Design and synthesis of proteoglycan analogues for tissue repair and regeneration

Jane Bramhill

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DESIGN AND SYNTHESIS OF PROTEOGLYCAN ANALOGUES FOR TISSUE REPAIR AND REGENERATION

By

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Design and Synthesis of Proteoglycan Analogues for Tissue Repair and Regeneration

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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
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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 \(\text{sp}^3\) carbon atoms to form a
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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.

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

1.2.3 Synthetic hydrogels

Figure 1.2.3.1 shows some common monomers used to make hydrogels, and polymers used as interpenetrants 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.
Introduction

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.
Introduction

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.
Introduction

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\textsuperscript{(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\textsuperscript{(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
Introduction

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**

**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
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\(^{(15)}\)](image)

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.
Introduction

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. Entrainment 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 ciliary 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 synovial 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 viability. 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 viability.

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 $\text{Na}^+$ 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\textsuperscript{(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\textsuperscript{(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 initiators 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 is shown below in figure 1.7.2.4.

*Fig 1.7.2.4 The action of the ascorbate radical*
Introduction

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\(^{(36)}\).

\[
\begin{align*}
I & \rightarrow 2R^• \\
R^• + M & \rightarrow RM^•
\end{align*}
\]

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

\[
\begin{align*}
RM^• + M & \rightarrow RM_2^• \\
RM_n^• + M & \rightarrow RM_{n+1}^•
\end{align*}
\]

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

\[
\begin{align*}
RM_n^• + RM_m^• & \rightarrow R_{2M_{n+m}}^• \\
RM_n^• + RM_m^• & \rightarrow RM_{n+} + RM_{m}
\end{align*}
\]

I= initiator  
R•= radical species  
M= monomer  
RM•= monomer with radical as chain end  
RM\(_2^•\)= 2 monomer units with radical as chain ends  
RM\(_n^•\)= n monomer units with radical as chain ends  
RM\(_m^•\)= 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.

1.7.4 Fotopolymerisation

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. This method of initiating polymerisation is predominantly used in the production of coatings and adhesives. 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. 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.

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.
Introduction

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.

\[ \tan \delta = \frac{G''}{G'} \]

\( G'' \) = 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° or 4° 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.
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.
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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Formula</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-ascorbic acid (AA)</td>
<td>C6H8O6</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Oxone</td>
<td>2KHSO5·KHSO4·K2SO4</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Potassium persulfate</td>
<td>K2S2O8</td>
<td>Sigma Aldrich</td>
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<td>Iron II lactate hydrate</td>
<td>C8H10FeO6aq</td>
<td>Fluka</td>
</tr>
<tr>
<td>Iron II gluconate</td>
<td>C12H22FeO14aq</td>
<td>Fluka</td>
</tr>
<tr>
<td>Dimethyl paratoluidine (DMT)</td>
<td>C9H13N</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>2,2 azo bis (2-methyl propionamide)</td>
<td>C8H18N6·2ClH</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>dihydrochloride</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N’N’N’N tetramethylene diamine</td>
<td>C6H16N2</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>(TEMED)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoyl peroxide (BP)</td>
<td>C14H10O4</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Tertiary-butyl hydroperoxide</td>
<td>C4H10O2</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>(NH4)2S2O8</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H2O2</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>
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]
Materials and Methods

1. Acryloylmorpholine (ACMO), [Sigma-Aldrich]

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

3. Nelfilcon - modified PVA [Cibavision]
Materials and Methods

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

2.2 Methods

2.2.1 Typical gel procedure

For example a typical composition consists of:

<table>
<thead>
<tr>
<th>Gel</th>
<th>NaAMPS (50%)</th>
<th>KSPA</th>
<th>Water</th>
<th>Crosslinking agent PEG575DA</th>
<th>Ascorbic acid 0.1M</th>
<th>Oxone 0.1M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rf1</td>
<td>3.7 ( 1.6 \times 10^{-3} ) mol</td>
<td>1.4g ( 0.6 \times 10^{-3} ) mol</td>
<td>3.6g ( 0.2 ) mol</td>
<td>0.2g ( 3.5 \times 10^{-4} ) mol</td>
<td>0.65g ( 6.5 \times 10^{-4} ) mol</td>
<td>0.65g ( 6.5 \times 10^{-4} ) mol</td>
</tr>
</tbody>
</table>

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 vials 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 viability 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°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 (\(\tan \delta\)) 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 2 weeks. 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>
<thead>
<tr>
<th>ELEMENT</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>S</th>
<th>Cl</th>
<th>Br</th>
<th>I</th>
<th>O</th>
<th>Na</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Theory</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Found 1</td>
<td>2.68</td>
<td>11.68</td>
<td>0.46</td>
<td>0.68</td>
<td>1.24</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Found 2</td>
<td>2.91</td>
<td>11.65</td>
<td>0.51</td>
<td>0.63</td>
<td>1.28</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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°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°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