Injectable Bioadhesive Hydrogels for Nucleus Pulposus Replacement and Repair of the Damaged Intervertebral Disc

A Thesis
Submitted to the Faculty of Drexel University
By Jennifer Vernengo
in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy January 2007
Dedications

To my parents,

MariaInez and Guillermo Vernengo
Acknowledgements

First and foremost, I would like to thank my parents, to whom I feel gratitude beyond what I can articulate. They have provided me with unwavering support, reassurance, generosity, and advice. Without them, I could not have completed this thesis. I could spend a lifetime trying to give back what my parents gave to me.

I am also very grateful to my advisor, Dr. Tony Lowman. Without having met him during my senior year at Drexel University, I would not be here today. He was an exceptional advisor and has given me constant encouragement, strong support, and invaluable guidance. I will always look back on my experience in graduate with fondness, and this is due, in large part, to having him as my advisor. Tony, thank you for giving me the opportunity to work in your laboratory.

I would also like to thank my committee members who have been advising me since my first proposal in 2004: Garland Fussell, Michele Marcolongo, Guiseppe Palmese, and Yossef Elabd. In particular, I would like to thank Garland for the great deal of guidance and humor he has provided over the years.

Speaking of humor, I would also like to acknowledge the current and former members of the Lowman Lab who have become my extended family. I hope these friendships will
extend beyond the time we shared together in the Lowman lab. Specifically, I would like to thank Jonathan Thomas, Tony Tuesca, Kara Spiller, Noelle Comolli, Vanessa Vardon, Sam Laurencin, Karri Momyer, Jason Colman, Jenna Tulskie, Erik Brewer, Kris Kita, Eric Perakslis, and Jamie Ostroha. Meredith Hans deserves a special thanks not only for her friendship, but for her technical contributions to my project as well. I would be remiss without mentioning Larry Matthews. Having him around to help during my last year has been priceless. I would also like to thank Dr. Hung Lee and Dr. Dunja Radisic for their assistance with NMR, as well as Amy Arthur for her guidance with cadaver testing and mechanics.
Table of Contents

List of Figures ......................................................................................................................... vi
List of Tables .............................................................................................................................. ix
Abstract ........................................................................................................................................ x

Chapter 1 - INTRODUCTION ............................................................................................... 1

Chapter 2 - BACKGROUND ................................................................................................. 4
  2.1 Anatomy of the Spine ........................................................................................................ 4
  2.2 Intervertebral Disc Biomechanics .................................................................................. 12
  2.3 Intervertebral Disc Degeneration .................................................................................. 14
  2.4 Treatment for Lower Back Pain .................................................................................... 18
    2.4.1 Conservative Treatments ........................................................................................... 18
    2.4.2 Current Surgical Interventions .................................................................................. 21
    2.4.3 Nucleus Pulposus Replacement ............................................................................... 39
  2.5 Poly(N-isopropylacrylamide) ....................................................................................... 47
    2.5.1 Thermodynamics of the Phase Transition .............................................................. 47
    2.5.2 LCST Determination ............................................................................................... 50
    2.5.3 LCST Modulation .................................................................................................... 53
    2.5.4 PNIPAAm-based Hydrogels ..................................................................................... 56
    2.5.5 PNIPAAm-based Systems for Drug Delivery ........................................................ 60
  2.6 Poly(ethylene glycol) .................................................................................................... 68
  2.7 Poly(ethylene imine) ..................................................................................................... 74
  2.8 Bioadhesive Polymers for Soft Tissue Repair ............................................................. 79

Chapter 3 - RESEARCH GOALS ......................................................................................... 105

Chapter 4 - SYNTHESIS OF PNIPAAm-PEG BRANCHED COPOLYMERS ........ 107
  4.1 Introduction .................................................................................................................... 107
  4.2 Experimental Section .................................................................................................... 109
    4.2.1 Materials .................................................................................................................. 109
    4.2.2 Methods .................................................................................................................. 109
4.2.2.1 Methacrylation of PEG ................................................................. 109
4.2.2.2 Copolymer Synthesis ................................................................. 110

4.3 Results and Discussion ........................................................................................................................................ 111
4.3.1 Methacrylation of PEG ................................................................. 111
4.3.2 Synthesis of PNIPAAm-PEG Branched Copolymers ................. 111

4.4 Conclusions .................................................................................................................................................... 113

Chapter 5 - CHARACTERIZATION OF PNIPAAM-PEG BRANCHED COPOLYMERS ........................................... 119

5.1 Introduction ................................................................................................................................................... 119
5.2 Experimental Section .................................................................................................................................. 120
  5.2.1 Methods ............................................................................................................................................... 120
    5.2.1.1 LCST Characterization ............................................................ 120
    5.2.1.2 Gel swelling and Dissolution .................................................. 121
    5.2.1.3 Mechanical Properties ............................................................ 122
      5.2.1.3.1 Compressive Studies ......................................................... 122
      5.2.1.3.2 Stress Relaxation Studies .................................................. 122
    5.2.2 Statistical Analysis ................................................................. 123

5.3 Results ......................................................................................................................................................... 123
  5.3.1 LCST Characterization ............................................................... 123
  5.3.2 Swelling and dissolution experiments ....................................... 124
  5.3.3 Compressive mechanical studies .......................................... 125
    5.3.3.1 Network Analysis with Rubber Elasticity Theory .................. 126
  5.3.4 Stress Relaxation Studies ......................................................... 128

5.4 Discussion ................................................................................................................................................. 128

5.5. Conclusions ................................................................................................................................................. 132

Chapter 6 - OPTIMIZATION STUDIES TO PREVENT WATER LOSS IN VIVO ................................................. 142

6.1 Introduction ............................................................................................................................................... 142

6.2 Experimental Section .................................................................................................................................. 143
  6.2.1 Materials ................................................................................................................................. 143
  6.2.2 Methods ................................................................................................................................. 143
  6.2.3 Statistical Analysis ................................................................................................................. 144

6.3 Results and Discussion ................................................................................................................................ 145
List of Figures

Figure 2.1 Anterior view of the vertebral column showing the cervical, thoracic, lumbar, and sacral regions................................................................. 90

Figure 2.2 The division of the vertebrae into three regions................................................. 91

Figure 2.3 (A) Lateral view, (B) superior view, (C) inferior view of the vertebra........... 92

Figure 2.4 The structure of the intervertebral disc. It consists of a nucleus pulposus (NP) surrounded by an annulus fibrosis (AF). Both are sandwiched between two vertebral endplates (VE). ........................................................................ 93

Figure 2.5 A cylindrical interbody fusion cage. A, anterior; P, posterior......................... 94

Figure 2.6 Photograph of the Type III SB Charité............................................................ 95

Figure 2.7 The Charité artificial disc consists of a free-floating biconvex sliding core encased in concave endplates......................................................... 96

Figure 2.8 Photograph of the Prodisc-L............................................................................ 97

Figure 2.9 Photograph showing the Maverick disc. ......................................................... 98

Figure 2.10 An implanted Prosthetic Disc Nucleus (PDN) device................................. 99

Figure 2.11 The Newcleus spiral implant ........................................................................ 100

Figure 2.12 The chemical structure of poly(N-isopropylacrylamide) (PNIPAAm)........ 101

Figure 2.13 The chemical structure of poly(ethylene glycol) (PEG)......................... 102

Figure 2.14 The chemical structure of poly(ethylene imine) (PEI)......................... 103

Figure 2.15 The chemical structure of glutaraldehyde. .............................................. 104

Figure 4.1 Schematic for the synthesis of PEGDM....................................................... 114

Figure 4.2 Schematic for the synthesis of PNIPAAm-PEG branched copolymers ...... 115

Figure 4.3 $^1$H NMR spectrum for PEGDM in deuterium oxide at 25°C ...................... 116
Figure 4.4 The changes in spectra over reaction time for PNIPAAm-PEG branched copolymers.
........................................................................................................................................ 117

Figure 4.5. NMR Spectra for purified PNIPAAm-PEG branched copolymer s in deuterium oxide at 25ºC.
........................................................................................................................................ 118

Figure 5.1 LCST and enthalpy of transition as a function of weight percent PEG in PNIPAAm-PEG branched copolymers.
........................................................................................................................................ 135

Figure 5.2 Effect of PEG block molecular weight on gel water content ......... 136

Figure 5.3 Effect of PEG block size on the equilibrium compressive modulus of PNIPAAm-PEG branched copolymers.
........................................................................................................................................ 137

Figure 5.4 Stress-strain behavior of PNIPAAm-PEG branched copolymers at 90 days immersion in PBS, normalized to account for the swelling of the gel .... 138

Figure 5.5 Modulus, $G$, for the high PEG content PNIPAAm-PEG branched copolymers at 90 days immersion in PBS, normalized to account for differences in the swelling behavior of the gels................................................................. 139

Figure 5.6 The effective molecular weight between crosslinks, $M_c$, for the high PEG content PNIPAAm-PEG branched copolymers at 90 days immersion in PBS .... 140

Figure 5.7 Effect of immersion time in vitro on the compressive modulus at 15% strain of high PEG content (a) and low PEG content (b) PNIPAAm-PEG branched copolymers................................................................. 141

Figure 6.1 Effect of immersion media osmotic pressure on the water content of PNIPAAm-PEG (4600 g/mol) ................................................................. 149

Figure 6.2 Effect of solution concentration at room temperature on equilibrium water content of PNIPAAm-PEG (4600 g/mol) gels......................................................... 150

Figure 6.3 Effect of thermally cycling the polymer solution by equilibrating in a 37 ºC osmotic environment, followed by cooling to room temperature............... 151

Figure 7.1 The $^1$H NMR spectrum for PNIPAAm-PEG/PEI ($M_n=60,000$ g/mol) blend in deuterium oxide at 25ºC................................................................. 173

Figure 7.2 The chemical stability of PNIPAAm-PEG/PEI ($M_n=10,000$) and PNIPAAm-PEG/PEI (60,000 g/mol) blends ................................................................. 174

Figure 7.3 The FTIR spectra for a dry (A) PNIPAAm-PEG/PEI gel, (B) glutaraldehyde/PEI mixture, (C) PNIPAAm-PEG/PEI gel injected with glutaraldehyde................................................................. 175
Figure 7.4 FTIR difference spectroscopy for the analysis of PNIPAAm-PEG/PEI
crosslinked gels............................................................................................................... 176

Figure 7.5 Release profile of free glutaraldehyde from the PNIPAAm-PEG/PEI network
........................................................................................................................................ 177

Figure 7.6 The water content over a 30 day immersion in PBS for PNIPAAm-PEG/PEI
blends with and without glutaraldehyde injection ....................................................... 178

Figure 7.7 The compressive modulus of PNIPAAm-PEG/PEI gels at 15% strain with and
without glutaraldehyde crosslinking at 7 days immersion in PBS .............................. 179

Figure 7.8 Typical stress strain curves for the in vitro bioadhesive force studies........ 180
List of Tables

Table 5.1 Equilibrium water contents and compressive moduli for PNIPAAm-PEG after 90 days immersion *in vitro* ...................................................................................................................... 134

Table 7.1 The mean maximum force and work of adhesion required to detach the PNIPAAm-PEG and PNIPAAm-PEG/PEI gels from the biological substrates..... 172
Researchers have begun to realize the potential benefits of treating intervertebral disc degeneration by replacing the nucleus pulposus. One of the material classes being studied for nucleus replacement is the hydrogel, a three-dimensional, hydrated polymer network. The development of an injectable hydrogel nucleus replacement would have important clinical consequences because it could be injected non-invasively using a needle. Aqueous solutions of poly(N-isopropylacrylamide), or PNIPAAm, have a lower critical solution temperature (LCST) between room and body temperature, making it suitable for an injectable implant material. Aqueous polymer solutions could be injected as a free flowing liquid at 25°C and solidify to a gel within the body at 37°C.

At physiological temperatures, PNIPAAm homopolymer gels hold little water and show poor elastic recovery. In the work, the swelling and mechanical properties of PNIPAAm gels are tailored by polymerizing NIPAAm in the presence of poly(ethylene) glycol dimethacrylate (PEGDM), thereby creating PNIPAAm-PEG branched copolymers. The effect of PEG molecular weight and NIPAAm/PEG molar ratio on the water content, stiffness, and elasticity of the hydrogels was determined. In addition, a suitable material candidate for nucleus pulposus replacement was chosen from this class of hydrogels. The copolymer formulation was optimized to minimize implant water loss following
implantation into the intradiscal environment, allowing the implant to remain space filling in the nuclear cavity. Bioadhesive properties were then imparted to the hydrogel system by blending it with the amine containing polymer poly(ethylene imine) (PEI). After gelation in the disc, the implant will be crosslinked with itself and with the surrounding tissues by the injection of a dialdehyde. This approach will help secure the implant in place, reducing the risk of implant migration or extrusion. The bioadhesive material also has the potential to function as tissue adhesive for the repair of the damaged annulus fibrosis.
Lower back pain, one of the most common pain conditions in the western world, is responsible for significant economic and social costs. In the United States alone, it accounts for more than sixty billion dollars annually in health care costs. One of the major causes of lower back pain is intervertebral disc degeneration. Disc degeneration is the result of damage to or dehydration of the nucleus pulposus, which reduces its hydrostatic pressure on the internal surface of the annulus fibrosis. This reduced hydrostatic pressure results in abnormal compressive stresses on the annulus, potentially causing tears, cracks and fissures after repeated loads. Back pain can develop as a result of nucleus tissue migrating through the annulus and impinging on nerve roots.

Currently, the most common surgical treatment for the degenerative disc is spinal fusion, but this causes loss of spinal mobility and increases stress on adjacent discs, accelerating degeneration of these joints. Total disc arthroplasty is emerging as a viable alternative to fusion because more spinal motion is retained. However, because these treatments involve highly invasive surgeries, a promising alternative is the replacement of the nucleus pulposus alone. There are several pre-formed polymeric implants currently under investigation for nucleus pulposus replacement. While some of these materials are promising, using an in situ forming nucleus replacement could have important clinical consequences, because it can be injected non-invasively using a small gauge needle.
In situ gel formation can be achieved using the thermo-responsive polymer poly(N-isopropylacrylamide) (PNIPAAm). Aqueous solutions of PNIPAAm undergo a phase transformation at its lower critical solution temperature (LCST), typically around 32°C, allowing it to form a free flowing solution in water at ambient temperatures and a gel at body temperature \(^5\). This property makes it ideal for an injectable nucleus pulposus replacement.

This work focuses on designing an in situ forming PNIPAAm-based polymeric nucleus pulposus replacement. Because the PNIPAAm homopolymer holds little water and shows poor elastic recovery, the swelling and mechanical properties of PNIPAAm gels are tailored by polymerizing NIPAAm in the presence of a small amount of poly(ethylene) glycol macromers, thereby creating PNIPAAm-PEG branched copolymers. It is hypothesized that a suitable candidate from this class of materials can be developed, which upon implantation, could prevent or postpone the annular degeneration process by restoring the healthy biomechanics of the intervertebral disc and alleviate the pain associated with degenerative disc disease.

The work will begin with the synthesis of a class of hydrogels composed of PNIPAAm and PEG. The molecular weight of the PEG chains and the relative proportions of NIPAAm and PEG units will be varied. Then, the range of pertinent material behavior for the PNIPAAm-PEG family of copolymers, and how it varies with polymeric structure will be examined. Based on these characterization studies, a material candidate which exhibits suitable, stable material properties will move on for further evaluation as a
material candidate for nucleus pulposus replacement. The copolymer formulation will then be optimized to minimize water loss and subsequent volume loss, following injection into the intradiscal environment due to the de-swelling of the polymer network. Finally, the copolymer chemistry will be modified to impart bioadhesive properties to the hydrogel system. This will help secure the implant in place, reducing the risk of implant migration or extrusion. With these bioadhesive characteristics, the material will also have the potential to perform as a closure material for the repair of annular defects.
Chapter 2 - BACKGROUND

2.1 Anatomy of the Spine

The back, or dorsum, is the posterior part of the body and includes the skin, muscles, vertebral column, spinal cord, nerves, and blood vessels. The vertebral column consists of 24 moveable vertebrae (Figure 2.1), 7 cervical, 12 thoracic, and 5 lumbar. Below the lumbar vertebrae are 5 sacral vertebrae, which in the adult body, fuse to form the sacrum. The three to five lowermost vertebrae fuse in late adult life to form the coccyx. These bones are joined together by a series of joints to form the vertebral column.

