65g 6.5×10^{-5} mol	0.65g 6.5×10^{-5} mol

An injectable gel was reproducibly formed with this composition. The ability of the gel to polymerise appeared greater than that synthesised from the HA previously used. Therefore it was advantageous to analyse the rheological behaviour of the gels. As before a 20mm disc of the gel was extracted for testing on the rheometer. In order to compare the rheological behaviour to that of the currently available IOLs the material was tested at a range of temperatures. The results, recorded at 10Hz are demonstrated in figure 6.4.1

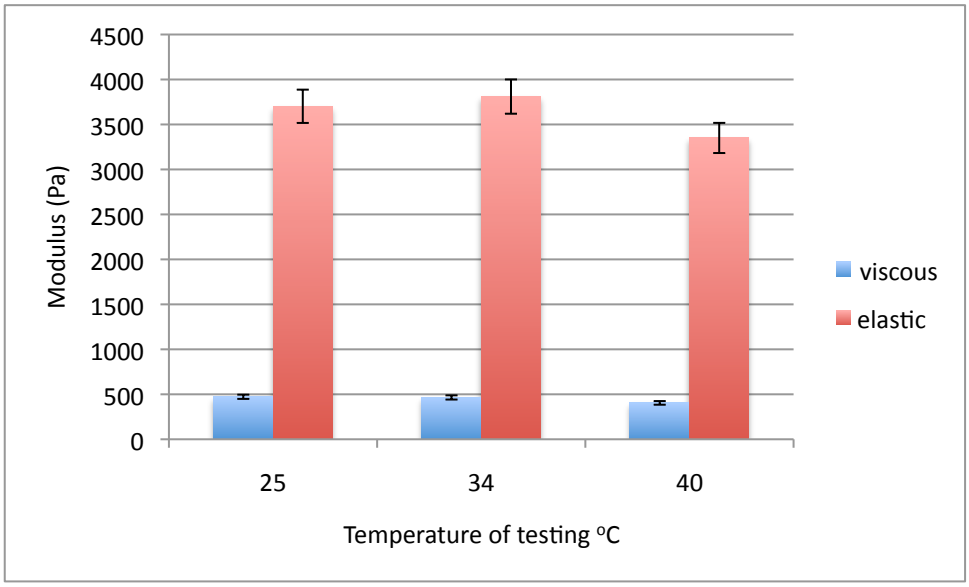


Figure 6.4.1 The rheological behaviour of gel I3 containing Healon, tested at a range of temperatures

It is clear to see that the material displays a much lesser dependence on temperature in comparison to currently available IOL materials. With the crosslinker level at 0.5% the material displays mechanical properties in line with that of natural lens tissue which is in the range of 308kPa.

The swell behaviour of the gel was investigates in both PBS and 10% PEG 35,000, the comparison with that of the gels containing solid HA is shown below in figure 6.4.2.

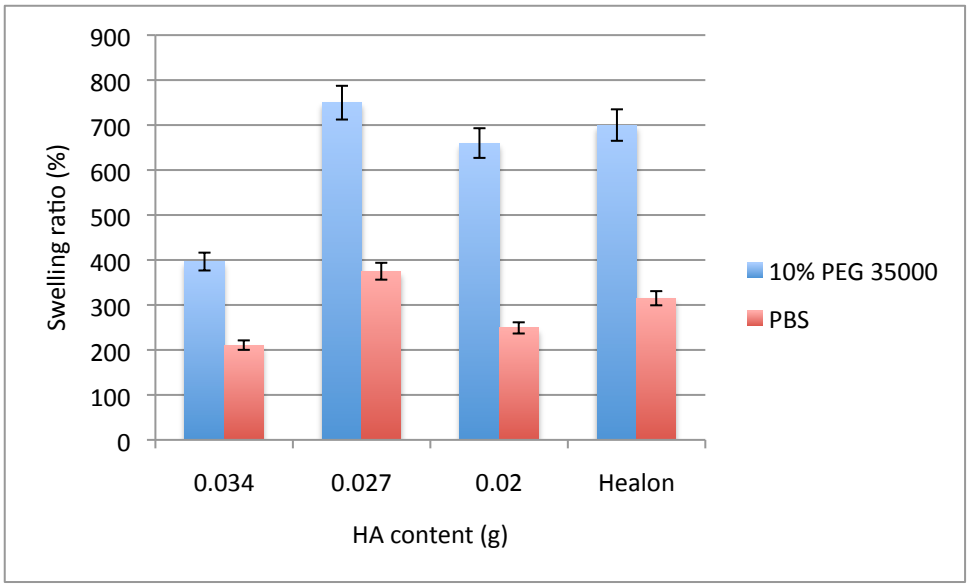


Fig 6.4.2 The swell capacity of gels I1a,b,c, in PBS and 10% PEG 35000 compared to I3 containing Healon.

The results show interestingly that the swell capacity is in between the 0.02g and 0.027 g inclusion. It would be expected that in the 3.6g Healon there was approximately 0.018g HA. The swell behaviour may be increased due to this being homogenised to a greater degree in the commercial solution than the laboratory prepared solution.

6.5 Discussion

The aim of the work detailed in this chapter was to adapt the existing proteoglycan analogue to the application of an injectable intraocular lens. The complications with current materials was first demonstrated. Understandably the properties of the acrylic IOL material were effected by testing temperature due to encroachment on the region of the glass transition temperature. It was proposed that this could be rectified by the use of the analogue material. The ability of HA to participate as part of the polymer structure was discovered to be more apparent when using the AA/Oxone initiator

system. This could be due to HA acting as an interpenetrant as opposed to just acting in bulk, which it appears to do when used in the TEMED/KPS system.

The material was demonstrated to still be osmotically responsive, although not to quite the same degree as the intervertebral disc analogue. This is probably advantageous, as excess pressure exerted once within the eye could be detrimental to the structure of the capsular bag.

A composition based on the general gel composition replacing the water content with commercially available Healon appears to offer the best solution. At 0.5% crosslinking agent this material has an elastic modulus well within the acceptable limits of elasticity in order to allow the eye to accommodate.

Chapter Seven

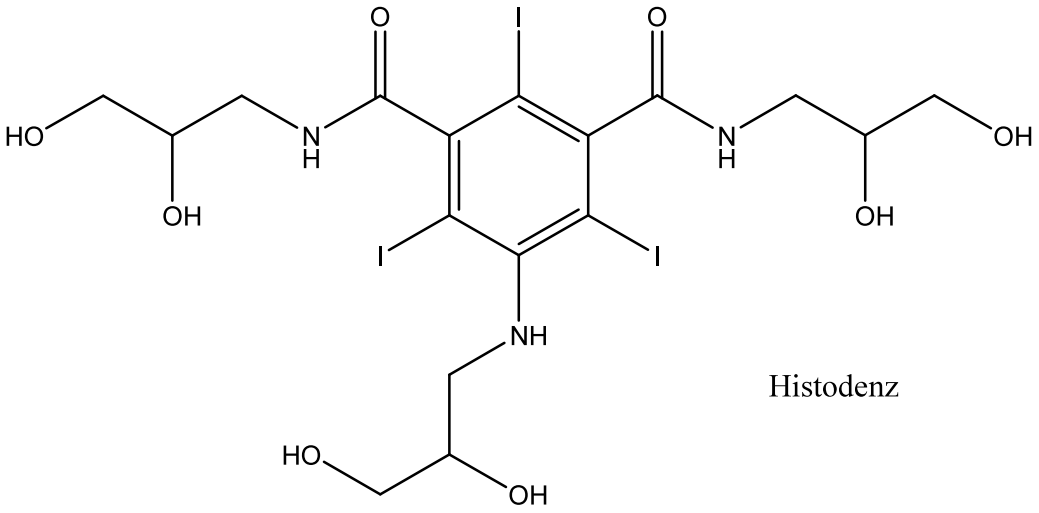
Development of a Radio-opaque Macromer for Incorporation into the Proteoglycan Analogue

7.1 Introduction

When injecting an implant into any body site it would of course be advantageous for the structure to be visible throughout its lifetime *in situ.* As has been detailed in section 4.6.1, free histodenz has been added to gels and does not appear to detrimentally effect the formation and mechanical properties used at 15% per total gel volume and below. However due to the hydrated nature of the gels, as fluid is exchanged once the gel is in its target implant area, the potential for the dye to leach out is quite high. This would prevent the visibility of the implant throughout its lifetime. Therefore the research detailed within this chapter focuses on the addition of a reactive double bond to the histodenz structure to enable it to form part of the polymer.