The main functions of the human spine are to support the body, protect the spinal cord and spinal nerve roots, and allow for movement of the trunk. These functions are carried out not only by the vertebrae, but the soft tissues surrounding them. The adult vertebral column has several visible curves. The cervical and lumbar regions are anteriorly convex, and the thoracic and sacral areas are posteriorly convex. The spinal curves are able to dissipate loads more efficiently than if the spine were a straight column.

A typical vertebra may be divided into three main regions: the vertebral body, the pedicles, and the posterior elements (Figure 2.2). The anterior part of each vertebra is a large block of bone called the vertebral body. The pedicles connect the back of the vertebral body to the posterior elements. The posterior elements consist of the lamina,
superior articular arch, inferior articular, transverse, and spinous processes (Figure 2.3). The lamina are formed from projections of bone extending from the pedicles. Two lamina meet one another to form the neural arch, which houses the neural elements of the vertebral column. The right and left inferolateral corners of each lamina extend downwards to form a large mass of bone called the inferior articular process. Similarly, the superior right and left portions of the lamina extends upwards to form the superior articular process. Each of these four processes possess a smooth region on the surface covered by articular cartilage, known as the articular facet of each process. Projecting posteriorly from the junction of the two lamina is a blade of bone called the spinous process. These bones form visual projections under the skin. Extending laterally from the junction of the pedicle and the lamina is a flat bar of bone called the transverse process.

The vertebral body is designed to impart load-bearing characteristics to the vertebrae. The flat superior and inferior surfaces of the vertebral bodies contribute to the stability of the vertebrae under longitudinal, or compressive, loads. Its internal architecture also contributes to its ability to support weight. The bone in this region is composed of an outer layer of dense cortical bone and a core of spongy cancellous bone. While the cortical bone allows the bone to sustain static loads, the cancellous bone makes the vertebral body resilient, or able to withstand sudden and dynamic loading. The cancellous bone is composed of thin rods of bone called trabeculae that extend in the vertical and transverse directions. Applied compressive loads are first supported by the vertical trabeculae and are then transferred to the horizontal trabeculae in transverse
tension. Both the vertical and transverse trabeculae increase in numbers after prolonged or increased compressive loading. Also, the transverse diameter of the vertebral bodies increases from the cervical to the lumbar regions of the spine, resulting from the fact that the latter carries a greater load. The width then diminishes from the first sacral segment to the tip of the coccyx.

The spaces between the trabeculae serve as channels for blood supply. Small veins are present in the superior and inferior parts of the vertebral body. These smaller veins enter into larger tributaries in the center of the vertebral body, forming a dense network of veins. The cortical bone also contains small veins and nutrient arteries, as well as some nerve endings.

Because there are no mechanisms on the surfaces of the vertebral bodies that prevent sliding and twisting motion, the vertebral bodies are dependent on the posterior elements for stabilization in the horizontal direction. The inferior articular processes form hooks that lock into the superior articular process of the vertebra below, forming a locking mechanism that prevents sliding. The posterior elements are designed to receive the dynamic forces placed on the spine. Loads on the processes are eventually transmitted to the pedicles and, eventually, the vertebral bodies. In addition, the posterior elements provide areas for muscle attachments, so muscle forces are transmitted to the posterior elements and then to the vertebral bodies through the pedicles.
Because the pedicles play such an important role in transmitting all forces to the vertebral bodies, they must possess a structure which enables them to resist bending, tension, and compression. Interestingly, the amount of cortical bone on the pedicles varies from one region of the spine to the other, being higher in regions which are subjected to more motion. The middle cervical and upper lumbar pedicle regions contain more cortical bone than the thoracic regions. The pedicles in the thoracic region are composed mostly of cancellous bone. These trends may be explained by the fact that there is less spinal motion in the thoracic region.

Adjacent vertebral bodies are separated by a layer of strong yet flexible soft tissue, called the intervertebral disc. This tissue is strong enough to sustain weight yet can deform to accommodate rocking motions. It also helps to carry compressive loads placed on the spine and transfer loads to the vertebral bodies. It has been suggested that its mechanical efficiency improves with use. Applied stresses affect cellular activity, so the disc can remodel itself to minimize that stress. However, the extent to which remodeling is possible may be limited, and high stresses are also thought to lead to disc degeneration. The shape of the disc is determined by the shape of the adjacent vertebral bodies. Also, the thickness of the discs varies with spinal level. The discs are thickest in the lumbar region and thinnest in the thoracic region. The discs of the cervical and lumbar regions are thicker anteriorly than posteriorly, while thoracic discs have a constant thickness throughout.
The main components of the disc are water, proteoglycans, and collagen. Proteoglycans are molecules consists of several glycosaminoglycan (GAG) chains covalently linked a core protein. GAGs are a class of chemicals present in most forms of connective tissue, such as skin, bone, cartilage, and tendons. They are long chains of polysaccharides, which consist of a repeating sequence of two molecules, a sugar and a sugar with an amine attached. The GAGs found in the intervertebral disc are principally chondroitin-6-sulphate, chondroitin-4-sulphate, keratin sulphate, and hyaluronic acid. As many as 60 GAGs can be attached to the core protein. Proteoglycans can aggregate when several of them link to a chain of hyaluronic acid. However, proteoglycans that do not aggregate with hyaluronic acid are the major proteoglycans that occur in the disc. These proteoglycans play a key role in allowing the disc tissues to attract and retain water.

The fundamental unit of collagen is the tropocollagen molecule. A tropocollagen molecule consists of three polypeptide chains that are twisted around one another in a helical fashion and held together by hydrogen bonding. When these molecules align side by side, a collagen fibril is formed. Aggregates of collagen fibrils form a collagen fibre. There are 19 different types of collagen, differing in chemical composition and structural arrangement. The types of collagen found in the disc are predominantly type I and II, which have the helical structure described above. These rope-like molecules give the disc its ability to withstand forces and loads.

The disc is composed of three basic structures: a central nucleus pulposus, a peripheral annulus fibrosis, and two layers of cartilage covering the top and bottom called vertebral
endplates (Figure 2.4)\(^8\). These components differ in the arrangement of proteoglycans and collagen in the tissue, as well as the relative concentrations of each. In healthy discs, the nucleus is a fluid like mass of mucoid material \(^8\). The nucleus is thickest in the lumbar region and thinnest in the thoracic region. It is most centrally placed in the cervical region and is more posteriorly placed in the lumbar region \(^1\). The nucleus pulposus is approximately 80% water in youth and gradually dehydrates beginning between ages 20 and 30 \(^2\). Proteoglycans comprise about 65% of the dry weight of the nucleus \(^1\). Only about 25% of these proteoglycans exist in the aggregate form. About 20% of the dry weight of the nucleus is composed of collagen. Bundles of type II collagen help to hold the proteoglycan matrix together \(^15\).

The main component of the annulus fibrosis is water, accounting for 70% of its weight. About 60% of the dry weight of the annulus is composed of type I and type II collagen fibres, arranged in concentric sheets called lamellae. The lamellae are thickest toward the center of the disc \(^16\). Posteriorly, they are finer and more tightly packed, so the posterior of the annulus is thinner that the anterior \(^1\). In the lumbar region, the fibres run parallel to one other at a 65° angle with the vertical plane, although the direction of the lamella can vary from person to person \(^10\). The spaces between lamellae are filled with proteoglycan gel which binds the collagen fibres together \(^8\). These proteoglycans make up about 20% of the dry weight of the annulus.

The vertebral endplates are attached to both the disc and the adjacent vertebral body. They are approximately 1mm thick peripherally and 3mm thick centrally \(^7\). The chemical
structure of these plates resembles that of the nucleus pulposus and the annulus fibrosis because they are composed of proteoglycans and collagen fibers. Directly above the nucleus, the endplate contains more proteoglycans than collagen. However, at the periphery of the endplates, the opposite is true. The inner lamellae of the annulus attach to the vertebral endplate and the outer lamellae anchors itself to the cortical bone that exists on the rim of the vertebral body. Even though the disc tissues are attached to the endplates, the endplates are only weakly attached to the vertebral bodies. For this reason, the endplates are considered part of the disc, instead of part of the vertebral body.

The mature human intervertebral disc has a very low density cell population compared to other tissues in the body. Cells occupy approximately 0.25-0.5% of the tissue volume in the disc. The cells in the nucleus and inner annulus are chondrocyte-like and round. On the other hand, the cells in the outer annulus are thin and extend along the collagen fibrils. In the endplates, chondrocytes are aligned along the collagen fibrils, too. The chondrocytic cells are responsible for the synthesis of proteoglycans and collagen in the disc, as well as proteases, which degrade old proteoglycans and collagen. The health of these cells is vital to the disc because degeneration will ultimately occur if the rate of tissue breakdown increases over the matrix synthesis rates.

Cell activity requires oxygen, glucose, and other substrates necessary for tissue synthesis. However, the disc is the largest avascular tissue in the body, since cells are located 6-8 mm from the nearest blood supply. Nutrients are supplied to the outer annulus via blood vessels at its periphery. The inner annulus relies on nutrients supplied from capillary
beds in the vertebral body, which terminate at the vertebral endplate. Small solutes such as oxygen, glucose, and lactate diffuse through the endplate in order to enter the center of the disc.

One key feature of the disc is its ability to maintain a high water content, even under compressive loads. This ability is attributed to the high osmotic pressure exerted by the proteoglycans in the tissue. The osmotic pressure of the proteoglycans is mainly attributed to the ionic carboxyl and sulfate radicals on the GAG chains, which impart a high negative fixed charge to the tissue matrix. Positively charged molecules are attracted into the tissue to balance the negative charge of the GAGs. The osmotic pressure generated by this ion flux is responsible for the ability of disc tissue to attract and retain water. The size of the GAG chains has little effect on the osmotic pressure of the disc tissues. In fact, the water binding capacity of the proteoglycans has been shown to be proportional to their concentration, which increases from the outer to inner annulus, and is highest in the nucleus pulposus.

The water content of the disc not only depends on proteoglycan concentration, but the pressure applied on it under loading. In human lumbar discs, the pressure inside the nucleus is lowest when laying prone, at 0.1-0.2 MPa, and increases by 5-8 times when standing or sitting. The disc fluid is expressed as pressure increases and under sustained loads, however this fluid loss occurs very slowly. The proteoglycans account for the fine pore size of the disc tissues, which restricts fluid flow under compression. Thus, the disc water content rarely achieves equilibrium. Approximately 20-25% of
the disc water is expressed during daily loading, but this water is regained during a night’s rest \textsuperscript{27}.

\textbf{2.2 Intervertebral Disc Biomechanics}

It has been shown experimentally that the annulus can support briefly applied compressive loads and transmit them to adjacent vertebrae without the nucleus pulposus \textsuperscript{10,28}. Its densely packed collagen lamellae make the annulus a relatively stiff body, and the collagen fibres are prevented from buckling due the presence of the proteoglycan gel. However, if the annulus is subjected to sustained loads, the proteoglycan gel will not be strong enough to prevent deformation. The fibres will start to buckle and annulus will be slowly squashed \textsuperscript{8}.

The primary function of the nucleus pulposus is to provide a bracing mechanism for the annulus under sustained loads. Because of its low hydraulic permeability, the nucleus volume initially will not be compressed under vertical loading. Instead, it deforms by expanding radially, exerting a hydrostatic pressure on the annulus, and pushing its collagen lamellae outwards. The annulus fibres resist this stretch, and exert an opposing force on the nucleus. Vertical loads are then transferred to the annulus by the nucleus in circumferential tension, preventing the fibers from buckling under sustained loads. \textsuperscript{2,8}. In addition, because the annulus is stiff enough to resist bulging, nucleus deformation in the radial direction is limited. This results in increased vertical pressure against the endplates. This pressure serves to transmit part of the applied load to the adjacent
vertebral bodies, lessening the weight on the annulus. Ultimately, however, the load is fully transmitted to the adjacent vertebrae. Another characteristic of the healthy disc is its elasticity. It has the capacity to store energy. When the expanding nucleus pushes the fibres in the annulus outwards, they stretch like springs due to the elasticity of the collagen. Once the load is released, the elastic recoil of the collagen fibers allows the energy stored in them to be transferred the nucleus, aiding it to restore any deformations caused by the loading.

Together, the nucleus and annulus also act as a shock absorbing system for the vertebrae. When a compressive force is rapidly applied to the disc, it is first transferred to the annulus in the tension, before being transmitted to the vertebral bodies. While this does not lessen the magnitude of the force, slowing the rate at which it is transferred to the vertebrae will help protect the bones. The shock absorbing capacity of the disc lessens when the same disc is loaded repetitively. Movements such as jumping or subtle bouncing, which may be experience during driving, are associated with disc injury.

The fluid property of the nucleus pulposus is essential for maintaining synergy between itself and the annulus fibrosis. Its high swelling pressure is the basis for the healthy biomechanical function of the disc. The normal mechanical function of the disc will depend on the ability of the nucleus to attract and maintain water. Any changes in the proteoglycan concentration or water content of the disc tissues will alter the mechanical properties of the disc.
In traction, sliding, and twisting movements the annulus acts as a ligament, helping to stabilize the spine by restraining movement. During traction, the collagen fibres in the annulus are all pulled an equal vertical distance from the vertebral body. The annulus resists this strain. The capacity of the disc to resist this strain is illustrated by how well the spine can sustain the load of the trunk and legs when one is hanging by the hands. Even under traction, however, the spine is still under some compressive load due to muscle action. In slide movements, the fibres that line parallel to the direction of movement will be strained and thus provide resistance to movement. Twisting is also only resisted by the collagen fibres inclined in the direction of movement. Movements such as these, which cause half of the annulus fibers to be stretched while the other half stays relaxed, are most likely to cause injury to the disc.

### 2.3 Intervertebral Disc Degeneration

Decreases in nutritional supply to the disc due to structural changes in the vertebral endplates are thought to be a major cause of disc degeneration. As a result of aging, the blood vessels in the vertebral bodies become gradually occluded, resulting in a decrease in nutrients delivery to the disc. Nutrient transport may also be inhibited by endplate calcification, which also naturally occurs in aging discs, as well smoking and vibration. This impaired nutrition to the cells in the disc may be the underlying cause for biochemical changes seen as the result of degeneration.
With aging, the rate of proteoglycan synthesis decreases. In early adulthood, proteoglycans comprise approximately 65% of the dry weight of the nucleus, but this drops to 30% by age 60. The proteoglycans that are synthesized are generally lower in molecular weight. In addition to their molecular weight, the chemical nature of the proteoglycans changes with age. The concentration of chondroitin sulphate GAGs falls, while keratin sulphate content stays the same. Because chondroitin sulfate possesses both sulphate and carboxyl radicals, it has twice the water binding capacity of keratin sulfate, which only carries one sulphate radical. The water binding capacity of the tissue therefore decreases with the drop in chondroitin sulphate content. In addition, the frequency of collagen-proteoglycan binding increases, leaving fewer ionic groups on the proteoglycans available to attract water. Because of these biochemical changes, the disc tissues dehydrate with age. In fact, by age 75, the water content of the nucleus can drop to as low as 65%. Added to this fluid loss, the disc tissues exhibit increasing hydraulic permeability with age, so water loss will occur more quickly under loading.

The overall collagen content of the disc also increases with age. Type X collagen is found in aged discs, and may be connected to calcification of the disc tissues. The fibre diameter of the type II collagen in the nucleus pulposus increases, making it resemble the type I collagen in the annulus. The collagen fibre diameter in the annulus decreases, making it resemble the type II collagen in the nucleus. This may be one reason that the boundary between the nucleus and the annulus become indistinguishable with age. Also, the annular rings thicken and become more disorganized. The tensile
strength of the collagen decreases as well \(^{41}\), making the disc tissues more susceptible to injuries associated with daily movements.

These fundamental biochemical changes alter the biomechanical behavior of the disc and can initiate a degenerative cascade, ultimately leading to back pain. The dehydration of the nucleus results in an overall volume loss, making it less able to exert a fluid pressure on the annulus when it is compressed \(^{42}\). In other words, the nucleus loses its ability to transmit weight directly to the annulus \(^{8}\), altering the uniform load distribution characteristic of healthy discs \(^{43}\). The annulus then bears a greater portion of any load placed on the disc \(^{8,43}\). Repeated stresses cause the lamellae to buckle \(^{8}\) and annular fissures to form \(^{2,8,21}\). These defects reduce the weight bearing capacity of the annulus, leading to further tearing and loss of tissue integrity \(^{44}\). Some finite element models have shown that loss of nucleus volume can lead to inward bulging of the annulus during compression \(^{45}\). This increases the shear stresses between the lamellae \(^{46}\) and tears the layers apart \(^{47}\).