7.2 The reaction of N-hydroxymethyl acrylamide (NMA) and histodenz

The structure of histodenz is shown in figure 7.2.1. As you can see it possesses six, almost identical hydroxyl groups. The idea was to create a structure that is capable of bonding with the polymer but does not have multifunctionality which has the potential to interfere with gelation time. Therefore *N*-hydroxymethyl acrylamide was added at a ratio of 1:6 to histodenz to facilitate the addition to only one hydroxyl group.



Histodenz

Fig 7.2.1 The structure of histodenz

In a 15ml glass sample *via*l 0.6g of histodenz was dissolved in 5ml 0.3% HCl solution (the acid catalyst). To this 0.21g 48% NMA in aqueous solution was added. The mixture was purged of oxygen with nitrogen for 10 minutes. After this was completed the *via*l was sealed, with a needle inserted into the lid to allow any gases created during the reaction to escape, and placed in a 60^{o}C waterbath for 2.5 hours. The proposed reaction scheme is detailed in figure 7.2.2

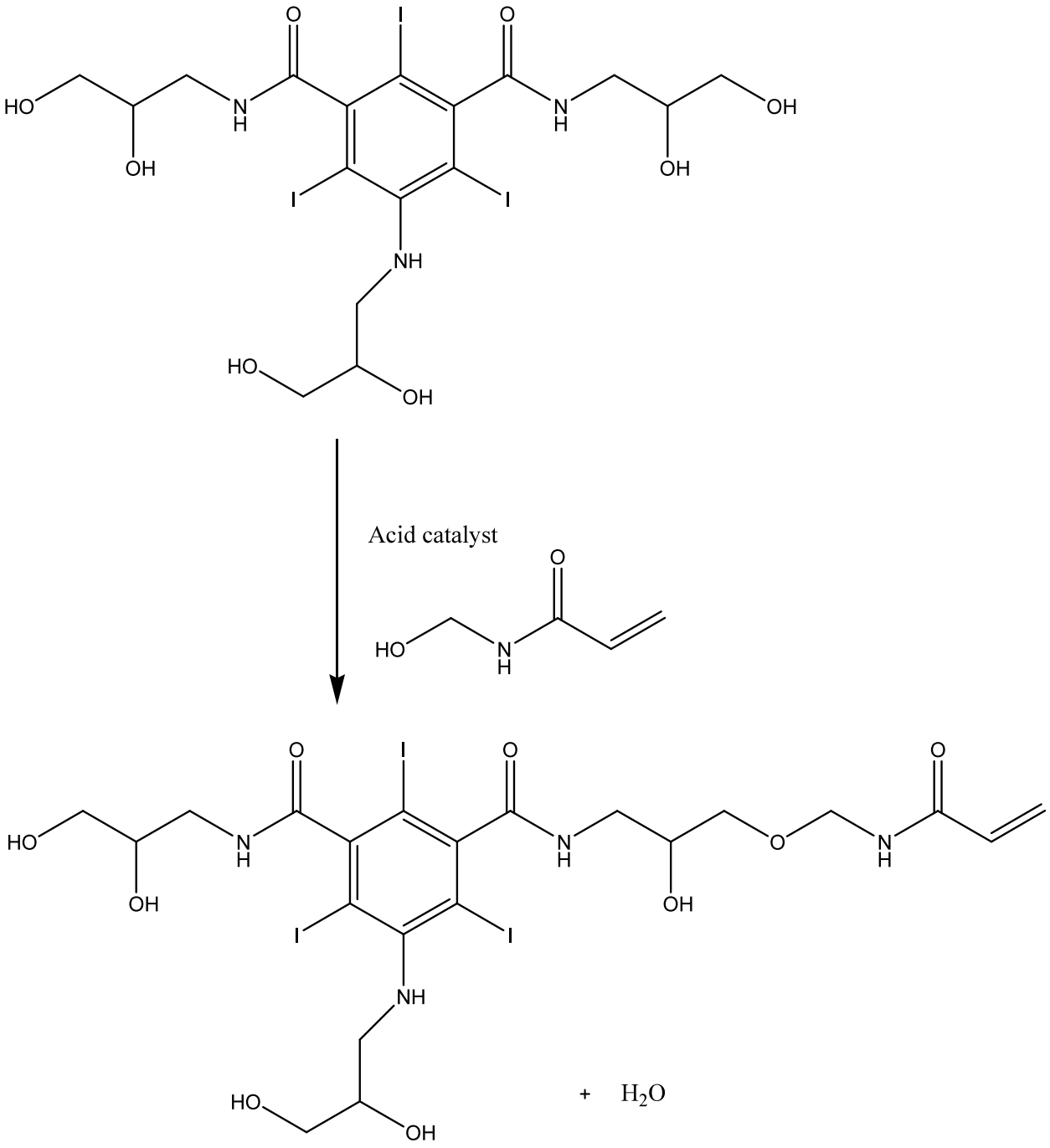


Fig 7.2.2 The reaction of histodenz with NMA

The FTIR spectra below, figure 7.2.3 illustrates histodenz in blue and NMA 48% aqueous solution in red.

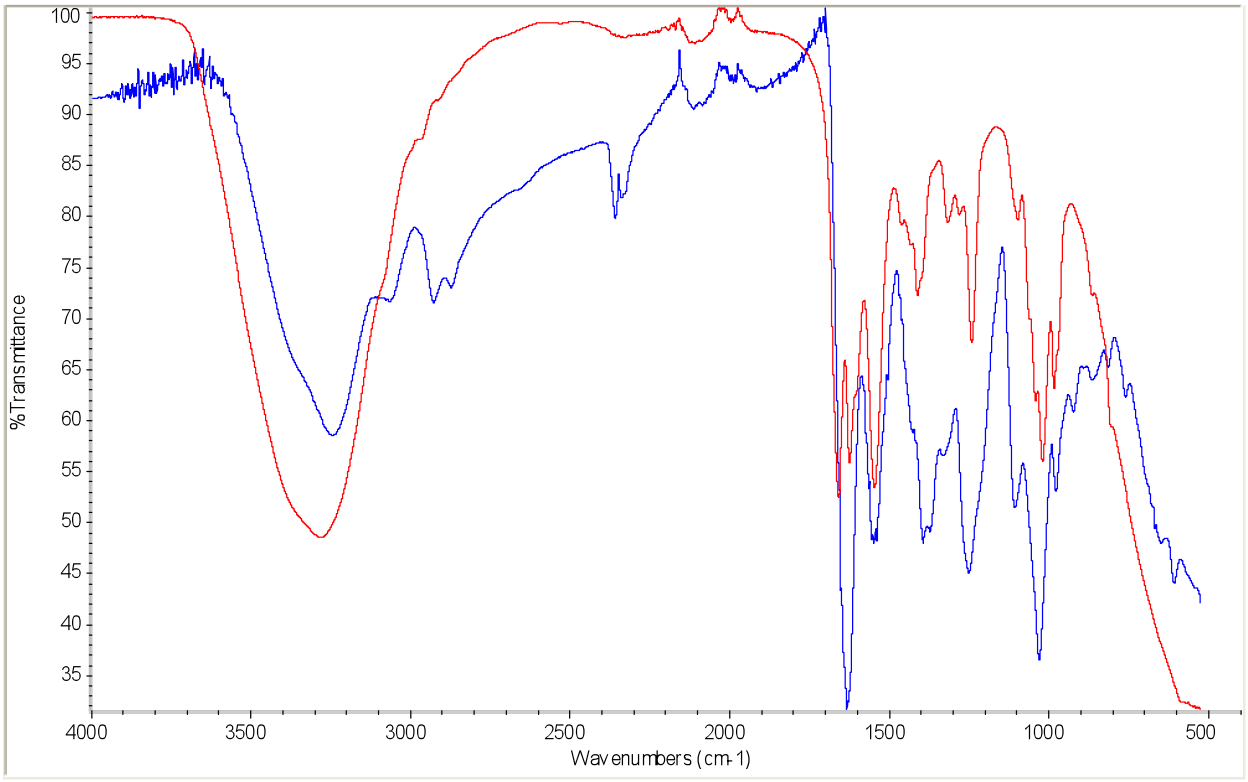


Fig 7.2.3 The FTIR spectrum of histodenz(blue) and NMA 48% solution (red)

The allocation of the important functional groups is shown below in table 7.2.1

Table 7.2.1 FTIR analysis of histodenz and NMA 48% solution

Chemical	***Peak position (cm^{-1})***	***Functional group***
Histodenz	3237	OH alcohol
Histodenz	3066	CONH amide
Histodenz	2930	NH amide
NMA	3283	OH alcohol
NMA	2977	CONH amide
NMA	1640	C=O amide
NMA	1020	C=CH deformation

FTIR analysis of the sample after reaction was conducted, the main area of interest being the presence or absence of an –O- linkage. The spectra is shown in figure 7.2.4.

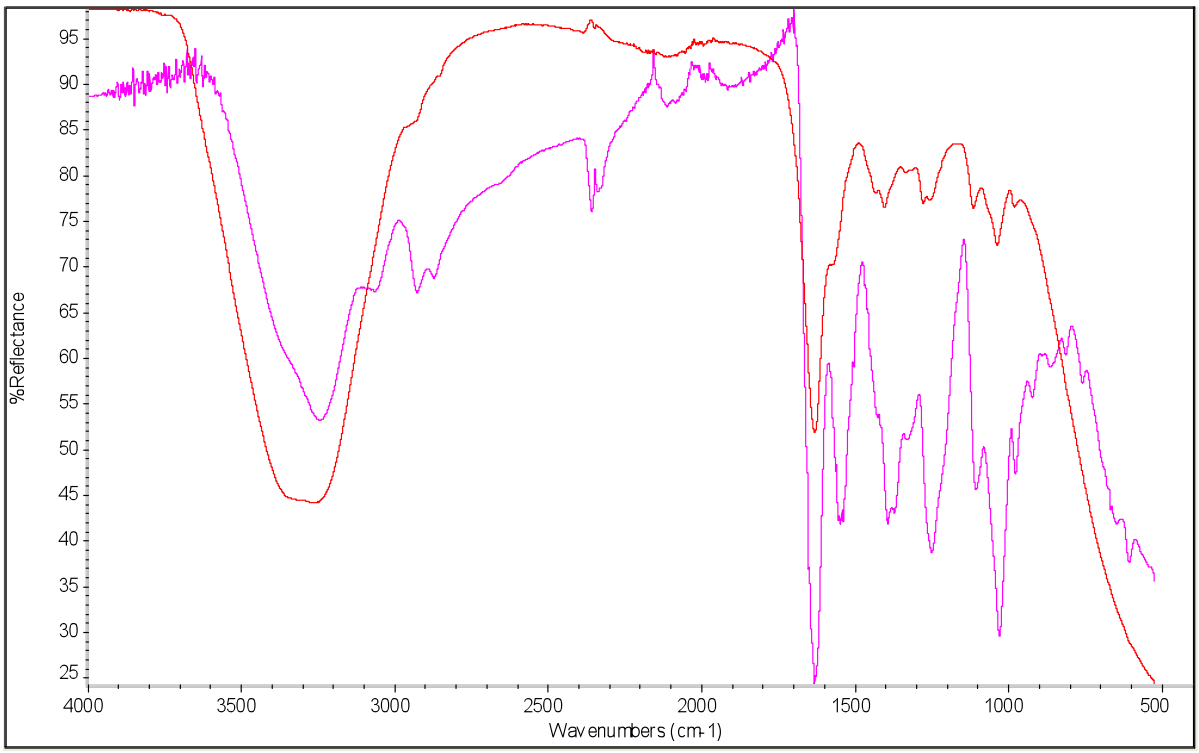


Fig 7.2.4 The spectrum of histodenz (pink) and histodenz-nma reaction (red)

Clearly as the reaction was conducted in aqueous solution, the detail of the spectra has been reduced. Unfortunately there appears to be no peak visible in the region of 1750-1730 or 1300-1000 that would demonstrate the formation of an ester linkage. The sharp peak at 1640 can be attributed to the presence of amide.

As NMA is known to react in both acidic and basic conditions, the experiment was repeated using potassium hydroxide at 0.3%, with all other conditions kept similar. The FTIR spectra of this reaction is shown in figure 7.2.5.

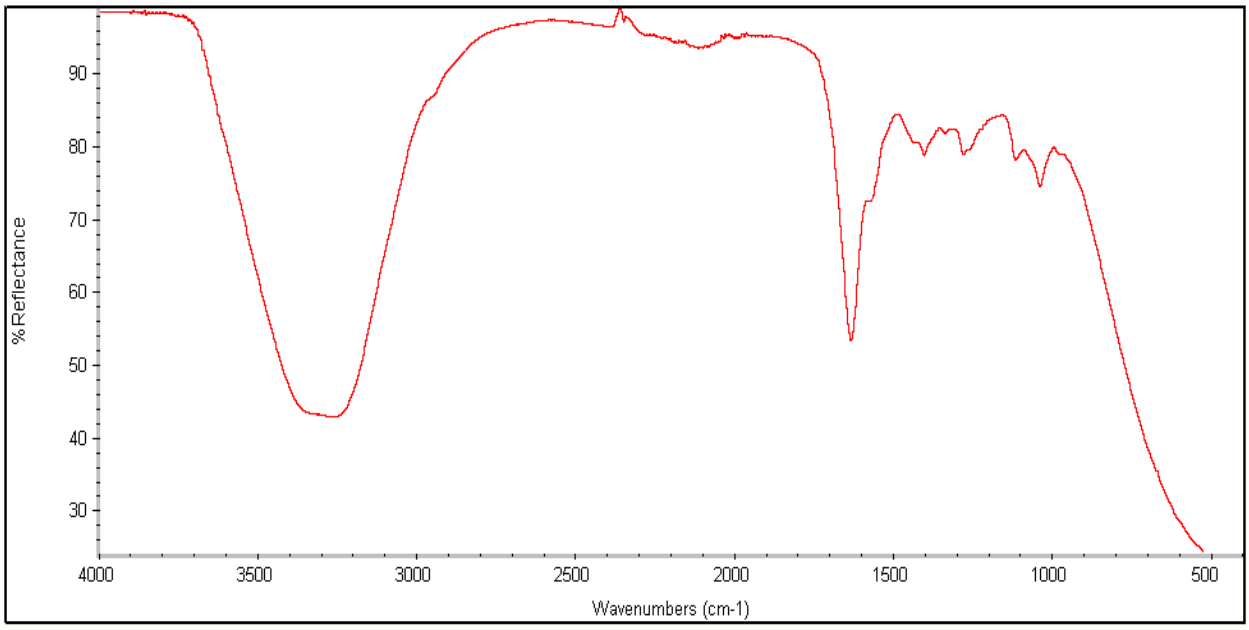


Fig 7.2.5 The spectra of histodenz –NMA reaction conducted in basic conditions

Again it the absence of a peak denoting the ester linkage is illustrated.