The nucleus may then migrate from the center of the disc, and perhaps eventually through all layers of the annulus, resulting in disc herniation. Consequently, the nucleus is no longer sheltered from the immune system, causing an inflammatory response from the body. Studies on human patients have shown that herniations attract macrophages, fibroblasts, and lymphocytes \(^{48}\). A variety of inflammatory chemicals are also produced, such as phospholipase A\(_2\) \(^{49}\) and interleukin 12 \(^{50}\). The presence of chemical pain
mediators activates nociceptors, which send the signals to the spinal cord and brain to cause back pain\textsuperscript{51}.

In the healthy human disc, nerves extend into the outer third of the annulus. However, degenerated discs often exhibit innervation that penetrates much deeper into the annulus, and in some extreme cases into the nucleus pulposus\textsuperscript{52,53}. The migrating nucleus also may impinge on nerve roots, leading to additional pain\textsuperscript{3}. Therefore, pain can occur even in discs where there is no protrusion of nuclear material into or beyond the periphery of the annulus fibrosis. Typically, fissures reaching into the inner third of the annulus are not painful, but 70\% of fissures extending into the outer third of the annulus cause pain for the patients\textsuperscript{54}.

With age, there is also an overall decrease in the bone density of the vertebral bodies and a corresponding decrease in bone strength\textsuperscript{7,55-57}. With ageing, the horizontal trabeculae are absorbed and not replaced\textsuperscript{57}. This phenomenon occurs most dramatically in the central region of the vertebral body, directly above or below the nucleus pulposus\textsuperscript{8}. The vertebral endplates then must share a greater portion of every load because it lacks support from the weakened underlying bone\textsuperscript{58}. Repeated loading causes microfractures to occur in the endplate and a gradual bow into the vertebral body\textsuperscript{57}. If the fracture in the endplate is large enough, nuclear material may migrate into the vertebral body, forming a Schmorl’s node. The incidence of these is highest in the thoracic region of the spine\textsuperscript{59}. The fracture of an endplate can also lead to the introduction of inflammatory cytokines into the nucleus. The presence of these chemical mediators actually slow metabolic and
reparative processes in the disc and lead to further degradation \(^\text{60}\). Cytokines can also be a direct source of pain \(^\text{61}\).

### 2.4 Treatment for Lower Back Pain

#### 2.4.1 Conservative Treatments

Current treatment for lower back pain begins with conservative interventions, the goal being to relieve pain and restore function quickly \(^\text{62}\). Conservative treatment consists of rest, medication, and physical therapy \(^\text{63}\). In a study on 71 athletes with disc herniation, it was found that conservative treatment was satisfactory in controlling symptoms. Approximately 79\% of the athletes were able to resume their sporting activities an average of 4.7 months after the start of treatment. Resumption of activity occurred when the subjective symptoms were reduced by at least 80\% \(^\text{64}\). Overall, however, the relative effectiveness of common conservative treatment strategies is not well understood \(^\text{65}\).

The most common initial treatment for lower back pain is rest. Two days of total bed rest is often prescribed after a back injury. This can help to reduce inflammation, relax muscles, and allow healing to occur. Once healing has begun, a patient may resume light activity. Although activity can increase the risk of relapse, periods of prolonged rest can lead to weakness and depression \(^\text{63}\). Four days or longer of bed rest has been reported to be detrimental to recovery \(^\text{66}\). Therefore, proper modifications to normal activities should be made to avoid recurrence of the injury \(^\text{62}\).
The benefit a patient gains from bed rest may depend on the type of low back pain being treated. Hagen et al. performed a randomized trial comparing the effect of two days of bed rest on the symptoms of acute lower back pain (without neurological deficits) and sciatica (nerve root compression). For patients with acute lower back pain, staying active was slightly more effective than staying in bed. For patients with sciatic back pain, there were no differences between staying active and bed resting.

There is substantial evidence that exercise helps in the management of lower back pain. Exercises can help regain range of motion, strengthen abdominal muscles to help compensate for injured spinal tissues, and increase endurance of spinal muscles. Exercise may also help prevent future episodes of chronic pain and modify pain perception.

There are several different modes of exercise and physical therapy. Cold therapy can reduce inflammation and pain, whereas hot therapy can aid in muscle relaxation and improve circulation. Transcutaneous electrical stimulation can block nerve impulses from being sent to the cerebral cortex that are interpreted as pain. Traction, where systems of weights, cables, and pulleys are used to reduce pressure on the vertebrae, is used by 41% of 1239 physical therapists interviewed in the United Kingdom. Clarke et al. reported results of a randomized trial of 2177 patients, approximately half of which received traction. There were no significant differences in the short or long-term outcomes of continuous or intermittent traction and placebo, with or without sciatica.
Many questions still remain regarding the exact application of exercise. Mannion maintains that an exercise program must target the special needs of the patient and be balanced with the healthcare budget of the provider. Hayden et al. demonstrated that individually designed exercise programs improved outcomes for non-specific chronic lower back pain patients over a 12 week period. Hurwitz et al. followed 681 patients with lower back pain for 18 months and found that focusing on recreational physical activity rather than specific lower back exercises was more effective at reducing pain and increasing psychological health. Linton et al. studied the effects of combining physical therapy with cognitive behavioral therapy on back pain related sick leave. They reported no significant differences in the recovery of patients who received cognitive behavioral therapy in addition to physical therapy compared to patients who received physical therapy alone.

If symptoms do not improve over 4 to 6 weeks, pharmacologic pain control can be considered based on symptoms and history. Muscle relaxants and oral nonsteroidal anti-inflammatory drugs are often prescribed. The nonsteroidal anti-inflammatory drugs are prescribed at a much higher frequency, however. They block prostaglandin production and reduce inflammation, effectively relieving pain. Epidural anesthetic injections are also used to minimize nerve irritation. A series of injections several weeks apart may be needed. The relief may last up to a few months. However, a risk associated with this procedure is accidental puncture of the dural sac, leading to the leakage of cerebral spinal fluid.
Only after exhaustive conservative treatments should surgical interventions be employed. In addition, surgery can only provide relief when the correct source of the pain is identified. For this reason, unresolved persistent back pain requires repeated, in depth evaluation. There are several diagnostic tests which can supplement a physical examination. Radiographs can be used to identify breaks in the vertebral body or posterior elements. They can also be used to detect spondylolisthesis, which is the slippage of one vertebral body over the other. Computed topography (CT) scans and magnetic resonance imaging (MRI) can be used to identify fractures, herniated discs, tumors, and infections. Discography can allow for the identification of a painful disc by following the injection of a contrast medium into the nucleus pulposus. Radiographic imaging will show leakage during a herniation. In addition, low injection pressures will invoke intense pain in degenerated discs \(^{61}\). Despite these tools, in 80% of patients, no obvious cause of back pain can be found \(^{80}\).

### 2.4.2 Current Surgical Interventions

Many current surgical interventions for back pain fall under one of two categories: decompression or stabilization procedures. Laminotomy, laminectomy, and discectomy are decompression procedures \(^{62}\). They involve relieving pressure on the nerve elements by excision of disc, bone, or ligament material \(^{2}\). In a laminotomy, the surgeon will attempt to relieve nerve compression by making a small hole in the disc material to free the nerve root. However, if this is not sufficient to free the nerve, a laminectomy can be
performed. This is the removal of a small part of the disc tissue, facet joints, or any portions of the disc impinging on nerves. This procedure will provide relief as soon as the inflammation subsides.\textsuperscript{79}

A discectomy is used in cases where complete herniation has occurred. The portion of the nucleus pulposus which is causing pressure on the nerve root is removed. Discectomy is sometimes performed in conjunction with laminectomy. Discectomy can be done via open, micro, or percutaneous routes or using a laser. In open discectomy, an incision over the posterior midline is made, and the ligaments and muscles are moved in order to access the affected disc. In microdiscectomy, this is done with a small incision. Percutaneous discectomy involves the use of a cannula to suction out the offending tissue, and the surgery is monitored with a fluoroscope.\textsuperscript{62} Laser discectomy is similar to percutaneous discectomy, however the tissue is removed with a laser, which burns the tissue for its removal.\textsuperscript{81}

To date, however, microdiscectomy has been used as the gold standard because no other discectomy technique has been able to match or exceed its outcomes.\textsuperscript{66} One popular microdiscectomy system is the METRx\textsuperscript{TM} (Medtronic Sofamor Danek, Memphis, TN). The necessary incision with this system is less than one inch long and damage to muscles is minimal compared to open discectomy. The surgery is made minimally invasive by accessing the surgical area with a series of tubes, called dilators, which increase in diameter. The tubes are sequentially inserted though the muscle to split the fibers, creating an opening large enough through which the disc tissue can be removed.\textsuperscript{66}
A review was performed on 873 consecutive patients who were treated for lumbar disc herniation with the METRx system. The Oswestry disability index was used to quantify pain. At 28 months post surgery, significant improvements were seen in the mean preoperative and postoperative score. The average hospital stay was 4.8 days and it took an average of 15 days to return to work. The average blood loss during the surgery was 44 mL.

Wu et al. performed one of the only studies which compares the outcome of percutaneous discectomy to that of conservative treatment. Surgery was performed on patients with herniations which were symptomatic for 6-12 weeks. At a 2 year follow up, there were no clinically significant differences in pain intensity and quality of life between the two groups. However, the discectomy group was associated with a more rapid initial recovery.

Another study involved a 25 year or longer follow-up on patients who received discectomy for lumber herniation. Patients who underwent surgery reported a higher quality of life and less pain than non-surgically treated patients. Despite these results, there has been little other research into the long term effects of discectomy, making long term outcomes of the treatment questionable. While the procedure is successful in relieving pain after surgery due to nerve root decompression, it does not restore the healthy biomechanics to the disc. The degenerative cascade can therefore continue, and has been reported to be accelerated due to alterations made to the disc.
Vertebroplasty, kyphoplasty, and spinal fusion are stabilization procedures. Vertebroplasty was developed in France in the 1980s, but it was not used in the United States until 1994. It involves the injection of bone cement into an area of vertebral compression fracture. By filling in a bone defect in the vertebral body, the vertebral body is stabilized because mechanical support is rapidly regained. A CT scan is used to verify that the void is completely filled.

Generally, curing poly(methylmethacrylate) is used as the augmentation material. The bone will not bond with the polymer and it will remain a foreign body in the vertebral body. Consequently, the patient may experience fever and increased pain from the inflammatory response. It is also possible to sustain tissue damage due to the exothermic polymerization reaction. Calcium phosphates have also been investigated as a bone replacement material because it is biocompatible, osteoconductive, nonexothermic and injectable. However, a bone substitute in the vertebral body must be able to withstand cyclic loading, so there are concerns over the brittleness of calcium phosphate cements. Wilke et al. investigated the fatigue behavior of calcium phosphate cement and poly(methylmethacrylate) in intact human osteoporotic specimens over 100,000 loading cycles. Both of the cements exhibited decreases in height with increasing number of load cycles. The poly(methylmethacrylate) lost 2.8 mm, while the calcium phosphate ceramic lost 4.2 mm. Cryosections revealed the presence of small cracks in the calcium phosphate, but there were no signs of fatigue for the poly(methylmethacrylate).
After vertebroplasty, patients have reported a decrease in pain and increase in function \textsuperscript{90,94}. However, for patients with malignancies, vertebroplasty has been shown to offer adequate vertebral stabilization, but inadequate pain relief \textsuperscript{90}. In addition, Berlemann and Polikeit et al. both found that vertebroplasty increases stress in adjacent vertebrae, leading to fracture \textsuperscript{95,96}. Another drawback to vertebroplasty is that vertebral height is not completely restored \textsuperscript{62}. Kyphoplasty involves the percutaneous insertion of a balloon into a vertebral defect. The balloon is inflated to compress the cancellous bone in order to create a cavity into which bone cement can be injected, thereby restoring height to the vertebral body \textsuperscript{97,98}. Kyphoplasty and vertebroplasty have been shown to provide similar pain relief. \textsuperscript{99} A correlation between vertebral body height restoration and pain relief was not found by Kasperk et al. \textsuperscript{99} However, a higher rate of cement leakage was reported for vertebroplasty. The rate of other adverse events, such as pulmonary embolism or neurological injury, was low for both procedures \textsuperscript{99}.

Spinal fusion, another stabilization procedure, is one of the most rapidly increasing forms of inpatient surgery in the United States. Currently, it is recognized as a treatment for degenerated discs and segmental instability \textsuperscript{100}. Between 1979 and 1990, the rate of lumbar spinal fusion doubled \textsuperscript{101}. Then, in the 1990s, the rate tripled \textsuperscript{102}. Possible reasons for this are changes in population, technological advances in the surgical methods, uncertainty about the indications, and financial incentives for doctors and hospitals \textsuperscript{103}. 
The basic idea behind fusion is to use arthrodesis to prevent motion across the pain generating disc. The disc material of the affected segment is removed, and the surface of the two opposing vertebral bodies are roughened and packed with bone material. This fills the gap between bones, allowing them to grow into a single segment. 3,103,104.

Today, metal implants are often used to stabilize the vertebrae until the fusion solidifies 62. However, uninstrumented fusions have been performed, and are indicated for spine conditions where there is no instability 104. In these fusions, bone grafts are place on the laminae, spinous processes, or transverse processes in order to eliminate motion. The patient would be required to wear a brace to provide additional stabilization to the segment 104.

Reported rates of internal fixation with instrumentation doubled from the 1980s to the 1990s 105. The most popular implants for internal fixation are metal pedicle screws 103. Using pedicle screws to stabilize the vertebral body provides significant immobilization, allowing for complete arthrodesis to occur 106-108. Wood et al. concluded that patients with degenerative discs who undergo uninstrumented fusion are 24 times more likely to have pseudoarthrosis 109, or incomplete fusions. The screws also provide continued support after the maturation of the fusion 110. One disadvantage of pedicle screws is that less space is made available for the bone graft material 104. An alternative method is to use facet screws for fixation. Here a screw is used to transverse the facet joints. While the procedure for using facet screws is more technically challenging than pedicle screws,
there is less need for soft tissue dissection. High fusion rates have also been reported for facet screws\textsuperscript{111}.

There has been some debate about the clinical value of internal fixation with screws. There have been several studies indicating that, while it may help accelerate fusion rates, there has been little impact on the clinical results of fusion\textsuperscript{112-115}. For instance, Thomsen et al conducted a randomized clinical trial which showed no significant differences in the functional outcomes of patients who received fusions with or without pedicle screw supplements\textsuperscript{114}. Moller et al. showed that the level of pain between the two groups 2 years after surgery was very similar\textsuperscript{112}. On the other hand, there have been other studies which showed a close relation between fusion rate and clinical outcome. One group reported an 80\% clinical success rate and 80\% successful fusion\textsuperscript{116}. Another group reported a 73\% successful fusion rate and a 74\% clinical success rate\textsuperscript{117}.

Fusion rates in the United States rose 77\% between 1996 and 2001. This is because in 1996 the Food and Drug Administration (FDA) approved intervertebral fusion cages (Figure 2.5)\textsuperscript{102}. Fusion cages are another alternative to providing stabilization to the fused segment. The advent of fusion cages reduced the incidence of graft collapse because they provide mechanical support while the bone matures\textsuperscript{100}. The bone graft is placed inside the cage\textsuperscript{3,118}. Cages are also associated with improved fusion rates over screw fixation and uninstrumented fusions\textsuperscript{119,120}. The high stiffness of some metal cages can cause endplate subsidence\textsuperscript{3}, therefore titanium or carbon cages have been
Carbon cages have an advantage over metal cages because they do not interfere with radiographs, tomography scans, or MRI\textsuperscript{100}.