Experiments were repeated adding NMA in excess of histodenz to prompt a reaction, however the analysis observed *via* FTIR did not differ. To ascertain whether the inconclusive nature of the FTIR spectra was due to the dilute nature of the reaction, the next process was the addition of the acid catalysed histodenz –NMA into a linear polymer.

The Histodenz- NMA sample was sent away for mass spectrometry analysis. The spectra is shown in figure 7.2.6.

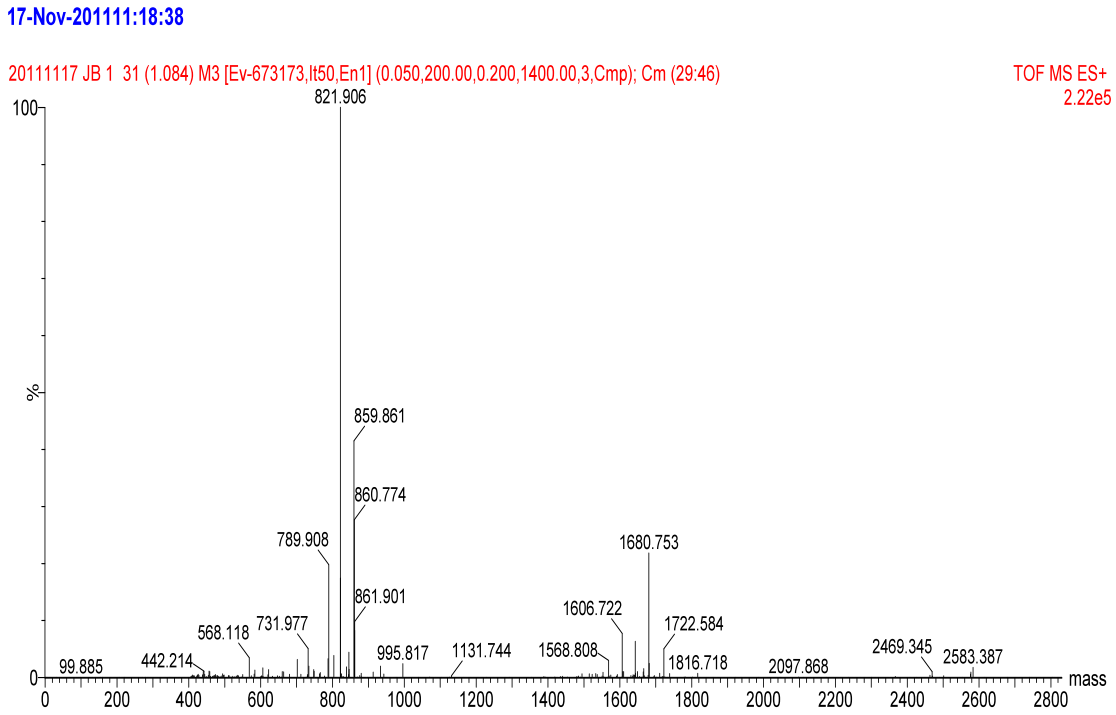


Fig 7.2.6 The mass spec analysis of the histodenz-NMA reaction

The relative molecular masses of histodenz and NMA are 821.14 and 101.10 respectively. The spectra above clearly shows a peak at 821.906 which can be assigned to unreacted histodenz. However there does not appear to be much evidence of the NMA on its own or reacting with itself. The next peak of relevance is at 1680.753 which could be histodenz reacting with itself with the addition of NMA.

The results are still inconclusive, for future work it may be advantageous to increase the level of NMA used in the reaction to maximise the potential of reaction with Histodenz. This is in contrast to the method used here where a 6:1 histodenz to NMA was used to facilitate addition to one OH group.

Alternative methods are mentioned in further work.

7.3 The addition of histodenz-NMA into a linear polymer

A typical linear polymer composition was used, consisting of 6g 3-sulphopropyl acrylate potassium salt (KSPA), 11.3g sodium 2-acrylamido-2-methylpropane sulphonic acid (NaAMPs)(58%) 6g *N*-methyl *N*-vinyl acetamide (NMVA) and 20g of a 20% aqueous mix of histodenz-NMA in the ratio of 1:1 w/w. All components were added to a 60:40 200ml solvent mix of acetonitrile to water (water content was adjusted to allow for the water in the composition and initiator) in a round bottom flask set up for a typical reflux reaction. The system was brought up to temperature ~60^{o}C whilst under mechanical stirring, and a constant stream of nitrogen was provided to the solution. Once temperature had been reached at stabilised a solution of potassium persulfate was added to correspond to 2% of the monomer content. The reaction was kept at 60^{o}C for 2.5 hours.

After the reaction had occurred the result was a highly viscous solution. This was allowed to cool and precipitated out using an excess of acetone. As histodenz is also not soluble in acetone, it was possible that some residual histodenz remained within the precipitated compound. To alle*via*te the possibility of this, the precipitate was washed with water, which may have reduced the yield, but hopefully removed any un-reacted histodenz. A sample of the solid was then sent to Medac for elemental analysis. The results are represented in table 7.3.1.

Table 7.3.1 The elemental analysis of a linear polymer containing histodenz

Element	Percentage (%)
C	2.91
H	11.65
N	0.51
S	0.68
I	1.28
Na	0.17

To calculate the efficacy of the reaction, 2g of the monomer mixture was histodenz. This corresponds to 9% of the overall composition. The molecular weight of a histodenz molecule is 821.4, with iodine being 126.9. There are three iodine per

molecule of histodenz, this corresponds to 380.7, which is 46% of 821.4. So 46% of 9% is 4.14%. This is the incorporation that would give a 100% success rate of inclusion in the polymer. The analysis results gave an average value of 1.28% which is approximately a 30% success rate.

These results proved that histodenz was able in some way to react with a polymer structure. It cannot be confirmed that this was entirely NMA or another structure. This is in part to the aqueous nature of the reactions, histodenz has poor solubility in organic media, and also due to the similarity between hydroxyl groups. This is illustrated by the NMR spectra of a 20% aqueous solution of histodenz, shown in figure 7.3.1.

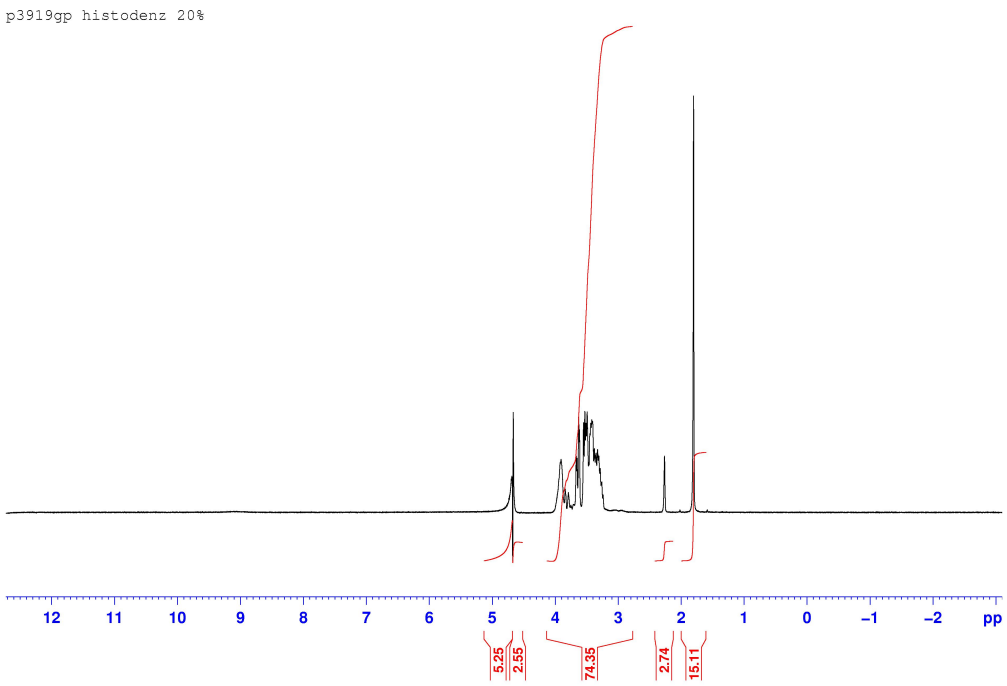


Figure 7.3.1 Water suppresion NMR analysis of histodenz

The OH groups are clearly represented between three and 4 ppm and it is impossible to distinguish between the six groups.