Spinal segments can be fused anteriorly, posteriorly, or circumferentially (360° fusion). The method employed will depend on the area of the defect. In anterior lumbar fusions, the surgeon retracts the abdominal contents and pulls the aorta and vena cava to the right side of the spine in order to access the anterior spinal area. The anterior approach is advantageous because the back muscles and nerves remain undisturbed, resulting in lower recovery time. In addition, Brady et al. maintain that fusing the anterior spine produces better results when the anterior portion of the disc is compressed\textsuperscript{118}. Still, the posterior approach has also gained wide acceptance as a method for lumbar fusion. It allows for the decompression of neural elements via a posterior midline incision\textsuperscript{118}. While both of these approaches have had clinical success\textsuperscript{122-125}, there is still debate regarding the effectiveness of an isolated arthrodesis because it may not totally eliminate motion through the segment\textsuperscript{126}. Posterior lumbar fusion can be supplemented by the addition of a posterolateral fusion, creating a 360° circumferential fusion\textsuperscript{100}. All of these techniques may or may not be assisted with internal fixation implants\textsuperscript{100}.

The bone graft used in spinal fusions can either be an autograft or allograft. Autografts are bone that is usually removed from the patient’s iliac crest, either requiring an additional incision or tunneling under the skin to reach the patient’s hip. Allograft is obtained from a donor cadaver. The bone is ground and applied over the area to be fused.
Autografts are generally considered the “golden standard” because of the availability and minimal risk of rejection. However, many patients elect to use bone from a donor because of the pain associated with an autograft. Patients have reported donor site pain to be greater than that of the spinal incision and it can persist for years after the surgery. It takes between three months and two years for the fusion to become solid.

The usage of osteoinductive agents can enhance clinical outcomes for patients. Bone morphogenetic protein (BMP) can be mixed with the bone in order to induce bone growth. Usage of these materials can also eliminate the need for autogenous bone harvesting all together. Recombinant BMP (rh-BMP) has been reported to give superior results to autogenous bone grafts and more rapid clinical improvement. Berkus et al. followed 46 patients who received spinal fusions with rh-BMP-2 threaded with cortical allograft. Clinical results were compared to patients who received autograft from the iliac crest. At 12 and 24 months, patients in the investigation group had higher rates of fusion and there were no unanticipated adverse events related to the rh-BMP-2.

While spinal fusion undoubtedly provides pain relief for some patients, its efficacy for treating degenerative disc disease remains unclear. Mofidi et al. surveyed 65 patients who received posterior lumbar fusion with carbon cages between 1993 and 2000. Clinical outcomes were assessed by measuring the postoperative Oswestry Disability Index, return to work, and overall satisfaction with the outcome. Approximately four years after the surgery, there was an 84% satisfaction rate and 61% of the patients were
able to return to pre-disease activity level, and there was a significant improvement in the Oswestry Disability Index. In another prospective assessment, 35 patients who received 360º fusion with screw fixation were asked about their outcome approximately 31 months after surgery. There was 71% overall satisfaction rate, but only 28.6% rated their outcome good or excellent. There was also a 75% reduction in medication usage.\(^{130}\)

The UK Medical Research Council followed 349 patients with a 12 month history of chronic low back pain. Approximately half of the patients received a fusion, though the surgical approach and instrumentation varied among patients. The other half received an intensive rehabilitation program for three weeks in addition to cognitive behavioral therapy. At 24 months after the surgery, the patients who received fusion had slightly better Oswestry scores than patients who participated in the rehabilitation program, but the groups did not differ in any other outcomes (anxiety, depression, or any adverse effects).\(^{131}\) In Norway, a similar study was done where 64 patients were studied after either receiving 360º fusion with pedicle screws or 3 weeks of physical exercises and cognitive behavioral treatment for lumbar degeneration. No differences were found between the Oswestry Disability Index for both groups one year after the surgery.\(^{132}\)

The Swedish lumbar Spine Study Group did a similar study where they followed 249 patients with chronic lower back pain and radiological evidence of lumbar degeneration. A 2 year study was done on a non surgical group, who received a variety of conservative treatments such as physical therapy, acupuncture, electrical nerve stimulation, and cognitive behavioral therapy, and a surgical group, who received three different fusion
techniques (posterior, anterior, and circumferential). Initially, the surgical group reported more pain relief and a quicker return to work. Pain and functioning improved by 33% for the surgical group, versus 7% for the non-surgical group. At the 2 year follow-up, however, the improvement gradually deteriorated for the surgical group and only 63% of the patients who received fusions considered themselves “better” or “much better”.

Spinal fusion is also associated with several surgical complications such as infection, nerve injury, and high blood loss. Complications at the donor site and vascular complications have also been reported. In addition, a Cochrane review of randomized controlled trials on the surgical treatment of lumbar disc degeneration concluded that there is no evidence for the efficacy of fusion for the treatment of spinal “instability”.

Like with discectomy, the long term results of fusion are questionable. Several biomechanical studies have shown that fusion causes increased stress to be experienced by spinal segments adjacent to the fusion site. This promotes degeneration of these segments and the cycle leading to back pain begins again. Miyakoshi et al. found that all intervertebral disc heights adjacent to the site of fusion decreased. Chen et al. used finite element modeling to confirm that the largest stress after fusion is placed on the disc adjacent to the surgical site. Then, Chosa et al. showed specifically that the stress increase in these discs is concentrated on the vertebral endplate and annulus during flexion and extension loadings. They also showed that firm fusions increased the stress imposed on adjacent vertebral segments. This has also been seen experimentally by other researchers.
Theoretically, replacing the diseased disc with a prosthesis would have an advantage over fusion because it could more closely mimic the loading and motion characteristics of a healthy spine. It is hypothesized that motion preservation will decrease stress on adjacent segments, leading to favorable long term results. Total disc replacement is therefore emerging as a viable alternative to fusion since it can be used to treat the same symptomatic pathology as fusion. In addition to allowing for the removal of the painful disc, restoring disc height, and improving stability, proponents of this method say a prosthetic disc may help restore a healthy pattern of load bearing to the spine.

The artificial disc with the longest clinical history is the Charité artificial disc. The prosthesis was developed in the early 1980s and it consisted of a polyethylene core sandwiched between to metal endplates. Since then, there have been three revisions to the implant, in order to minimize complications such as subsidence and fatigue failure. The current generation of the product is called the Type III SB Charité or the LINK SB Charité (Depuy Spine, Johnson & Johnson, Raynham, MA) (Figure 2.6). This version of the Charité was the first total disc implant for the lumbar spine to gain approval from the FDA. It is indicated for patients between the ages of 18-60 with symptomatic single-level degeneration and failure to achieve pain relief for at least six months. Patients who have spinal fracture, bone disease, or spinal tumors should not receive the implant.
The implant consists of two concave metal endplates made from a cobalt chromium molybdenum alloy. The surfaces of the endplates facing the vertebral endplate are covered in porous titanium and coated with calcium phosphate to encourage bonding with the bone. In addition, the endplate surfaces also contain six teeth which are used to physically anchor the implant into the vertebral body. Encased between the two endplates, there is a free-floating biconvex sliding core made of ultra-high-molecular-weight polyethylene (Figure 2.7). The mechanical behavior of the core mimics the major movements of the intervertebral segment: flexion, extension, and translation.

The technique for the surgical implantation of all artificial discs is very similar to anterior lumbar fusion. An anterior midline incision, approximately 5 cm in length, is made in order to access the spine and remove the diseased disc tissue. A spreader instrument is used to distract the disc space. The surfaces of the vertebral endplates are smoothed in order to maximize contact area with the prosthesis. The artificial endplates are then positioned so that the inner surfaces of the plates are parallel and then they are implanted with the aid of a mallet to secure the teeth in the bone. The polyethylene core is then inserted in place between the endplates.

An FDA regulated prospective, randomized study was performed for the purpose of gaining FDA approval for the Charité. The objective of the study was to compare the safety and effectiveness of the Charité artificial disc to anterior lumbar fusion for the treatment of single level lumbar degeneration and determine if there exists a correlation between implant placement and clinical outcome. A total of 304 patients were followed...
over 24 months, 205 being in the investigational group, and the remainder receiving fusion with a BAK cage and an iliac crest bone graft. Overall, patients in both groups improved significantly after surgery. However, patients who received the Charité recovered faster, had lower disability levels, and statistically lower pain scores. The hospital stay was also significantly shorter for the disc replacement group. Patients in the investigational group had a 13.6% mean increase in mean flexion/extension ROM, while the fusion group had an 82.5% decrease. The investigational group also had significantly better restoration of disc height and less subsidence. The flexion/extension ROM and prosthesis function improved with surgical technical accuracy of placement. At the 24 month follow up, no implant wear and creep was found.

The study has received major criticism because the reference procedure, fusion with the BAK cage, has a very poor clinical history and is rarely used by surgeons due to its high failure rate. This control was chosen because it was the only FDA approved anterior cage at the time of the study. Currently there are other cages available that could have produced better scores for the fusion group. It has also been suggested that the claim that the Charité disc produces less subsidence is not a valid argument for the superiority of disc replacement. The BAK cage rests on the vertebral endplate, and because the area of the cage is small, this produces a stress point that leads to subsidence. The Charité endplates are much larger, so it is only logical that less subsidence would occur. In addition, despite the improvements in pain scores for both groups, 64% of the people in the investigational group and 80% of the people in the fusion group remained on narcotics 2 years after the surgery.
While the purported advantage of disc replacement technology is the preservation of adjacent vertebral segments, there was no conclusive evidence presented in the FDA regulated study to support this hypothesis. Van Ooij et al.\textsuperscript{142} reported on 27 patients who received the Charité disc replacement and were suffering from complications. At the 53 month follow-up, 7 patients exhibited degeneration at another spinal level that was not present prior to surgery, despite accurate placement of the prosthesis. The authors conjecture that it is unlikely that this degeneration was simply caused by the degenerative disease itself. They maintain that it is highly unlikely that the Charité or other disc replacements can completely mimic the normal movement of the disc, making adjacent segment degeneration inevitable. Physiologically, the center of rotation in normally functioning segments is positioned posteriorly in the disc, and in the Charité this is centrally or anteriorly located\textsuperscript{142}. In addition, the authors claim that the metal and polyethylene do not possess the same shock absorbing capacity as the healthy disc.

Van Ooij et al.\textsuperscript{142} also reported 16 cases of subsidence into the vertebral bone. Because the central endplate is weaker than the outer rim, the metal plates of the implant must be large enough to rest on the periphery of the endplate. Several of the patients who experienced subsidence had too small of a prosthesis inserted. However, a disadvantage to larger plates is that there is a greater chance of nerve impingement. Because a great many blood vessels must be mobilized for correct insertion of the implant, this poses bleeding and thromboembolic risks\textsuperscript{151}, which increase with implant size\textsuperscript{142}. Van Ooij et al. also reported anterior migration of the implant, causing iliac vein compression\textsuperscript{142}. 
The ProDisc-L (Synthes Spine, West Chester, PA) (Figure 2.8) was developed in the late 1980s and the second generation, the Prodisc, has been available since 1999. The implant consists of two metal endplates made from cobalt chromium molybdenum alloy, the surface of which is coated with porous titanium. Implant stability is achieved by a spike located on each endplate, called a keel. During surgery, keel cuts in the bone are made with a chisel. The ultra-high-molecular-weight polyethylene core is snapped into the lower endplate during the operation after endplate distraction. Unlike the Charité, which has a free floating biconvex sliding core, the Prodisc inlay does not move, thus the center of rotation is fixed. In 2006, the FDA approved the ProDisc-L for lumbar disc replacement in skeletally mature patients with discogenic back pain and degeneration.

Chung et al. reported positive clinical outcomes for 36 patients who received a lumbar total disc replacement with the ProDisc. The mean score for lower back pain improved significantly and 7 out of 10 preoperatively unemployed patients were able to return to work. The Oswestry disability index scores also improved. Mean range of motion and disc height also significantly increased. There were no significant complications in this study. Similar results were reported by Bertagnoli et al. At 3 months postoperatively, statistical improvements were reported in Oswestry scores and patient satisfaction, which were maintained at 24 months. There were increases in disc height and range of motion. There were no significant complications or the need for any surgical revisions. Mayer et. al. followed 37 patients postoperatively after receiving the ProDisc prosthesis. After an average of 5.8 months, 76% of the patients had no low
back pain. In addition, 61% of the patients were “completely satisfied” and 21.7% were “satisfied”. Radiographs showed no changes in function of the implant over time and there were no incidences of subsidence.

Zigler et al. report the early results of an ongoing FDA trial at 19 US centers. One report concerns 39 patients at a center in Plano, TX who received either the Prodisc or a 360º lumbar fusion. The fusion was a more substantial operation involving anterior fusion with femoral allograft followed by posterior fixation with pedicle screws and an iliac bone graft. It is not surprising, then, that lower blood loss, shorter operating times, and briefer hospital stays were reported for the disc replacement group. Zigler et al. also reports that, at three months postoperatively, the Oswestry disability scores were significantly lower for the disc replacement group and they exhibited significantly improved range of motion.

Delamarter et al. reported 30 month follow-up data on 78 patients in Santa Monica, CA, who were participating in the same FDA trial. Early results were similar to those described above; the disc replacement group had an early advantage in pain and recovery. However, at 12, 18, and 24 months, there were no significant differences in the pain, disability or mobility for the two groups.

The complications associated with surgical implantation of the ProDisc appear similar to those associated with the Charité. Those include nerve root impingement, endplate subsidence, malpositioning, dislodgement of the polyethylene core, and iliac
vein laceration\textsuperscript{156}. Shim et al.\textsuperscript{159} reported a split fracture of the vertebral body, which was attributed to the keel design of the ProDisc. In the author’s opinion, the need for chiseling increases the risk of vertebral fracture, and the height of the keel is unnecessarily high, especially for Asian people who have smaller vertebral bodies than Caucasians.

There are other total disc replacements besides the Charité and ProDisc under investigation, though the literature data concerning them is sparser. The Maverick disc (Medtronic, Minneapolis, MN) (Figure 2.9) has similar design to the ProDisc, but is a metal-on-metal implant. It consists of two flat metal endplates with a ball and socket-joint sandwiched between them. The center of rotation is fixed in the posterior of the disc space. Stabilization in the vertebral bones is achieved by two large keels, similar to those on the ProDisc. Due to the height of the keels, usage of the disc may be limited to larger vertebral bodies\textsuperscript{144}. Barzilay et al.\textsuperscript{160} reported 1 year follow-up results on the first 30 consecutive patients to receive single level Maverick discs. At 6 months and 1 year, patients experienced an improvement in back pain and achieved a normal range of motion. There were no major device related complications. The Flexicore disc (Stryker Corp, Kalamazoo, MI), is also a metal-on-metal implant with a ball-and-socket joint. The endplates are dome-shaped to adapt to the concavity of the endplates. Because of this shape, the implant can be implanted either anteriorly or anterolaterally. Also, the device is implanted as one single unit\textsuperscript{144}. 
2.4.3 Nucleus Pulposus Replacement

Based on the clinical outcomes of spinal fusion and total disc replacement, it appears that re-establishing the healthy biomechanics of the degenerated spine is still a challenge that needs to be realized by spine medicine. Other than total disc replacement, another non-fusion alternative being investigated is the replacement of the nucleus pulposus alone. A synthetic nucleus replacement could be designed to recreate the biomechanical function of the healthy nucleus pulposus by applying tension to the annulus under compressive loads 161,162. In doing so, a nucleus replacement would restore stability to the spinal segment 162. In addition, motion could be preserved and disc height could also be restored 161-163, the latter helping to lessen compressive forces on the facet joints 163,164. Further tearing and nerve irritation could be prevented or postponed by the nucleus replacement relieving or lessening the shear forces on the annulus 165. In addition, the implantation procedure for a nucleus replacement has the potential to be less invasive than a total disc replacement or spinal fusion 166, so the morbidity associated with those surgeries can be avoided.

There are no clear standards establishing the degree of degeneration for which nucleus replacement is acceptable 167. Some propose that nucleus replacement be intended for people in the earlier stages of degeneration, when the annulus is still intact 168 and there have been no previous operations 167. Proponents of this philosophy view nucleus replacement as an early therapy, rather than a replacement for spinal fusion or a
prosthetic disc. An intact annulus would be better able to contain the implant, reducing the risk of migration. This is especially important for *in situ* forming nucleus replacement materials, which are injected as liquid and form a solid in the body. A competent annulus is necessary to prevent the spread of liquid beyond its periphery.