Due to this encountered difficulty, coupled with the apparent undersirability in the US of having a radio-opaque implant, alternative methods were proposed.

Chapter Eight

Summary and Conclusions

8.0 Summary and conclusions

The central aim of the work detailed in this thesis was to design a tissue analogue material based on the natural sulfated proteoglycan structure. The material had to be deliverable *via* injection to the required site of application. The advantages of an injectable repair system for the nucleus pulposus of the intervertebral disc is the ability for the procedure to be carried out in a similar manner to that of a typical discogram. This has the potential to save time as a typical discogram procedure requires a minimum of 15 minutes for completion. The availability of a minimally invasive alternative to surgical disc repair would make the procedure available to those not able to withstand major surgery. It is important to note however that the procedure is only suitable if the annulus fibrosus of the intervertebral disc is intact as this would need to contain the prostheses.

Requirements of the system

With respect to the injectable intraocular lens application, the delivery of a material that is capable of polymerising *in situ* as oppose to a solid lens, results in the availability of a much more elastic material. Current materials have high stiffness imposed by their manufacturing constraints, in particular their ability to withstand lathe cutting. This stiffness means that whilst the opacified lens can be removed, the ability of the eye to accommodate is never restored and the result is usually the patient still requiring glasses and/or contact lenses. The injection of an osmotically responsive proteglycan analogue which has been modelled on the natural tissue, has the potential to amend the issue of accommodation. The ability of the material to incorporate hyaluronic acid, which is the most commonly used viscoelastic to maintain cavity *via*bility during surgery, is an added advantage as it would allow a much more simplified procedure. The incision size would be minimal, required only to allow phacoemulsification to occur, so has the potential to be less that 1.8mm, which is currently the smallest *via*ble incision to deliver a hydrophilic foldable acrylic lens. The pre-gel would be a substitute for the viscoelastic prior to polymerisation *in situ.* The use of an under hydrated pre-gel would enable the polymer to be hydrated *in situ* thus allowing it to swell to fill the cavity, yielding the potential to minimise posterior capsular opacification. As with the intervertebral disc application,

simplifying the procedure and lessening the size of the required incision would improve available of the operation to those unsuitable for traditional cataract removal and intra-ocular lens insertion.

This required a material for both applications, that was deliverable *via* a low viscosity pre-gel through a small bore needle, which would then polymerise efficiently and completely *in situ.* The pre-gel system must be stable for storage within a surgical environment for at least 1 month, and once polymerised within the target tissue, it must be stable to both enzymatic and hydrolytic degradation in addition to the physical forces it is subjected to over an extended time period. The resultant material must be biomimetic and exhibit mechanical properties similar to those of the target tissue. A particularly important requirement is that the material must be osmotically responsive and must maintain hydration levels under load. Residual monomer content after polymerisation must be minimal and no displacement or leaching of the material must occur throughout its lifetime once implanted.

The biomimetic target and synthetic analogues

The specific proteoglycan target structures -the nucleus pulposus and crystalline lens- that will be replaced by the analogue are chondroitin sulfate and keratan sulfate. Proteoglycans are protein structures which are covalently bound to glycosaminoglycans that consist of a disaccharide repeat unit containing either glucosamine or galactosamine. Chondroitin sulfate in particular consists of a chain of alternating sugars *N*-acetylgalactosamine and glucuronic acid. A chondroitin chain can be variably sulfonated along its structure, and is a major component in the ability of the target tissue to withstand compression.

The monomers used to mimic these structures were sodium 2-acrylamido 2- methyl propane sulfonic acid (NaAMPs) and the potassium salt of 3-sulphopropyl acrylate (KSPA). The presence of a sulfonate group within their structure supplies the ability of the polymer material to mimic the behaviour of the sulfate group within natural tissue. Initial investigations to produce a homopolymer of NaAMPs produced a material with an extremely high swelling capacity, typically 400% in water and 250% in phosphate buffered saline. This can be attributed to the presence of both the amide

and the sulfonate groups. Acrylamide monomers are known to polymerise effectively and efficiently and this was illustrated *via* the rapid polymerisation, ~2 minutes in the presence of ambient oxygen at room temperature.

Due to the commercial availability of NaAMPs as only a 50% or 58% solution this affects the w/w solid content of the resultant gel, for this reason the addition of a comonomer was investigated. Inclusion of a neutral monomer such as acryloylmorpholine or poly (ethylene glycol) acrylate with NaAMPs as a comonomer successfully results in gelation. However the swell capacity of the resultant gel is diminished due to the reduction in its ionic nature. This affects both the percentage of water that can be polymerised as part of the gel, and the subsequent ability of the gel to imbibe water. This is also apparent in the rheological behaviour of the materials, resulting in a much higher stiffness in comparison to a NaAMPs homopolymer. This can in part be attributed to the increase in monomer content and the reduction of water content. Not only is there less water included to behave as a plasticiser, but also not enough to act as a spacing device between crosslinks.

Therefore it was logical to investigate the inclusion of another sulfonated monomer. 3-Sulfopropyl acrylate potassium salt (KSPA) was an ideal candidate due to its commercial availability and use in prior hydrogel applications such as wound dressings. As an acrylate, KSPA is also readily polymerisable, although not as reactive as NaAMPs. A copolymer composition of the order 1:1 NaAMPs:KSPA produced the most reproducible polymerisation under redox conditions. The reactivity ratios of NaAMPs:KSPA have been reported by Boote as 0.55:1.70[53] in photopolymerisation. KSPA is less reactive than NaAMPs, demonstrated by its inability to form a homopolymer under redox conditions. However in a copolymer system KSPA is incorporated into the chain under mild redox conditions so its inclusion controls the time taken for complete polymerisation to ocurr. When more aggressive initiating systems were utilised such as transition metals and peroxides, often an instantaneous nucleus of gelation was formed.

In this case it can be proposed that NaAMPs was rapidly polymerised excluding both KSPA and some water from the polymerisation resulting in an unsuitable system. When KSPA content was increased to a ratio of 3:2 to NaAMPs, although gelation

was effective, it was not reproducible. Therefore in an ascorbic acid oxone system the ratio is kept similar to 1:1 depending on the concentration of the NaAMPs used (58% or 50%).

The swell capacity of the copolymer was slightly less than that of the NaAMPs homopolymer. This is an ideal result as a less aggressive swell capacity reduces the possibility of damage to the surrounding tissues once the prostheses is *in situ,* this is particularly important in the IOL application. The reduction can be attributed to the absence of an amine group on the KSPA structure. To improve responsiveness and functionality further it may have been ideal to include a monomer which was disulfonated such as sulphopropyl itaconate dipotassium salt (KSPI). The addition of two sulfonate groups per monomer unit would have allowed the natural structure to be more successfully mimicked. However, as was expected after the lesser reactivity of KSPA, it was not possible to copolymerise with KSPI under redox conditions.

Initiators and crosslinking agents

The preferred initiator systems were ascorbic acid and Oxone and *N'N'N'N* tetramethylenediamine and potassium persulfate pair. This latter system successfully and reproducibly produced gels in a similar manner to the ascorbic acid and oxone system. Three possible crosslinking agents were investigated for use in the copolymer system. Poly(ethylene glycol) diacrylate Mn 575 (PEG575DA), poly (ethylene glycol) dimethacrylate Mn 1000 (PEG1000DMA), and Nelfilcon; a modified poly (vinyl alcohol). The latter is a multifunctional macromer, which has more reactive sites per unit weight in comparison to previous di-functional crosslinking agents. The copolymerisation reactions were carried out using PEG diacrylate, demonstrating its effectiveness as a crosslinking agent. Due to its relatively short chain length, it was possible to incorporate quite small percentages circa. 0.5% w/w in comparison to that of PEG dimethacrylate which with a chain length of 1000, it was not possible to include at such low levels. Both di-functional crosslinking agents produced efficient reproducible gels and their inclusion did not appear to alter the polymerisation time in any way. However in the case of the macromer, not only was it difficult to handle due to its viscous nature, it tended to induce a nucleus of gelation almost immediately even in small quantities circa 0.5%

under mild initiation conditions. Its use often resulted in incomplete gelation as the macromer rapidly reacted with NaAMPs, excluding some KSPA and water. This was caused by the increased available sites for propagating radicals on the macromer chain in comparison to a difunctional crosslinking agent. At higher levels of macromer, complete gels were formed, with much higher mechanical strength due to the rapid creation of short chains. Synuresis was a common occurrence during polymerisation due to the increased rate of polymerisation and the subsequent initial exclusion of water from the polymer structure. As expected the swell behaviour of a macromer containing copolymer was diminished due to the increased number of crosslinks and thus reduced mobility of the chains to swell. All of these factors make nelfilcon an unsuitable crosslinking agent for this application.

An important factor affected by composition and initiator system is the residual monomer concentration in the remaining polymer. The two systems investigated were the AA/ Oxone system and the TEMED potassium persulfate. Both systems produce a complete gel in approximately 5 mins. The residual monomer content as analysed by refractive index appears to be slightly lower at 0.27% for the TEMED initiated system than the ascorbic acid/oxone system at 0.4%. This could be due to the former being a more effective initiation system which is also demonstrated by its slightly faster polymerisation time.

Stability considerations

When refining the system for application in the intervertebral disc, the main factors to consider were as follows:

- Efficient and reproducible gelation
- Polymerisable in a timescale which allows for injection
- Stable under storage for at least one month
- Exhibits the required mechanical properties

The two systems to be further refined were both NaAMPs/KSPA copolymers but with different initiation systems. One the AA/ Oxone pair, and the other the TEMED and potassium persulfate pair. Prior to these investigations the gel components had been

assembled as one when required. To be deliverable *via* injection, whether added to a single syringe prior to use or utilising a dual syringe, need to be stored as a two-pack pregel system. To focus first on the ascorbic acid and Oxone system, it was discovered early on that as Oxone is the more aggressive initiator of the pair it could not be kept in a solution with NaAMPs as even refrigerated in the absence of light, premature initiation occurred within the storage vessel in excess of 7 days. This did not occur if stored with an aqueous solution of KSPA, not surprising giving its lesser reactivity. Therefore ascorbic acid was combined with NaAMPs, this was stable as long as the solution was protected from light and refrigerated, this was as expected as ascorbic acid is known to be photoactive. The next factor was the inclusion of the crosslinking agent. It was discovered that this could not be stored with NaAMPs as the combination of both a quite reactive monomer and possible oxidative degradation of the ascorbic acid often resulted in gelation within the storage vessel. This could not be reproducibly controlled, resulting in the incorporation of the crosslinking agent into the aqueous KSPA solution containing oxone. This produced two equal volume stable pregel solutions, redox A and redox B.

To summarise redox A was comprised of:

- Ascorbic acid 0.1M
- NaAMPs (50%)
- Water

Redox B:

- Oxone 0.1M
- KSPA
- Water
- Crosslinking agent ~PEG575DA

These solutions were prepared in the presence of ambient oxygen and not degassed. Degassing of components was found to induce polymerisation in less than one minute, entirely insufficient for the injectable disc application. Under ambient oxygen conditions polymerisation of this system after injection using a 19gauge

needle and 2.5ml syringes was in the region of 5-7 minutes with a storage stability of 1 month.

Understandably the exact amount of oxygen dissolved within a system cannot be controlled so there is a potential for the reproducibility of the stored systems to be in doubt. For this reason the use of a stabiliser such as dipicolinic acid was investigated to ascertain whether solutions could be purged of oxygen and their polymerisation controlled by other means. This was not successful, the additive appeared to alter the polymerisation, often hindering it all together.

A similar process was adopted with the TEMED and potassium persulfate initiated system. Due to the higher concentration of persulfate in comparison to oxone (potassium persulfate was used at 0.18M and oxone at 0.1M) on occasion there was a small nucleus of gelation formed within the KSPA and crosslinker solutions. For that reason the redox A and redox B pregel solutions for this composition was as follows.

Redox A:

- NaAMPs (50%)
- KSPA
- Water
- TEMED 0.4M

Redox B:

- Water
- KPS 0.18M
- Crosslinking agent ~ PEG1000DMA

Unlike the ascorbic acid and oxone system this cannot be used as a 1:1 ratio, instead it is used at a ration of 3:2 redox A to redox B

This system is stable under refrigerated storage in the absence of light for circa 6 weeks. As expected this system has slightly better storage stability due to the replacement of ascorbic acid with an amine initiator which is capable of producing

radicals in a much more controlled manner. This system gels within 3-5 minutes, allowing for injection during surgical manipulation of intervertebral disc repair.

Whilst investigating the injection of pre-gel solutions, it was discover that syringe size and needle size has an effect on gelation time. In general the longer the needle and the smaller the diameter, the quicker the polymerisation occurs. This was attributed to both shear forces and potentially metal mediated catalysis by the needle and was clearly demonstrated by polymerisation occurring in the region of 3 minutes with the use of a 22 gauge 178mm length discography needle. This was in comparison to a needle with a diameter of 0.5mm and a length of 16mm producing gelation within 30 seconds, an unsuitable time frame for the application.

Mechanical property considerations

The mechanical properties of the materials were investigated thoroughly *via* rheology. The properties of the analogue can be manipulated *via* three main factors, monomer composition, crosslink ratio, and water content. With respect to the intervertebral disc application, the elastic modulus of the intervertebral disc has been reported in the literature to range between 7 and 20kPa. Both gel compositions have been reported to have an elastic modulus within this range. Ascorbic acid and oxone redox pair produce the most uniform gel and reproducible elastic behaviour when used at 0.1M, which supports both the initiation studies and subsequent stability studies. The use of TEMED and KPS at 0.4M and 0.18M respectively visually appeared to produce the most efficient and reproducible gelation. This was supported by the recorded mechanical properties.

The use of PEG 575 diacrylate as a crosslinking agent was ranged from 0.25% to 2% and produced gels with elastic modulii ranging between 4kPa and 29kPa. The use of PEG 1000 dimethacrylate between 0.5% and 2% resulted in a range between 3.5kPa and 11kPa. Mechanically there is little preference between the two crosslinking agents, similar low stiffness can be obtained from the use of both just at 0.25% and 0.5% respectively.

The effect of polymerised water content also has an effect on the mechanical properties of the gel, a 20% increase in water content was reported to have the potential to reduced mechanical strength by 2kPa.

Injection into excised disc samples which were then analysed *via* DMA for comparison to the properties of the disc sample with intact nucleus and removed nucleus were conducted. The disc segment containing the gel showed some restoration of properties close to that of the segments containing the natural disc. This suggests than the gel does have the potential to restore disc height and function.

Adaptations to the system

In order to adapt the intervertebral disc analogue for suitability as an injectable IOL, it was necessary to incorporate the viscoelastic material that is required during surgery. A viscous material is necessary to prevent the surfaces of the lens capsule from coming into contact with each other and causing damage. This was initially investigated using HA derived from bovine vitreous humour. HA appeared to behave differently within the hydrogel depending on the nature of the intiation system. When gels were polymerised using ascorbic acid and oxone, HA appeared to become part of the structure, as an interpentrant. This was illustrated by the recorded increase in elastic behaviour of the gels in response to the amount of HA added. In the case of the TEMED potassium persulfate initiated system, there appeared to be less of a dependence on the amount of HA used within the gel composition. This was also apparent in the material's swell capacity. The HA gel displayed a lesser degree of swelling pressure in comparison to a similar composition in the absence of HA. This is advantageous as any excess pressure within the lens capsule has the potential to do damage to the tissue. Overall the most successful composition appeared to be one which incorporated the surgical viscoelastic Healon in place of water. This gel exhibited elastic behaviour in the region of 3kPa, flexible enough to allow natural accommodation to occur once injected into the eye.