The problem with this approach is that by the time discogenic symptoms occur, the annulus may already be in the later stages of degeneration. Nucleus replacement has also been proposed as an adjunct to discectomy or nucleotomy. Removing offending disc material and filling the space with a synthetic material could help to restore function to the disrupted disc. The nucleus replacement would lessen the load on the remaining disc tissues and transfer compressive forces to the annulus in tension again, preventing or postponing future degeneration. However, the disc height must still be sufficient to allow insertion of the implant. One major consideration to be taken into account when using nucleus replacements for this indication is that, by the time the patient becomes symptomatic, nerve impingement may not be the only cause of back pain. Pain can be generated by inflammatory mediators which can access and stimulate the nociceptors in the tears of the outer annulus. Mayer et al. maintains that replacing the nucleus in these situations would not bring relief to the patient, because the annulus fibrosis will remain insufficient. However, if a soft tissue adhesive were used to close the annular fissures, and no vertebral fractures are present, the patient theoretically should not feel this pain anymore, because inflammatory chemical mediators would not be able to reach the nociceptors in the outer annulus.
There are several material requirements for a synthetic nucleus replacement. First, it must be able to endure cyclic fatigue without failure or formation of significant particle debris\textsuperscript{163,166,169}. An implant is expected to withstand approximately 100 million cycles over 40 years\textsuperscript{170}. It must also possess elastic characteristics which would allow for shape memory under repetitive loading. The stiffness of the material must be such that the load distribution on the endplates and vertebral bodies will not cause subsidence or stress shielding, leading to bone resorption\textsuperscript{47,169}. The material should completely fill the disc space in order to avoid excessive movement of the implant\textsuperscript{169}. In addition, intimate contact between a nucleus replacement and the inner annulus is necessary for the full restoration of biomechanical function\textsuperscript{171}. Furthermore, the implant should be delivered to disc non-invasively, with minimal tissue trauma, which would reduce the risk of implant migration or expulsion\textsuperscript{166,169}.

Currently, the most widely studied material candidates for nucleus replacement are hydrogels. Hydrogel-based materials have been investigated for this application for over 15 years\textsuperscript{168}. In the literature, these three dimensional hydrated polymer networks have been favored for nucleus replacement because they have the ability to exude water under loads and re-imbibe it when the load the released\textsuperscript{161,172}. Authors often note that this behavior is similar to the healthy nucleus pulposus\textsuperscript{161,166,169}. Under sustained loading, however, the nucleus only undergoes a gradual fluid loss\textsuperscript{173}. To more closely mimic the mechanical behavior of the disc, it may be favorable to design a material with a low hydraulic permeability, which would allow it to maintain a hydrostatic pressure on the annulus under sustained loading.
Currently, hydrogel materials for nucleus pulposus replacement can be categorized as either preformed or *in situ* curing. Preformed hydrogels are of a predetermined size and shape. In order to minimize the invasiveness of the implantation procedure, preformed hydrogels are often implanted in a dehydrated or partially dehydrated state. They then expand in the nuclear cavity due to the presence of physiological fluids. The main challenge associated with using preformed materials is matching the final size and shape of the implant to the cavity in the disc.\(^{169}\)

The largest clinical experience with a preformed hydrogel nucleus replacement comes from the Prosthetic Disc Nucleus (PDN) (Raymedica, Inc., Bloomington, MN). This copolymer hydrogel is composed of polyacrylamide and polyacrylonitrile, encased in a polyethylene jacket which constrains the swelling of the hydrogel pellet, reducing risk of endplate fracture.\(^{174}\) Earlier versions of the device were implanted as two separate units; a tapered anterior unit and a rectangular posterior unit. The two-unit system allowed the device to be implanted less invasively than a larger single unit. The two units are connected with a tethering suture in the disc.\(^{162}\) (Figure 2.10). Currently, the invasiveness of the implantation is thought to fall between a discectomy and fusion or total disc replacement.\(^{162}\) The product is indicated for patients with degenerative disc disease and at least 5mm residual disc height. Patients with annular defects are excluded as candidates for receiving the PDN.\(^{162}\)
Biocompatibility testing of the device revealed no systemic toxicity, carcinogenicity, or genotoxicity \(^{175}\). Scanning and transmission electron microscope examinations demonstrate no evidence of a giant-cell reaction or evidence of wear debris \(^{176}\). Fatigue testing revealed that the device is able to continue functioning as designed after 50 million cycles \(^{175}\). In addition, Eysel et al. \(^{177}\) implanted the PDN in nucleotomized cadaveric lumbar spinal segments and found that the device restored disc height and stabilized the motion segment to the intact pre-nucleotomized condition.

In 1996, the first feasibility study for the PDN device was conducted. Twenty four patients received the implant. With the hydrogel pillow positioned perpendicular to the anteroposterior dimension of the disc, a success rate of 83\% was seen. Then, a second series of studies were performed on 17 additional patients in 1997 and 1998. They tested an angle shaped hydrogel which was meant to conform better to the concavity of the endplates. They also used a softer polymer to reduce stress on the endplates. Unfortunately, this version only produced a 62\% success rate. There was a high rate of device expulsion and several revision surgeries to remove the implant and perform a fusion. In 1998-1999, a new trapezoid shaped model, designed to reduce the migration rate, was implanted in 26 patients. The success rate improved to 79\% \(^{162}\).

In 1999, surgical instrumentation was modified to facilitate implantation of the device. A series of dilators were developed to help stretch the annulus fibers, rather than cut them. Fluoroscopic imaging was used to ensure proper placement of the device.
restrictive post-operative procedure was developed which required patients to wear a brace for 6 weeks. With these changes in place, the success rate increased to 91% \(^{162}\).

The clinical results have been encouraging. After four years, patients report significant improvements in Oswestry scores. Disc heights increased and implanted segments were stabilized \(^{162}\). However, there was still problems with device migration, the average migration rate being 12 percent \(^{178}\). For this reason, a new surgical technique was introduced. While the device was traditionally implanted via a posterior approach, similar to discectomy, Bertagnoli et al. \(^{179}\) developed an anterolateral transpsoatic approach. An incision is made in the lateral region overlaying the disc, and the lateroabdominal muscles are dissected. The disc can be accessed without disrupting the posterior structures of the spine. A small study group of 8 patients were implanted with this approach. While the Oswestry scores improved and disc heights increased, there was an increase in the migration rate. The magnitude of the increase was not disclosed in the report, but the authors note that it may have been an artifact of the small patient population.

The Newcleus Spiral Implant (Centerpulse Orthopedics, Winterthur, Switzerland) has also been implanted in humans as a treatment for the degenerative disc \(^{163}\). The implant is a polycarbonate urethane having the shape of a memory coiling spiral (Figure 2.11). The device is intended to be implanted into the disc after a discectomy, performed via a microsurgical posterior approach. During the discectomy, the nucleus material is removed to create space for the implant. The spiral can then be inserted into the disc
space through the same opening in the annulus created by the discectomy. The spiral is loaded into a special insertion instrument and pressure is applied to advance it into the disc. Gradually, a spiral forms in the disc space. The volume of the implant is not fixed. When the cavity is filled, the surgeon can cut the spiral and close the wound.

Fatigue tests were run up to 50 million cycles with no significant wear observed. The device was also implanted into human cadaveric specimens. Flexion and extension were measured in the intact condition, after the nucleotomy, and after insertion of the spiral. After the nucleotomy, an increase in rotation and facet joint translation was seen (decrease in spinal stability). After implantation, a return to the intact condition was seen.

Five patients have been implanted with the Newcleus thus far. Low back pain decreased postoperatively in all the patients. Four out the five people were able to return to employment and there were no intraoperative or postoperative complications that occurred. MRI pictures show no abnormal displacements. Five years post-implantation, disc height and rotational mobility were maintained. Long term clinical studies are now under way.

The Aquarelle (Stryker Spine, Allendale, NJ) is another preformed nucleus pulposus replacement. It is primarily composed of poly(vinyl alcohol). The implant is inserted laterally or posteriorly through an annulotomy using a 4 to 5mm tapered cannula. The
implanted hydrogel contains approximately 80% water. It has shown good biocompatibility in animal models and mechanical durability up to 40 million cycles\textsuperscript{166}.

\textit{In situ} forming hydrogels are injectable materials which can be delivered into the disc space as a liquid through a cannula or needle and harden in the body\textsuperscript{166}. This minimally invasive approach reduces the risk of implant migration or expulsion\textsuperscript{166,169}. These materials can conform to the shape of the cavity, completely filling the disc space, allowing for better stability of the vertebral segment\textsuperscript{166}. The most common injectable nucleus replacements are self-curing elastomers. The drawback to these systems is the potential for leakage of unreacted monomer into the physiological environment. It is critical that the polymerization reaches completion, eliminating the presence of toxic leachables\textsuperscript{180}. In addition, the concentration of unreacted monomer necessary to achieve the mechanical strength necessary for a nucleus replacement is quite high\textsuperscript{181}.

The DASCOR Disc Arthroplasty Device (Disc Dynamics, Inc., Eden Prairie, MN) is a nucleus replacement consisting of self curing polyurethane. An 18°C monomer mixture is injected into the disc via a catheter and cures in 12-15 minutes to form an elastomer\textsuperscript{168}. The reaction mixture is contained in a polyurethane balloon\textsuperscript{166}. The balloon also serves to create a space in the disc to accommodate the injectable material\textsuperscript{168}. The device has been implanted in 16 patients in Europe, but the clinical results have not been published yet\textsuperscript{166}. 
Also being developed is the Biodisc (Cryolife, Kennesaw, GA). It is an injectable protein hydrogel based on Cryolife’s surgical adhesive BioGlue. The Biodisc material, the detailed components of which have not been reported, is injected into the disc space and cures within 2 minutes. The polymerization process occurs with negligible exothermic heat production. This produces a solid with properties similar to the natural nucleus pulposus. Cadaver tests on calf lumbar segments showed that the posterior injection of the hydrogels restored denucleated disc height and segment stability. Minimal reduction in height was seen after 10 million loading cycles\textsuperscript{182}.

Another injectable nucleus replacement device is the IDN (Injectable Disc Nucleus) (Spine Wave, Shelton, CT). The hydrogel is composed of a synthetic silk-elastin copolymer created through DNA bacterial synthesis fermentation. It is injected through a needle and forms a gel within a few minutes with no measurable exotherm. Preliminary testing indicates that the IDN is able to restore disc height under loads, and cadaveric tests have shown no evidence of extrusion\textsuperscript{168}.

2.5 Poly(N-isopropylacrylamide)

2.5.1 Thermodynamics of the Phase Transition

It is common for the miscibility of a polymer-solvent system to increase with increasing temperature. Complete miscibility is reached at an upper critical solution temperature (UCST). This phase behavior can be used to describe several binary mixtures, often in
systems composed of a polar liquid and paraffin hydrocarbon. The miscibility of such a system can be explained in terms of fundamental thermodynamics. A system tends to minimize its free energy, which can be described by Equation 2.1. At low temperatures, a system minimizes its free energy by minimizing its enthalpy. However, at high temperatures, increasing the entropy by a small amount can result in a large decrease of free energy of the system. At high temperatures, binary systems with a UCST tend to maximize their compositional entropy by distributing the molecules randomly, therefore forming a miscible mixture.

\[ \Delta G = \Delta H - T \Delta S \]  

Equation 2.1

There are some polymer-solvent mixtures, however, which exhibit increased solubility with decreasing temperature. This phase equilibrium results in a lower critical solution temperature (LCST), below which complete miscibility occurs. LCST behavior occurs in systems where both components have polar groups which interact through hydrogen bonding at lower temperatures. This hydrogen bonding lowers the enthalpy of the system, since molecules that are bound together are at a lower energy state than free molecules. However, hydrogen bonds have very low orientational entropy because the bonds between the polar groups on the polymer and the solvent can only be formed when the molecules are in specific orientations. Hydrogen bonds have an angular spread of 10 degrees, so if the orientation of the molecules relative to one another changes by more than 10 degrees, the bond will break. At increased temperatures, the hydrogen bonds are not thermodynamically favored because of the high losses in orientational entropy. The increase in molecular rotations caused by the temperature increase will break the
hydrogen bonds between the water and polymer. It will be replaced by van der Waals attractions between like polymer chains. The solute then segregates itself from the solvent, forming an immiscible phase. The enthalpy of the system is minimized by the aggregation of like molecules, and this loss in enthalpy outweighs associated gains in orientational entropy associated with this conformation. Some systems exhibit both LCST and UCST behavior, meaning that the miscible phase may once again reappear at higher temperatures. This is because the high orientational entropy associated with the randomly distributed molecules minimizes the free energy of the system, causing miscibility to be thermodynamically favored.

Aqueous solutions of poly(N-isopropylacrylamide) (PNIPAAm) exhibit an LCST, typically occurring around 32-34°C. The chemical structure of PNIPAAm is shown in Figure 2.12. Below the LCST, water hydrates PNIPAAm chains by forming hydrogen bonds with its polar amide and carbonyl groups. Above the transition temperature, the polymer becomes hydrophobic, so its behavior is dominated by the isopropyl group and backbone, resulting in a dramatic phase separation. In linear PNIPAAm systems which are soluble in water below the LCST, the phase transition will result in a sol to gel transition. In crosslinked systems, hydrated PNIPAAm networks exhibit a swelling-shrinking transition at the LCST. It has also been observed that the phase transition of PNIPAAm is reversible.
2.5.2 LCST Determination

The first reported value of the LCST of PNIPAAm was in 1967 by Scarpa et al. They reported a dramatic precipitation of the polymer when a 2% solution was heated to 31ºC. This temperature was determined by simple visual observation of the phase separation upon heating. Because this method is simple and convenient, other researchers have employed this method of LCST determination, and it has become known as the cloud point method. Since then, efforts have been directed at developing more quantitative techniques for cloud point determination, such as UV-Vis spectrophotometry. Fundueanu et al. measured the temperature dependence of the absorbance at 450nm using a UV-Vis coupled to a temperature controller. The group used 1 w/v% aqueous solutions of poly(N-isopropylacrylamide-co-methyl methacrylate-co-methacrylic acid) and heated the solutions at 0.2ºC/min and 0.02ºC/min in the vicinity of the LCST. The cloud point, found to be 36ºC, was taken as the temperature at the inflection point of the absorbance versus temperature curve. This method of analysis is consistent with that of Feil et al.

Differential scanning calorimetry (DSC) is another very common method of LCST determination. Heskins and Guillet first reported that the transition temperature can be detected on a DSC thermogram by an endothermic peak. The heat of phase separation, ΔH, can also be determined from the thermogram. This is the energy required to break the hydrogen bonds formed between water and the amide and carbonyl groups on PNIPAAm. Schild and Tirrell reported a value of 6.3 kJ/mol for the
enthalpy, which is consistent with those found from other researchers and in the typical range for hydrogen bonded interactions. The entropy changes associated with the transition can also be calculated using Equation 2.1, where $\Delta G$ is taken as zero at the transition.

Schild and Tirrell determined that LCST of aqueous PNIPAAm solutions determined with DSC is consistent with results from cloud point measurements. Transition temperatures were found to be independent of heating rate in the range below 30ºC/hr. While the shape of the endotherm varied with molecular weight of the PNIPAAm, it was independent of polymer concentration in water in range 0.4-4.0mg/mL. These results are in contrast to those of Boutris et al., who varied the polymer concentration in water between 0.5 and 22 wt%. There was a significant dependence of transition temperature on polymer weight fraction in the range below 5 wt%; however the phase diagram tended to plateau in the range of 15-20%. This group also compared the phase transition temperatures obtained from DSC and cloud point measurements with UV. They reported that the techniques yielded similar values, but those obtained from DSC were slightly lower. This was attributed to the fact that DSC detects the energy change associated with the destabilization of hydrogen bonds, which is the initial step in the phase separation process. Cloud point techniques only detect the macroscopic phase separation which follows.

In the literature, when DSC is used to detect the LCST of PNIPAAm, the temperature has been defined as either the onset of the endotherm or the temperature at the peak.
minimum \(^{187,200}\). Boutris et al. reported that the peak minimum can shift to higher temperatures with increasing sample mass, while the peak onset is not affected by this variable. However, the peak onset can depend on the polydispersity of the polymer. For these reasons, the group defined the transition temperature as the temperature corresponding to the maximum of the first derivative of the thermogram \(^{201}\).

The LCST of PNIPAAm has also been studied through light scattering techniques. This route may be considered advantageous over DSC and cloud point techniques because it reveals information about the size of the polymer chains \(^{206}\). Light scattering will detect the change in chain conformation from coil to globule as the polymer is heated above its LCST \(^{206,207}\). Dilute solutions must be used in order to accurately measure the radius of gyration of a single coil. Because the phase transition begins with the collapse of individual chains, followed by chain aggregation, decreasing the concentration in solution will decrease interchain aggregation, making the measured size changes for a single polymer chain more precise \(^{207}\). Kubota et al. measured changes in the hydrodynamic radius and radius of gyration of linear PNIPAAm chains of varying molecular weights between 1.63E6 and 2.52E7 g/mol \(^{206}\). They found a sharp decrease in the dimensions of the polymer at the LCST. They were also one of the first groups to report a polydispersity for the polymers, 1.3-1.4, making the measured changes in chain dimensions more reliable. Longer chains will precipitate first, perturbing experimental results \(^{207}\).
There are a variety of other LCST characterization techniques that have been used in the literature. The changes in solution viscosity associated with PNIPAAm chain aggregation have been studied. Heskins and Guillet have reported a decrease in viscosity with increasing temperature for dilute solutions. IR spectroscopy has also been used to detect changes in inter- and intramolecular bonding that occur at the LCST. A study on aqueous PNIPAAm solutions using Fourier Transform Infrared Spectroscopy (FTIR) revealed that the peak associated with the polymer hydrogen bond with water (C=O-HO-H) at 1625 cm\(^{-1}\) decreases as temperature increases. In addition, a peak at 1650 cm\(^{-1}\), representing intersegment hydrogen bonding (C=O-H-N) appears at temperatures above the LCST. This group also reported that the intersegmental hydrogen bonds act as crosslinking points, allowing the chain aggregates to swell like a gel.

### 2.5.3 LCST Modulation

Many studies have focused on modulating the LCST by modifying PNIPAAm with hydrophilic, hydrophobic, or ionic comonomers. Acrylic acid is one of the most commonly used comonomers for increasing the LCST of PNIPAAm. The LCST of random block copolymers of PNIPAAm and acrylic acid were characterized by DSC and cloud point techniques. The copolymers composed of 2 mol% acrylic acid exhibited a 3.6°C increase in transition temperature compared to the PNIPAAm homopolymer. Furthermore, the LCST of the copolymer composed of 7 mol% acrylic acid was found to be approximately 60°C. The group asserts that this increase in transition temperature is tied to increase in hydrophilicity of the copolymers due to the presence of acrylic acid.
As the temperature is raised, the hydrogen bonds between the water and polymer are weakened, and eventually broken, when the polymer precipitates. When the hydrophilic acrylic acid chains are present, these losses in hydrogen bonding are offset by the hydrogen bonds between the acrylic acid units and the water. This results in an increase in the LCST.

The above hypothesis was also put forth by Feil et al, who synthesized poly(NIPAAm-co-butyl methacrylate) copolymers with butyl methacrylate (hydrophobic), acrylamide (hydrophilic), and acrylic acid (anionic) \(^{200}\). It was found that the changes in LCST were proportional to comonomer content. The increase in LCST was largest for the anionic comonomer, followed by the cationic and hydrophilic comonomers. The group also varied the charge density on the anionic and cationic copolymers by changing the pH of the swelling medium. They reported an increase in LCST with increasing charge density, and attributed this to the same mechanism by which hydrophilic components increase the transition temperature.

Hoffman et al. reported similar increases in the LCST of PNIPAAm as a result of copolymerization with acrylic acid \(^{210}\). In addition, Brazel et al. reported increases in the LCST of PNIPAAm as a result of copolymerization with methacrylic acid \(^{211}\). David et al. created block copolymers of PNIPAAm and poly(n-acetylimino)ethylene. They showed that increasing the chain length and frequency of the hydrophilic blocks increased the LCST \(^{212}\).
By the same token, incorporating hydrophobic comonomers into PNIPAAm networks decreases the LCST. Ma et al. showed that creating PNIPAAm copolymers with the hydrophobic isopropyl methacrylate lowered the transition temperature. The experimental results showed a linear correlation between the LCST and the amount (mol %) of isopropyl methacrylate \(^{213}\). Xue et al. prepared hydrogels composed of PNIPAAm with hydrophobic di-\(n\)-propylacrylamide, di-\(n\)-octylacrylamide, or di-dodecylacrylamide. The group hypothesized that use of comonomers containing two alkyl chains would impart stronger hydrophobic interactions to the polymer network. It was found that the incorporation of the comonomers lowered the LCST of the PNIPAAm. However, the temperature was independent of hydrophobic comonomer. Also characterized was the transition temperature of gels formed from aqueous solutions containing the anionic surfactant sodium dodecyl sulfate. The surfactant causes an appreciable increase in the LCST of the polymer, this being attributed to electrostatic repulsions between polymer chains due to the binding of the sodium dodecyl sulfate to the PNIPAAm chains \(^{192}\).

Shin et al. observed anomalous swelling behavior for an interpenetrating network of PNIPAAm and hydrophilic poly(acrylic acid). They observed a decrease in the LCST of PNIPAAm as a result of the formation of the interpenetrating network and hypothesized that the free carbonyl groups on the PNIPAAm hydrogen bonded with the acid group on the poly(acrylic acid), leaving less polar groups on the PNIPAAm available for hydrogen bonding with water. This increased the overall hydrophobicity of the PNIPAAm, lowering its LCST \(^{214}\).
Han and Bae \textsuperscript{215} performed an extensive study on the precipitation of a high molecular weight PNIPAAm block copolymer with 2 mol\% acrylic acid. The LCST of the copolymer, determined by cloud point measurements, was 32\(^\circ\)C. Solutions composed of 5 wt\% polymer were dissolved in phosphate-buffered saline (pH 7.4). The solutions, which were initially clear, turned cloudy when heated to 27\(^\circ\)C. When the temperature was increased to 35\(^\circ\)C, an immobile, bulk phase appeared. Upon heating to 43\(^\circ\)C, the solid began to shrink due to the expulsion of water from the network. The authors noted that this gel deformed without shape memory. In other words, it exhibited very poor elastic properties. Based on these visual observations, it was speculated that polymer chains with lower acrylic acid contents precipitated first, forming globules, but those with higher acrylic acid contents remain in a partially collapsed conformation. The globules become entangled with the partially collapsed chains, forming hydrophobic regions with physical junctions \textsuperscript{215}.

\subsection*{2.5.4 PNIPAAm-based Hydrogels}

In the past several years, temperature sensitive and \textit{in situ}-forming hydrogel systems have gained extensive interest for biomedical and pharmaceutical applications. PNIPAAm-based systems have been one of the most commonly proposed thermosensitive materials, not only because its phase transition conveniently occurs between ambient and body temperature, but because copolymers of NIPAAm with different types of monomers can result in materials with a range of different properties. For instance, copolymers of thermosensitive N-isopropylacrylamide and gelatin were prepared which form an elastic
hydrogel above 34°C or below 10°C \(^{216}\). In another study, functionalized PNIPAAm chains were grafted onto alginate to form a comb-like polymeric structure which, when heated above the LCST, formed extremely porous hydrogels, allowing for rapid deswelling kinetics. These materials were investigated as a rapid stimuli-responsive drug delivery system \(^{217}\).

Some researchers have investigated copolymeric hydrogels of NIPAAm with a hydrophobic comonomer by characterizing the effect that the hydrophobic component has on the swelling and mechanical properties of the hydrogels. Hydrogels composed of PNIPAAm and hydrophobic comonomers 2,2,3,3,4,4,5,5-octafluoropentyl methacrylate and n-butyl methacrylate were evaluated by Lee et al. It was hypothesized that the presence of the hydrophobic comonomer would increase the deswelling kinetics of hydrogel system, decrease the swelling capacity of the gel network, and increase the mechanical properties. The group found that the gel swelling ratio decreased and the stiffness increased with increasing content of the hydrophobic comonomer. This effect was more pronounced in the hydrogels composed of NIPAAm and 2,2,3,3,4,4,5,5-octafluoropentyl methacrylate, attributing this to the fact that the atomic size of the fluorine is much larger than hydrogen, causing the molecular chain packing in these gels to be much denser than that of the n-butyl methacrylate gels \(^{218}\).

Conversely, the incorporation of hydrophilic comonomers tends to increase the swelling capacity of PNIPAAm networks. For example, PNIPAAm copolymers with dextran were synthesized by free radical polymerization of NIPAAm and a dextran macromer
containing multiple hydrolytically degradable oligolactate-2-hydroxyethyl methacrylate units. It was found that the swelling capacity of the gels was strongly dependent on temperature and copolymer composition. While all of the copolymers exhibited decreases in water content as the temperature was raised above the LCST, the swelling ratios increased with increasing dextran content. This was attributed to overall increase in hydrophilicity of the network due to the presence of the dextran. After six days immersion in PBS, the gels began de-swelling at a higher rate, due to the degradation of the dextran within the gel work, resulting in an overall increase in the hydrophobicity of the polymer.

In addition, Lin et al. investigated the structure-property relationship of block or star copolymers of PNIPAAm and poly(ethylene glycol) (PEG) hydrogels. The PEG lengths used in this study were approximately 2000 Da. These copolymers formed injectable solutions at 5°C and at physiological temperatures formed strongly associated networks. The group reported that these networks were stabilized by either crosslinking, in the case of the networks formed from multiple armed PEG, or micellar packing and entanglements in the case of the linear block copolymers. The gelation kinetics of the hydrogel system were rapid, occurring in less than one minute, and there was no significant syneresis after 2 months. The networks formed from the four-armed PEG exhibited the highest strength and deformability. These materials are being investigated for use as a drug delivery or cell encapsulation system.
The incorporation of ionizable groups into PNIPAAm hydrogels can improve the swelling of the network more effectively than non-electrolyte hydrophilic components. For instance, Shin et al. synthesized interpenetrating networks of PNIPAAm and poly(acrylic acid). They found the swelling behavior to be highest above pH 5.3, conditions under which poly(acrylic acid) is charged. They also found that the compressive stiffness of the networks was directly related to the swelling of the network, and therefore pH of the immersion media. Gels swelled at lower pH, such as 3, were stiffer than those swelled at pH 4.6 and 5.3.

In another study, block copolymers of PNIPAAm and acrylic acid were loosely crosslinked with N-N’-methylenebis(acrylamide), forming a transparent, pliable solution at ambient temperatures. These gels possessed improved volume retention capabilities compared to gel networks composed of the PNIPAAm homopolymer. This was attributed to the fact that the acrylic acid containing networks exhibited 75% water retention after 6 days immersion in phosphate buffered saline, whereas the homopolymer network lost half of its water during that time period. The authors postulated that the improved swelling properties of the acrylic acid containing networks were due to repulsions of the –COO groups, whereas the homopolymer chains can collapse substantially and expel pore water above the transition temperature.

PNIPAAm-based hydrogels have also been investigated as three dimensional matrices for cell encapsulation. The acrylic acid copolymers described above were shown to support chondrocyte viability, resulting in the formation of cartilage-like tissue inside the
matrix. Consequently, the hydrogels are a viable material for the repair of cartilage defects. Cho et al. encapsulated human mesenchymal stem cells in a PNIPAAm matrix grafted with chitosan. The cell-thermosensitive gel complex was injected into the submucosal layer of the bladder of a rabbit, resulting in cartilage growth. This system could be used as a treatment for vesicoureteral reflux or reflux esophagitis.\(^{221}\)

Solutions of PNIPAAm were also proposed as an extracellular matrix for an artificial pancreas. It has been shown that islets of Langerhans, groups of pancreatic cells that produce and secrete hormones, need to be repeatedly replaced after implantation to avoid malfunction. Solutions of PNIPAAm loaded with islets can be placed in a pouch and implanted into diabetic patients. The PNIPAAm gel, formed at body temperature, has been shown to immobilize islets in rats without impairing insulin secretion. Periodically, the pouches can be cooled, allowing the solutions to be withdrawn, and refilled with a fresh polymer/cell suspension\(^{222-224}\).

### 2.5.5 PNIPAAm-based Systems for Drug Delivery

PNIPAAm-based hydrogels can also be used to immobilize drugs, enzymes, antibodies, and other biomolecules. The LCST phase transition can be used as trigger for drug release. In many of these cases, therapeutics are loaded inside a swollen matrix at \(T<\) LCST and is released when placed in a medium with \(T>\) LCST\(^{225}\). An initial burst release of surface drug, or other biologically active agent, can occur. This is followed by the formation of a dense skin on the outside of the hydrogel as a result of the temperature
gradient across the gel. Drug from within the matrix is squeezed out due to a buildup of hydrostatic pressure inside the gel. Lastly, as PNIPAAm chains throughout the entire network collapse, drug release can occur via a diffusion process through the newly formed gel.

Interestingly, the LCST phase transition can be used as an on or off switch for drug release. Hoffman et al. have studied the release rate of methylene blue from copolymers of NIPAAm and methacrylic acid, as well as from PNIPAAm homopolymer gels. In both cases, above the LCST, there were two distinct regions of release where the amount of methylene blue liberated was proportional to the square root of time. The first region was the initial rapid release and it was followed by a slow Fickian diffusion through the network at long times. Gutowska et al. synthesized PNIPAAm copolymers with acrylic acid which were crosslinked with ethylene glycol dimethacrylate. They reported a zero order release profile for acetaminophen at pH of 7.4, which was dictated by the des-welling kinetics of the gel network. This release occurred as a response to heating the polymer above its LCST, in contrast to the NIPAAm copolymer system with butyl methacrylate studied by Bae et al. They reported that the release of indomethacin occurred at temperatures lower than the gel LCST. Above the LCST, the release was inhibited by the formation of a dense outer layer on the polymer surface. The formation of this layer was found to be controlled by the concentration of hydrophobic comonomer. The presence of the hydrophilic component in the PNIPAAm copolymers with acrylic acid prevented the dense skin formation and allowed for release above the phase transition of the gel.
Drug loading into, and release from, a hydrogel network will be controlled by pore
volume fraction, pore size and their interconnections, the size of the drug, interactions
between the polymer and the drug, among other factors. Some research efforts have
focused controlling the release rate of certain drugs from PNIPAAm systems. Specifically, efforts have been directed into understand how to reduce a burst release of
drug, which could lead to toxic levels of drug in the blood plasma, as well as
modifying the system to prolong dosing of the drug.

For instance, Zhang et al. prepared hydroxyl-functionalized glycerol poly(ε-
caprolactone) based microspheres loaded with ovalbumin and physically entrapped them
within a crosslinked PNIPAAm hydrogel. At physiological temperature, the PNIPAAm
hydrogel alone release 90% of the ovalbumin within 24 hours, but only 40% of the model
drug was released after 60 days from the combined microsphere/hydrogel delivery
system. The release curve for the latter had a typical biphasic pattern, with an initial
burst release followed by a slow sustained release. Release from the combined system
was also examined at 22°C, and the release characteristics were similar to those at 37°C,
but the burst release was smaller in magnitude. This was attributed to the collapse of the
PNIPAAm chains extruding ovalbumin from the network. A more sustained release was
achieved at 37°C because the collapsed PNIPAAm matrix slowed the release of the drug.

In another study, interpenetrating networks of temperature sensitive PNIPAAm and
hydrophobic poly(ethylene acrylate) were investigated for the release of hydrophobic
drug daidzein. The release profiles were compared to those from interpenetrating networks composed of the PNIPAAm homopolymer. The poly(ethylene acrylate) created hydrophobic reservoirs, present even below the LCST when the gel is in the hydrated state. This increased the diffusion resistance of the drug through the network and slowed its release rate, thereby avoiding the burst release seen with the homopolymer network.

Wu et al. evaluated the release of bovine serum albumin (BSA) and insulin from a crosslinked PNIPAAm network. Drug loading was achieved by soaking the crosslinked gels in aqueous solutions of the proteins for 4 days, and it was found that loading decreased with increased network density, which limited the adsorption of the protein. It was found that the insulin was released in its entirety within 48 hours, while the release of the encapsulated bovine serum albumin was incomplete at the end of the study. The group hypothesized that the release of the BSA was slowed by a strong protein-gel interaction due to hydrogen bonding or hydrophobic interactions. Other researchers have also reported an association between PNIPAAm and BSA which can be described as an intramolecular complex of a PNIPAAm chain with several bound albumin molecules.