Surgical procedure would ideally require the injectable gel to be radio-opaque to allow its visualisation during surgery and its lifetime. There are many radio-opaque agents however the only commercially available to us was histodenz. Initially

attempts were made to modify this into a radioopaque macromer which would also have the potential to act as a crosslinking agent. Due to the symmetrical nature of the histodenz structure and its difficult solubility, this was not successful. Currently it is hoped that the histodenz will be visible at 5% within the gel and it will remain in the gel long enough to see the gel outline for as long as is needed. Current US opinion from interested parties has suggested preference for the gel to be available without a radio-opaque.

Further work

With respect to the intervertebral disc the natural progression would be the development of a suitable surgical delivery system, prior to testing in animal subjects. For the IOL application, the next step would involve an *in-vivo* eye model to ascertain the ability of the pre-gel to remain within the capsular bag prior to its complete gelation. The addition of an optional UV initiation step to completely cure the gel could also be explored in a similar manner to the way crosslinking can occur within the eye.

Further work is being carried out within the Biomaterials Research Unit to functionalise a radio-opaque macromer based on the addition of *N*-methylol acrylamide to diatrizoic acid and or 2,5 iodobenzoic acid. It is also proposed to synthesise a new radio opaque from an iodinated benzoyl chloride as is shown in figure 8.1

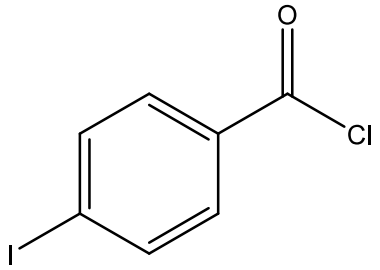


Fig 8.1 Iodinated benzoyl chloride

In order to create a more iodinated structure a di-iodinated benzoic acid shown below, can be converted to a benzoyl chloride by reaction with $CHCl_3$ and $SOCl_2$.

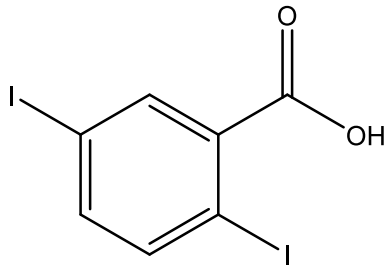


Fig 8.2 Diiodobenzoic acid

The project will involve reaction of an iodinated benzoyl chloride with a monomer containing a hydroxyl group to yield an ester under pyridine or NaOH (to react with the HCl formed). This would then need to be purified and the yield assessed, then characterised using FTIR and NMR.

Another approach involves the reaction of the iodinated benzoyl chloride with NaAMPs either in the ratio of 1:2 or using NaOH to 'mop up' the HCl produced, to synthesise a benzamide.

The second stage of the project will involve the conversion of the synthesised iodinated ester or amide into a macromer. Primarily NMA N-(hydroxyl methyl)-acrylamide, is attached to the ester by heating in solution, at 60^{o}C for 2hrs under an acid or base catalyst to provide a reactive double bond.

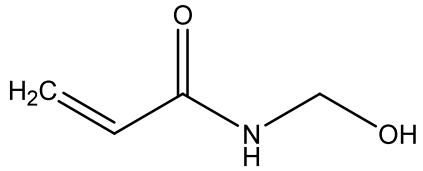


Fig 8.3 N- (hydroxy methyl) acrylamide

Via the selection of appropriate hydrophilic monomers such as tris hydroxyl methyl methyl acrylamide, this is then reacted to form the macromer. This macromer will then need to be purified and characterised again by FTIR and NMR and its radio-opacity assessed.

A further alternative would involve the direct addition of NMA to 2,5, -diiodobenzoic shown in figure 8.1 and/or ditriazoic acid the structure of which is shown below in figure 8.4

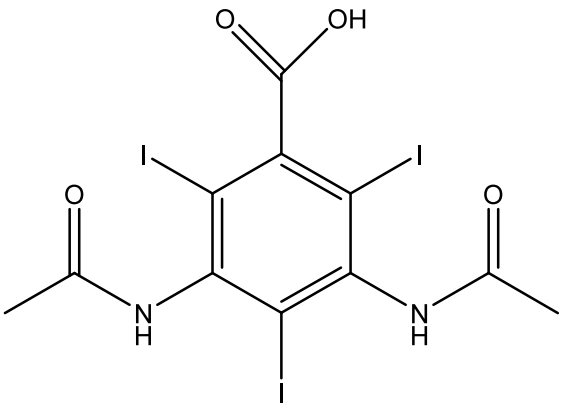


Fig 8.4 Diatrizoic acid

The functionalisation reactions are extremely time consuming and were not a major focus of the work detailed in this thesis, more an advantageous addition. Hence they feature significantly in the suggestions for further work.

References

(1) Apple D.J., Mamalis M., Olson R.J., Kincard M.C. (1989) Intraocular Lenses: Evolution, Designs, Complications, and Pathology. Baltimore, MD. Williams and Wilkins

(2) Wichterle, O and Lim D, (1960) Hydrophillic gels for biological use. *Nature*, 185, 117-118

(3) Berger J., Reist M., May JM., Felt O., Gurny R., (2004) Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. *European Journal of Pharmaceutics and Biopharmaceutics*. 57, 1035-52

(4) Peppas NA., Bures P., Leobanding W., Ichikawa H., (2000) Hydrogels in pharmaceutical formulations. *European Journal of Pharmaceutics and Biopharmaceutics* 50 (1) 27-46

(5) Tighe B.J., (2002) Chapter 5 "Softlens Materials in Efron N., (Ed) Contact Lens Practice, Butterworth Heineman

(6) Tonodekauiu S., Prasitsilp M., Thakornyuitkam B., Pothsree T., Pateepasen R., (2004) Preparation of acrylic grafted chitin for wound dressing applications. *Biomaterials,* 25, (7-8) 1453-1460

(7) Chen J., Yong Y., Qian P., Ma Z., Wu W., Sing P., Wang X., Li J., (1993) Drug carrying hydrogel base wound dressing. *Radiation Physics and Chemistry* 42 (4-6) 915-918

(8) Hoffman A.S., (2002) Hydrogels for biomedical applications. Advanced Drug Delivery Review, 43, 3-12

(9) Corkhill P.H., Hamilton C.J., Tighe B.J., (1989) Synthetic Hydrogels VI. Hydrogel composites as wound dressings and implant materials, *Biomaterials* 10 (1) pp.3-10

(10) Eidelson S.G., (1999) Available at: http://static.spineuniverse.com/displaygraphic.php/137/dp_latcutaway-BB.jpg Date accessed 9/12/2010

(11) University of Virginia Health System: Spinal Centre (2010) Available at: http://www.uvaspine.com/images/syndicated/AxialViewOfDisc_labeled.jpg Accessed 9/12/2010

(12) Stoeckelhuber M., Brueckner S., Spohr G., Welsch U., (2005) Proteoglycans and collagen in the intervertebral disc of the rhesus monkey (Macaca mulatta) *Ann Anat* 187 pp 35.42

(13) Berthet Colominas C., Miller A., Herbage D., Ronziere M.C., Tocchetti D., (1982) Structural studies of collagen fibres from intervertebral disc. *Biocheimica et Biophysica Acta* 706 (1) pp. 50-64)

(14) Ljubimov, A.V., Alba, S.A., Burgenson, R.E., Ninomiya, Y., Sado, Y., Sun, T-T., Nesburn A.B., Kenney, M.C., Magues, E., (1998) Extracellular matrix changes in human corneas after radial keratotomy. *Exp Eye Res* 67, pp.265-272.