Zhang et al. demonstrated that release bovine serum albumin from interpenetrating polymer networks of PNIPAAm lowered the burst release compared to non-interpenetrating PNIPAAm networks. This is due to the higher polymer density in the interpenetrating network, inhibiting protein diffusion and increasing its interaction with the BSA. In addition, they also noted an incomplete release of the albumin.
Other studies on drug release from PNIPAAm networks have focused on the use of micro or nanosized PNIPAAm-based gel carriers. Microgels composed of PNIPAAm copolymers with acrylic acid were prepared. BSA was loaded into the microgels by incubating them in a solution of BSA and phosphate buffered saline for 12 hours, allowing the albumin to adsorb onto the particles. It was found that without the acrylic acid component in the copolymer, albumin adsorption did occur, though in small quantities. The authors maintain that the driving force for the adsorption was the hydrogen bonding interaction occurring between the carboxyl groups on the albumin and the amide groups on the PNIPAAm. However, adsorption was enhanced considerably by the presence of acrylic acid, due to the affinity between the carboxyl groups on the acrylic acid and the –CONH- groups on the protein. BSA loading was maximum at pH between 4 and 5, since the acrylic acid dissociates, initiating an electrostatic attraction with the positive charges on the protein molecule \(^{234}\).

Another group studied the potential for using PNIPAAm-based nanogels to prevent proteins against denaturation agents. Their studies demonstrated that nanospheres of various sizes between 765 and 1146 nm composed of PNIPAAm crosslinked with PEG dimethacrylate 400 and 1000 had the ability to protect insulin against high temperature. After 12 hours at 80ºC, 25% of the insulin loaded into the nanoparticles could still be detected by reverse phase high pressure liquid chromatography, compared to 0% for the control, which was an insulin solution in phosphate buffered saline \(^{235}\).
Nanoparticles consisting of interpenetrating polymer networks of poly(acrylic acid) and PNIPAAm were also investigated as a potential drug delivery system. The nanoparticles, loaded with dextran markers of various molecular weights, can be dispersed in an aqueous medium at ambient temperatures and injected into the body, where the dispersion becomes a gel as a result of the phase transition of PNIPAAm. The resulting gel was described by the authors as a physically crosslinked system of nanoparticles. The bonding between nanoparticles can be turned on or off using the gelation characteristics of PNIPAAm. The release studies, conducted at 37°C, indicated that lower molecular weight dextrans were released from the network much faster than the higher molecular weight markers. Decreasing the particle radius did not have a significant impact on release. Additionally, decreasing the weight percent of copolymer in the particles increased the release rate due to the formation of pores through which the model drug molecules could diffuse.

Hsieu et al. studied two PNIPAAM-based systems designed for the controlled release of ophthalmic agents for drug release. The first system was composed of linear PNIPAAm chains, which formed an entangled network above 32°C through which the drug could diffuse. The second was a mixture of linear PNIPAAm chains combined with physically entrapped crosslinked PNIPAAm nanoparticles. The intraocular pressure-lowering effect was measured in order to evaluate the release characteristics of the systems. It was found that the composite system produced a longer, sustained release. The intraocular pressure drop lasted 32 hours for the composite system, compared to 24
for the linear PNIPAAm network. Both delivery systems produced an improved effect over traditional eye drops, the pressure drop duration of which only lasts 6 hours.

One of the most interesting applications for nano and microscale PNIPAAm delivery systems is targeted cancer treatment based on magnetic particles. Magnetite nanoparticles were conjugated to functionalized PNIPAAm chains \(^{238}\). Because the magnetite particles generate heat in response to an alternating magnetic field, the system exhibited a temperature-induced aggregation in response to the magnetic field. In addition, the nanoparticles also possessed free carboxyl groups, which could be used as a conjugation point for drugs.

Muller-Schulte et al. \(^{239}\) studied the release characteristics of a similar system. Magnetic colloids and model drugs rhodamine B or methylene blue were encapsulated together inside spherical micro and nanoparticles composed of crosslinked PNIPAAm. Those model drugs were chosen due to similarities in molecular mass and structure to the anticancer drug doxorubicin. When an alternating magnetic field was applied, the particles were heated above the transition temperature of the PNIPAAm, due to heat transfer between the magnetic colloid and the polymer matrix, resulting in the complete release of the model drugs within 16 minutes, in concentrations that would be adequate for cancer therapy. Methylene blue had a slightly slower release, attributed to an interaction between the positive charge on the methylene blue and the negative charge on the magnetite.
Nanoscale delivery systems composed on polymeric micelles have gained significant importance in biomaterials over the last two decades. In an aqueous environment, amphiphilic block copolymers can form micelles, with the hydrophobic block becoming the hydrophobic corona of the micelle, and they hydrophilic forming the outer shell, or corona. The highly hydrated corona of the micelle reduces recognition of the particle by phagocytic cells, increasing circulation time in the bloodstream. In addition, the hydrophobic core can serve an environment for the incorporation hydrophobic drugs, which protected against external stresses by the outer shell. These hydrophobic drugs would otherwise have a very low bioavailability in the body.

Temperature-sensitive PNIPAAm copolymer-based micelles all function by a similar mechanism. At ambient temperatures, hydrated PNIPAAm chains for the micelle corona, while a hydrophobic comonomer, conjugated the PNIPAAm, stabilizes a hydrophobic drug in the core. Drug release occurs as a result of increasing temperature, which causes the PNIPAAm to become hydrophobic, causing structural changes in the polymeric micelles. For instance, amphiphilic graft polyphosphazene, constructed with hydrophilic PNIPAAm oligomers and hydrophobic ethyl 4-aminobenzoate side groups can assemble into micelles at 25°C. Above the LCST of PNIPAAm, occurring at 32.6 °C, the micelles aggregate due to interactions with neighboring PNIPAAm chains, resulting in an abrupt increase in the measured particle size.

Temperature and pH responsive micelles were constructed from a diblock copolymer of poly(t-butyl acrylate-co-acrylic acid) and PNIPAAm. At room temperature and pH 5.8,
the copolymers form micelles with the t-butyl acrylate as the core, and the hydrophilic acrylic acid and PNIPAAm chains stretched out to form a two component shell. When the temperature is raised from 25°C to 32.4°C, the LCST of the PNIPAAm, water progressively becomes a poorer solvent for the PNIPAAm, so these chains collapse into the core and the acrylic acid composes the corona. At this temperature, a significant decrease in the hydrodynamic size distribution of the particles could be measured. As the temperature is raised further to 50°C, the size of the particles reaches a constant value because the collapse of the PNIPAAm chains stop. In addition, with the temperature held constant at 25°C, and the pH lowered, the 3.0, the acrylic acid chains collapse to the core and PNIPAAm becomes the corona. The solubility of the acrylic acid depends on the degree of ionization and is enhanced at increasing pH values.\textsuperscript{243}

\textbf{2.6 Poly(ethylene glycol)}

Poly(ethylene glycol) (PEG) is a hydrophilic, non-ionic, crystalline polymer. Its chemical structure is shown in Figure 2.13. It is soluble in water via hydrogen bonding interactions.\textsuperscript{244} PEG is known for its biocompatibility. It has been approved by the FDA for internal consumption.\textsuperscript{245}

Because of its biocompatibility, PEG has received ample attention in the biomaterials community of recent years. PEG is a component in several injectable biodegradable polymer systems intended for drug delivery, such as ABA or BAB block copolymers, where A is PEG and B is poly(DL-lactide-co-glycolide). These systems are soluble in
water just below room temperature due to the dominating behavior of hydrogen bonding that exists between the PEG and water. At slightly higher temperatures, typically above 30°C, the behavior of the hydrophobic poly(DL-lactide-co-glycolide) dominates, allowing it to form associative physical crosslinks, causing a sol to gel transition. These systems have been shown to be effective carriers for both hydrophilic and hydrophobic drugs, such as 5-fluorouracil and indomethacin\textsuperscript{246}. Other PEG based sol to gel systems have also been investigated for biomedical applications, such as copolymers with polyglycolide, ε-caprolactone, sulfamethazine\textsuperscript{247}, poly(butylene oxide)\textsuperscript{248}, and poly(propylene oxide)\textsuperscript{249}.

In many cases, research has been directed at using PEG-based materials for scaffolds that support cell growth and function. For instance, Sims et al.\textsuperscript{250} developed a technique for delivering a mixture of bovine chondrocytes and PEG subcutaneously into rats. The cells were suspended in a 20 wt/vol% solution of PEG 100,000 g/mol. It was hypothesized that the PEG could provide a biocompatible scaffold in which the chondrocytes could proliferate and secrete extracellular components. The researchers saw an increase in the glycosaminoglycan content of the rat cartilage. However, the PEG scaffold exhibited rapid dissolution and poor mechanical properties\textsuperscript{251}.

Subsequent work showed that the properties of these networks can be enhanced by the preparation of crosslinked or interpenetrating PEG networks. For example, PEG dimethacrylate-based hydrogels were prepared by UV photopolymerization. By varying the crosslinking density of the network, hydrogels were prepared with shear moduli ranging from 10 kPa to 1 MPa. In addition, supplementing the reaction mixture with a
small amount of glycidyl methacrylate comonomer created reactive sites within the network through which biomolecules could covalently bond with the PEG network. Textured surface topographies, in the form of parallel grooves, were created on the hydrogels by casting them on microstructured silicon wafers. The cellular response to the surface topography and varying mechanical properties will be investigated.

Crosslinked PEG scaffolds can also be rendered biodegradable by the incorporation of hydrolytically degradable components into the hydrogel matrix. Crosslinked hydrogels were prepared from photopolymerization of PEG dimethacrylate. Three degradable lactide units were grafted to each PEG molecule. Additionally, neural cells were photoencapsulated into the network, which were shown to differentiate, proliferate, and generate neural tissue which penetrated through the open space in the network. The distance between crosslinks in the network, or the mesh size, was shown to be a controlling factor in the rate of tissue ingrowth. If the average mesh size was too narrow, neural growth was inhibited. Because the network mesh size increased as the lactide units degraded, one way to control the rate of tissue ingrowth is to vary the lactide content of the network. This work also demonstrated that the PEG-based material was well tolerated by the neural cells, making it a viable carrier for cell transplantation into the central nervous system.

Conventional tissue engineering scaffolds which only provide a temporary, 3-D structural support are being replaced with scaffolds which are composed of more interactive biomaterials, such as biomolecules, which not only support cell migration and
proliferation, but initiate it. In this new direction in tissue engineering, PEG still remains a major component in the hybrid biomaterials. For instance, PEG-protein hydrogels have been studied for applications in tissue engineering because the protein sequences naturally possess cell signaling domains for adhesion and protease degradation. Meanwhile, the PEG domains impart structural integrity to the scaffold.

Benoit et al. created PEG hydrogels functionalized with heparin to use as a culture system for human mesenchymal stem cells. Heparin has been shown to promote cell adhesion and interact with proteins associated with osteoblast and osteoblast progenitor cell adhesion. In this study, 94% of the stem cells survived over the five week study, compared to only 47% in previous studies with unfunctionalized PEG hydrogels. This improvement was attributed to the ability of heparin to bind with fibronectin and bone morphogenic protein. Hwang et al. incorporated glucosamine into embryonic stem cell-containing photocrosslinked PEG hydrogels. Glucosamine has been shown to play a role in enhancing the synthesis of cartilage, tendons, ligaments, bone, and other connective tissues. The researchers found that the presence of glucosamine enhanced extracellular matrix production and, in doing so, helped improve the structural integrity of the scaffold.

PEG has also been shown to be a beneficial component in mucoadhesive drug delivery systems. Mucoadhesive devices can be applied to, and deliver drugs locally to, mucosal tissues, including the gastrointestinal, nasal, and buccal areas. Mucoadhesion is the attachment of a natural or synthetic polymer to the mucosa. This occurs via the
interpenetration, and subsequent hydrogen bonding, between the polymer chains in the chemical carrier with the glycoproteins present in the mucus \(^{263}\). Acrylic–based polymers have been extensively used in mucoadhesive applications due to their high adhesive bond strength with the tissue \(^{264,265}\). However, PEG can be used as a mucoadhesion promoter, though the polymer is not mucoadhesive itself. PEG chains that are grafted onto the chemical carrier will enhance interpenetration of the material into the mucosa, increasing mucoadhesive strength \(^{262,263,266-268}\). For instance, Serra et al. \(^{263}\) showed a 480% increase in the work of adhesion value for a poly(acrylic acid) copolymer with PEG 1000 g/mol tethers compared to pure poly(acrylic acid).

Arguably, the most important and extensive use of PEG is in colloidal polymer systems for biological and pharmaceutical applications. In fact, PEG is used universally as the hydrophilic block in polymeric micelles \(^{241,269}\). There are several reasons for its widespread use. Firstly, PEG provides steric stabilization to colloidal particles such as liposomes or micelles \(^{241}\). The hydrated PEG chains create steric forces which compete with interparticle van der Waals attractive forces. Particle coagulation is prevented because the repulsive forces overwhelm the attractive forces between particles \(^{270}\). The larger particle sizes caused by coagulation would increase the chances of the drug delivery vehicle being cleared by the immune system, reducing circulation time in the body \(^{241}\).

Secondly, PEG inhibits the surface adsorption of biological molecules \(^{241}\), such as proteins, which typically occurs within the first few minutes of exposure to physiological
fluids. As proteins approach a PEG surface, the PEG chains become compressed, reducing their conformational entropy and increasing their enthalpy, making protein adsorption thermodynamically unfavorable. Also, the increase in particle concentration at the PEG surface due to the presence of proteins causes an influx of water caused by an osmotic pressure gradient, forcing the protein to separate from the surface \(^{271}\). Protein adsorption onto drug delivery vehicles could damage the particle or interfere in biochemical pathways \(^{241}\). Moreover, PEG chains prevent surface adsorption of proteins which belong to the reticuloendothelial system. The adsorption of these proteins would attract phagocytic macrophages to ingest the drug delivery vehicle, clearing it from the body. Grafting PEG onto particle surfaces will therefore impart “stealth” characteristics to the drug delivery system, increasing circulation time in the body \(^{272}\).

The work of Tobio et al. \(^{273}\) is a prime example of how PEG can improve the stability and bioavailability of nanoparticle delivery systems. The work investigated the influence of a PEG coating around poly(lactic acid) nanoparticles on their stability in GI fluids and their ability to transport proteins though the intestinal mucosa. The authors hypothesized the PEG coating would help to overcome the challenges involved in oral application of drug carriers, specifically the instability of the particles in the GI tract and low intestinal uptake of the drugs. PLA nanoparticles have been shown to degrade significantly in intestinal fluids \(^{274,275}\), but the protein repellent properties of PEG could help overcome this. In addition, the ability of PEG to improve mucoadhesion could increase transport through the intestinal mucosa. Their studies revealed that, after four hours of incubation in digestive fluids the PEG coated particles were only slightly degraded (3% of the
poly(lactic acid) was converted to lactate, compared to 9% for the uncoated particles). Additionally, the levels of radioactive antigen in the blood stream, which was encapsulated in the nanoparticles, were higher for the coated system.

Similarly, in another study, using a hydrophilic PEG corona in polymeric micelles (with cyanoacrylate-co-hexadecyl cyanoacrylate as the hydrophobic block) for the delivery of active therapeutic molecules increased the half life of the particles in the blood. Results also showed a higher uptake of the drug by the target organs, the spleen and brain, compared to non-PEGylated micelles, the composition of which was not expressly stated.

### 2.7 Poly(ethylene imine)

Poly(ethylene imine) or PEI is a cationic polymer consisting of primary, secondary and tertiary amines. Its chemical structure is shown in Figure 2.14. It is available in a wide variety of molecular weights and degrees of branching. It exhibits a very high charge density over a large range of pH values. In fact, PEI exhibits one of the highest charge densities among organic molecules. The pKa value of the primary amine is around 9, the secondary amine around 8 and the tertiary amine around 6–7. At pH of 10, the amine groups are uncharged, and the swelling of PEI under these conditions can be mainly attributed to the hydrophilicity of the amine groups. Lowering the pH results in protonation of the amine groups. In salt solutions, swelling under these conditions is determined by a balance between the polymer network osmotic pressure and elasticity.
The osmotic pressure resulting from the high concentration of counterions in the interior of the PEI network causes an influx of water which swells the polymer. If the PEI chains are crosslinked, they will oppose this expansion \(^{279}\).