(15)http://en.wikipedia.org/wiki/Image:Schematic_diagram_of_the_human_eye_en.svg (2010) Date accessed 9/12/2010

(16) Abolmaali, A., Schachar, R.A., Le, T., (2007) Sensitivity study of human crystalline lens accommodation *Computer Methods and Programs in Biomedicene* 85, 77-90

(17) Augusteyn R.C., (2008). Growth of the lens: in vitro observations. *Clin. Exp. Optom.* 91.3.pp226-239

(18) Coghlan S.D., Augusteyn R.C. (1977) Changes in the distribution of proteins in the aging human lens. *Exp. Eye. Res* 25.pp603-611

(19) Fagerholm PP., Philipson BT., Lindstrom B., (1981) Normal human lens: the distribution of protein. *Exp Eye Res*, 31, pp.615-620

(20) Tones C., Atchinson DA., Meder R., Pope JM., (2005) Refractive index distribution and optical properties of the isolated human lens measured using magnetic resonance imaging (MRI) *Vision Research* 45 2352-2366

(21) Torzilli, P.A., Arduino, J.M., Gregory, J.D., Bansal, M., (1997) Effect of proteoglycan removal on solute mobility in articular cartilage. *J. Biomechanics* 30(9) pp.895-90

(22) Bullough, P., Fellow, J., (1968) The significance of the fine structure of articular cartilage. *J.Bone and Joint Surgery.* 50B pp.852-857.

(23)http://www.google.co.uk/imgres?imgurl=http://kentsimmons.uwinnipeg.ca/cm1504/Image122.gif&imgrefurl=http://kentsimmons.uwinnipeg.ca/cm1504/cellwall.htm&h=600&w=749&sz=98&tbnid=jXpr-uo4-0T2_M:&tbnh=113&tbnw=141&prev=/images%3Fq%3Dextracellular%2Bmatrix&zoom=1&q=extracellular+matrix&usg=__J41a0-

DzYvbq4HJU3zPCKy-MP7Y=&sa=X&ei=_uCPTeX4KqiJ4gb0jKX9Cw&ved=0CEoQ9QEwBA Accessed 8/11/2010

(24) Rostand K.S., and Esko J.D., (1997) Microbial adherence to and invasion through proteoglycans' *Infect.Immun.,* 65, 1-8

(25) Schmidtchen, A., Frick, I. and Bjorck L., (2001) Dermatan sulfate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha fusion. *Mol. Microbiol.* 39, 708- 713

(26) Iatridis JC., Gwynn I., (2004) Mechanisms for mechanical damage in the intervertebral disc annulus fibrosus *Journal of Biomechanics* 37 pp.1165-1175

(27) Adams, M.A., Dolan, P., (2005). Perspective: Spine biomechanics. *Biomechanics* 38 p.1972-1983

(28) Maniadakis, N., and Gray, A., (2000). The economic burden of back pain in the UK *Pain,* 84 p.95-103

(29) Moffett, KJ., Richardson, G., Sheldon, T., Maynard, A. (1995) Back pain: its management and cost to society. Discussion Paper 129, York: York University, Centre for Health Economics

(30) Strasser A., (1999) Available at :http://www.spineuniverse.com/conditions/herniated-disc/chiropractic-care-back-pain-non-invasive-treatment Date accessed 9/12/10

(31) St John Providence Health System (2010) Available at: http://www.stjohnprovidence.org/StaywellTesting/img.aspx?7481 Date accessed 09/10/2010

(32) Pope R.E., (2010) The ProDisc-L. Available at: http://www.spinesurgeon.com.au/modalities/artifiscial_discs/ProDiscL_combined.jpg Date accessed 09/12/2010

(33) Malhotra, R., (ed.) (2008) Cataract, Edinburgh, Butterworth Heinemann

(34) Lloyd, A.W., Dropcova, S., Mikhalovsky, S.V., Denyer, S.P., Gard P.R., Hanolan, G.W., Ollift. C., Muir, A., Jones. S., Rosen. P., Riding, M.A., (1996) Biocompatible Coating for IOLs. *Vision Research* 36, 73

(35) Available from http://doctor-hill.com/iol-main/polypseudophakia.htm, Accessed 7/7/2010

(36) Walling, C., (1957) Free radicals in solution, New York, Wiley; Chapman and Hall

(37) Sarac A.S., (2009) Redox Polymerisation *Prog Polym Sci* 24 pp.1149-1204

(38) Narain H., Jagadale S.M., Ghatage N.D., (1981) Studies of redox polymerisatio I. Aqueous polymerisation of acrylamide by an ascorbic acid peroxydisulfate system. *J Polym Sci* 19:1225

(39) Wayne, C.E., Wayne, R.P., (1996) In Photochemistry, New York, Oxford University Press

(40) Decker, C., (2002) Kinetic Study and New applications of UV Radiation Curing. *Macramol. Rapid. Commun.* 23, pp1067-1093.

(41) Dietliker, K.K., (1991) Chemistry and Technology of UV and EB formulations for coatings, inks and paints, vol 3 Photoinitiators for radical and cationic polymerisation. SITA Technology Ltd London (3)

(42) Decker, C., (2002) Kinetic Study and New applications of UV Radiation Curing. *Macramol. Rapid. Commun.* 23, pp1067-1093.

(43) Sovak, M.,(Ed) (1984) Radiocontrast Agents. Springer Verlag, Berlin Germany

(44) Nukina et al Sanyo Chemical Industries Ltd (1973) *Method for preparing salts of sulfoalkyl esters* US Patent 3,759,985

(45) IARC (1974) Some aromatic amines, hydrazine and related substances, N-nitroso compounds and miscellaneous alkylating agents. *IARC monographs on the evaluation of carcinogenic risk to humans Vol 4*. International Agency for Research on Cancer, Lyon, France pp.286

(46) IARC (1999), Hormonal contraception and post menopausal hormonal therapy. *IARC monographs on the evaluation of carcinogenic risk to humans Vol 71*. International Agency for Research on Cancer, Lyon, France pp.1095

(47) Harrison et al, Haas Company (1970) *Process for manufacturing sulfonated amides* US Patent 3,544,597

(48) Narain H., Jagadale S.M., Ghatage N.D., (1981) Studies of redox polymerisatio I. Aqueous polymerisation of acrylamide by an ascorbic acid peroxydisulfate system. *J Polym Sci* 19:1225

(49) DuPont (2010) Denture Cleaners. Available at: http://www2.dupont.com/Oxone/en_US/uses_apps/denture_cleansers.html Accessed 21/09/2010

(50) Buhler, N., Haerri, H.P., Hofmann, M., Irrgang, C., Muhlebach, A., Muller, B., and Stockinger, F., (1999) Nelfilcon A, A New Material for Contact Lenses, *Chimia* 53, 269-274

(51) Bilmeyer F.W., (1984) *Textbook of Polymer Science.* Wiley

(52) Flory P.J. (1953) *Principles of Polymer Chemistry.* Cornell University Press, London pp.77

(53) Boote, N.D., (2007) *Photopolymerisation of Sheet Hydrogels: A Mechanistic Study.* PhD. Birmingham:Aston University

(54) Miller, C.H., Arenberg, J.K., Altshuluer, J.H., (1989) Fibrin glue delivery system US004874368

(55) Anipsitakis, G.P., Dionysiou, D.D., (2004) Radical generation by the interaction of transition metals with common oxidants. *Environ Sci Technol* 38 (13) 3705-3712

(56) Dada et al. (2010) Dilute aqueous peracid solutions and stabilisation method US 2010/0021558

(57) Tehrani, M., Dick, H.B., Paklua, T., Wolf, E., (2004) Material Properties of Various Intraocular Lenses in an Experimental Study. *Ophthalmologica* 218 (1)

(58) Young, N., (2003) The Development of a Biomimetic Repair System for IVD, BRU Aston University MSc project