At the present time, the biological properties of PEI are not completely understood \(^{280}\), as there have been few investigations into the biocompatibility of PEI toward human cell lines. Larkard et al. \(^{281}\) coated semiconducting electrodes with polymer films and examined the adhesion, proliferation, and morphology of rat neuronal cell lines on the polymer surfaces. After 8 hours in a cell culture medium, a high percentage of the cells were found to be attached to the PEI. After 72 hours, the cells were found to proliferate faster on PEI than on the other polymers (polypropyleneimine and polypyrrole). It was therefore determined by this group that PEI is a satisfactory coating for the cultivation of neuronal cells. In another study, the adhesion and proliferation of human Schwann cells was compared on substrates composed of fibronectin, laminin, crosslinked gelatin, or PEI. It was found that adhesion and proliferation was satisfactory on all coatings, but the relative performance of PEI was poor. Cell proliferation was moderate compared to the other polyelectrolytes, and while choice of substrate was deemed not to be an important consideration in the culture of Schwann cells, PEI was not recommended.

Based upon these studies, it may be inferred that the biocompatibility of PEI may depend on cell type. However, the molecular weight of PEI used in the aforementioned research articles was not reported by the authors. Morimoto et al. \(^{282}\) reported the cytotoxicity of
PEI to be molecular weight dependent. A comparison among the cytotoxicity of PEI 1800, 10,000, and 70,000 Da showed increasing cytotoxicity with molecular weight.

In another study, PEI 70,000 Da was deposited onto a titanium alloy surface, and it was found that osteoblasts and fibroblast proliferation and adhesion decreased over a 7 day period. A group who studied PEI 750,000 Da as a potential coating for bone implants evaluated the response of human osteoblasts-like cells and periodontal ligament cells. While maximum adhesion occurred within twenty minutes for the coatings composed of poly(sodium 4-styrenesulfonate), poly(allylamine hydrochloride), poly(L-glutamic acid), or poly(L-lysine), cell adhesion to PEI was similar to that of glass or plastic. In addition, no proliferation was seen for either cell type on the PEI substrate. The cell nuclei became condensed or fragmented, and after 24 hours, cell apoptosis was observed. These results are consistent with those of Moghimi et al., who showed that branched PEI of molecular weight 25,000 and 750,000 Da induced necrotic changes within 30 minutes in human umbilical vein endothelial cells and hepatocyte-like cells. Within 24 hours, cell apoptosis was initiated. While the authors note that the molecular basis for the cytotoxicity is not understood, this property of PEI may have beneficial aspects when applied to tumor gene therapy.

Despite its potential cytotoxicity, the presence of the ionizable groups on PEI has motivated researchers to investigate its potential in drug delivery. Vinegradov’s group developed nanosized gel networks composed of crosslinked PEG and PEI. While the PEG aids in the stabilization of the aqueous nanogel dispersions, the cationic PEI chains
allow for the immobilization of negatively charged biologically active molecules, such as indomethacin. Prior to drug loading, the nanogels form expanded networks due to the osmotic pressure generated by the fixed positive charges on the PEI. After drug loading occurs, due to the formation of an electrostatic complex with the charged drug molecule, the nanogels dehydrate, resulting in network collapse and decrease in particle size. Release studies showed that 17.5% of the indomethacin was released during the first hour, followed by an 82% release by 24 hours. Interestingly, the release of the drug causes the nanogel to swell again, due to the increase in net ion concentration inside the network.²⁷⁹

Some researchers have diverged from using PEG as the hydrophilic corona for micellar structures in favor of using PEI. The cationic nature of PEI allows the micelles to interact with negative charges on cell surfaces²⁸⁴,²⁸⁵, helping to increase cell uptake. Tian et al.²⁸⁴ synthesized novel amphiphilic biodegradable block copolymers based on PEG, hyperbranched PEI, and poly(γ-benzyl L-glutamate). In aqueous solutions, these copolymers self-assembled to micellar structures. Characterization studies on the micelles revealed that particle size was dependent on the ionic state of PEI. As its charge density increased, the particle size decreased. The authors attribute this change to an increase in macromolecular aggregation, which impeded hydrophobic block interactions. In addition, micellar solutions were added to plasmid DNA, which readily became immobilized on the micelles.
Another group prepared micelles composed of PEI and poly(D,L-lactide-co-glycolide). They also found that the micelle size depended on the charge density of the PEI, the size decreasing as charge density increased. This was once again attributed to the inhibition of hydrophobic block interactions. This group incubated micellar solutions with human keratinocyte cells and found that the particles not only adsorbed onto the cell membranes, but translocated into the nucleus. PLGA nanoparticles were also studied as a control, and minimal adsorption and diffusion into the cell surface was exhibited.

Based on the above findings, it is obvious why the most prominent application of PEI is gene delivery. DNA is condensed with PEI due to its electrostatic interaction with the positive charges on PEI. In the condensed form, DNA is protected against digestion of enzymes and can be delivered toward a targeted cell, and internalized via endocytosis. Polycations destabilize cell membranes, causing their rupture, releasing the PEI/DNA complex into the nucleus, allowing it to be expressed. It has been reported that the size of the PEI has a strongly affects the transfection efficiency. Low molecular weight PEI (less than 2000 Da), though proven to be non-toxic, produces low transfection activity. High molecular weight PEI (greater than 25 kDa) has shown high transfection activity combined with significant cytotoxicity.

While the reason for its low transfection activity remains unclear, there have been some efforts to chemically modify low molecular PEI in order to increase its transfection efficiency while retaining its low cytotoxicity. Peterson et al. created PEI block copolymers with star-shaped PEG molecules. They hypothesized that chemically
modifying low molecular weight PEI to increase its size would increase the transfection efficiency, and that the charges on PEI do not critically contribute to condensation. Based on the obtained results, the authors maintain that low molecular weight PEI exhibits poor transfection efficiency because it only moderately condenses the DNA, insufficiently protecting it. However, its condensation potential was enhanced by coupling it to star PEG molecules with branch sizes of 10 or 15 kDa. These conjugates had similar size to high molecular PEI, and formed compact, stable complexes upon mixing with plasmid DNA. The transfection activity and cytotoxicity of these complexes are still under investigation.

Similar copolymers, based on linear copolymers of PEG 5 kDa and PEI 22 kDa, have been complexed with DNA to deliver genes to tumors which encode for the tumor necrosis factor. Tumor growth inhibition was shown to occur in three murine tumor models. It has also been demonstrated that the PEG molecules prevent non-specific interactions with the biological environment and prolong the circulation time of the DNA complex.

2.8 Bioadhesive Polymers for Soft Tissue Repair

Bioadhesive polymers are natural or synthetic materials that can be used for soft tissue repair, including wound closure, achieving hemostasis after a surgical procedure, or
fistula repair. Bioadhesive materials can supplement the use of sutures or replace them altogether.

Cyanoacrylates are a widely used class of bioadhesive compounds. The use of these adhesives in medicine began in the 1960s. Cyanoacrylates are synthetic glues that polymerize in the presence of water or blood. N-butyl-2-cyanoacrylate (Histoacryl, B Braun, Melsungen, Germany) and N-butyl-2-cyanoacrylate (Glubran, GEM S.r.l., Viareggio, Italy) were recently approved for endoscopic use in Europe. Although Histoacryl and Glubran are not commercially available in the United States, 2-octyl-cyanoacrylate (Dermabond, Ethicon, Inc., Somerville, NJ) was approved by the FDA for superficial wound closure, but not for endoscopic applications.

Cyanoacrylate sealants have had wide ranging clinical applications. For example, injection therapy with cyanoacrylate adhesives has shown to be an effective treatment for gastric varices. In one clinical study, this treatment was shown to be superior to band ligation, a widely accepted treatment for gastric variceal bleeding. The use of cyanoacrylates for the treatment of gastric varices has been widely adopted outside of the United States. In addition, N-butyl-2-cyanoacrylate was used to repair esophageal variceal rupture, and this method was found to be superior to sclerotherapy with ethanolamine olate solution. The same adhesive was used to repair a peptic ulcer, and it was shown to be superior to using hypertonic saline. While the two treatments showed similar initial bleeding rates, the re-bleeding rate was lower for the cyanoacrylate adhesive. In another study, 8 out 12 pancreatic fistulas were successfully closed with
N-butyl-2-cyanoacrylate. 2-octyl-cyanoacrylate was used to close a corneal perforation, and the patient went on to full visual recovery.

Mixing cyanoacrylates with lipid soluble contrast agent lipidol increases the radiopacity of the adhesive, enhancing imaging. Lipidol also retards the polymerization process, allowing the adhesive to be applied endoscopically via needle, with less risk of premature solidification. When closing superficial wounds, Chigira et al. recommends combining 2-octyl-cyanoacrylate with reinforcing skin closure tape, which significantly increases its tensile strength. Another group proposes preparing composites of 2-octyl-cyanoacrylate and poly(L-lactic-co-glycolic acid) to increase tensile strength.

Cyanoacrylates form solid impermeable plastics in situ after polymerization is complete. Therefore, possible complications include inflammatory response to a foreign body and local tissue necrosis. Wippermann et al. created distal coronary anastomoses in porcine hearts and subsequently sealed with butyl cyanoacrylate. Immediate hemostasis was obtained with the application of the cyanoacrylate adhesive. Histological examinations were performed to determine the immunologic response to the adhesive. The inflammatory response to the adhesive was found to be moderate. Microscopic evaluation revealed the presence of foreign body giant cells. Three months after the surgery, evidence of an inflammatory response still existed and glue fragments were still present, though there was no scar tissue. Another group used cyanoacrylate to close anastomotic blood vessels in adult dogs. They reported necrosis rapidly occurring in the region surrounding the glue, which was later replaced by fibrous tissue. They propose
that this was caused by thermal injury as a consequence of the heat generated from the exothermic polymerization 306.

Fibrin adhesives have been investigated experimentally since the 1970s 307. They act as a hemostatic plug by mimicking the last stage of blood clotting. Fibrin sealants contain components such as fibrinogen, factor XIII, and thrombin. Thrombin catalyzes the conversion of fibrinogen to fibrin, and factor XIII catalyzes the formation of crosslinks within fibrin to form an insoluble fibrin clot 308. The clot is resorbed within days or weeks by macrophages and fibroblasts 309, allowing healing to occur at the site of adhesion. Because they are natural materials, fibrin sealants are completely biocompatible 310.

They are available commercially from Tisseel (Baxter, Westlake Village, CA) and Hemaseel (Hemacure, Sarasota, FL) 311. They are approved for topical application and the closure of anastomoses in cardiovascular and colorectal surgery 297. Tisseel is composed of four main components. Fibrinogen, which is obtained from human donor plasma, is dissolved in bovine aprotinin. Dry human thrombin is reconstituted in calcium chloride, and the two solutions are mixed and applied simultaneously 312.

On the whole, studies comparing this fibrin adhesive to other hemostatic agents show fibrin to be equivalent or superior 297. One such trial compared the use of the fibrin sealant to the injection of polidocanol for the repair of peptic ulcers. Patients who received daily applications of the fibrin sealant had less re-bleeding and fewer acute
treatment failures. However, patients who received only a single fibrin treatment did not experience significantly different outcomes than those in the control group. In a randomized trial involving patients with esophageal variceal bleeding, the outcome of using sclerotherapy with ethanolamine in addition to human thrombin was compared to using sclerotherapy alone. All clinically important outcomes were deemed to be equivalent. Fibrin adhesives have also been concluded to be effective sealants for corneal incisions, LASIK flaps, and conjunctival and skin grafts.

Although fibrin sealants are not associated with inflammatory or foreign body reactions, some components in fibrin adhesives may present complications to patients. Because the fibrinogen is obtained from human plasma, theoretically there is the risk of viral transmission. Because aprotinin is extracted from bovines, there is risk of immunologic or allergic response when administered to humans.

Glutaraldehyde (Figure 2.15) has achieved widespread acceptance as a successful chemical agent for tissue crosslinking. Glutaraldehyde is inexpensive, can form miscible solutions with water, and reacts with tissue in a short period of time. Glutaraldehyde is an aliphatic organic molecule with aldehyde groups at each end. The di-aldehydes are able to react readily with the amines on proteins of the tissue extracellular matrix, via a Schiff’s base reaction, resulting in covalent crosslinks. Lower concentrations of glutaraldehyde, rather than high concentrations, have been shown to more effective for bulk tissue crosslinking. High concentrations produce rapid
crosslinking of the tissue surface and inhibit diffusion of glutaraldehyde into the tissue.

Because of its ability to rapidly react with tissues, a major group of tissue adhesives are based on glutaraldehyde. BioGlue (CryoLife Europa Ltd, Hampshire, United Kingdom) is composed of BSA and glutaraldehyde. The material does not become active until the two components are mixed at the point of application. A rigid mechanical seal forms within seconds. The glutaraldehyde forms covalent crosslinks between the BSA molecules and also reacts with the tissue at the location of application, forming an adhesive bond at the repair site. Currently, BioGlue is approved in the United States to repair aortic dissection. However, in Australia, the Therapeutic Goods Administration has approved BioGlue for all surgical specialties, including cardiac care. The glue exhibits very slow biodegradation, as remnants have been shown to exist in the body as long as two years after surgery. The cohesive strength of BioGlue is higher than the fibrin glue Tisseel. As a consequence of this strength, Bioglue is not recommended for use in children because it could inhibit growth when applied circumferentially.

Passage et al. produced a large scale evaluation of 115 consecutive patients who underwent a range of cardiac procedures such as aortic root, aortic wall, or valve replacement, and coronary artery bypass grafting. BioGlue was used for the achievement of hemostasis, tissue adherence, or tissue strengthening. All of the surgeons believed that
BioGlue facilitated the operation. However, they recommended more studies to assess the long term patient outcomes \(^{321}\).

BioGlue was also investigated for use after a thoracotomy to reduce alveolar air leaks. These air leaks could not be repaired by conventional tissue closure techniques. It was found that BioGlue reduced air leak duration and resultant chest drainage, as well as hospital stay. The surgeons recommended that the volume of BioGlue applied be kept to a minimum, while, of course, ensuring tight closure of the air leaks \(^{323}\). BioGlue was also applied for the achievement of hemostasis after a partial nephrectomy. Adequate hemostasis was achieved, in addition to decreased blood loss and transfusion rate.

The authors recommended investigation into using BioGlue in renal surgery \(^{324}\). The adhesive was also used to repair a dural tear which occurred during lumbar decompressive surgery. Two years after application, during re-operation for an onset of sciatica, fragments of the glue were found adjacent to the dura. They were not encased in fibrous tissue and there was no evidence of an inflammatory response. Complete dural healing occurred \(^{322}\).

Despite its high adhesive strength, the potential toxicity of glutaraldehyde based adhesives needs to be considered \(^{318}\). Inflammatory responses have been associated with glutaraldehyde application and have been ascribed to its cytotoxicity \(^{325}\). Application of BioGlue to the bovine pericardium resulted in the presence of inflammatory cells surrounding the fixed tissue for as long as 26 weeks \(^{326}\). In another study, BioGlue was
used to repair bronchial anastomosis in sheep. While tight closures were obtained with the adhesive, microscopic evaluation revealed the presence of polymorphonuclear neutrophils, macrophages, granulation tissue, and foreign body giant cells after two weeks. This response did not occur in the sutured group.

A valuable study was performed by Fürst et al. The group cured portions of BioGlue and overlaid it with saline solution. The supernatant was analyzed for glutaraldehyde content and applied to human embryo fibroblasts or mouse myoblasts, so the in vitro cytotoxic effects of the glue could be evaluated. In addition, the in vivo toxicity of BioGlue was evaluated in the lung, liver, and aortic tissues of rabbits. The application of the supernatant to both cell lines caused changes in the morphology and density of the cells. Even diluted supernatants, which contained 10% of the initial concentration of glutaraldehyde, resulted in a complete loss of cell viability. The in vivo studies revealed severe inflammatory responses in the lung and liver tissues, characterized by necrosis, edema, granulation tissue, and detected giant cells. In contrast, the aortic model showed no significant differences between the tissue reactions to BioGlue and the biocompatible fibrin sealant.

While several authors have demonstrated that inflammatory effects are a potential consequence of glutaraldehyde-based adhesives, this is the only study which systematically compares the physiological response of different tissues. The results in this study indicate that the consequences of glutaraldehyde toxicity are strongly dependent on the location of application. The authors postulate that the inflammatory
response was less severe in the aortic tissues than in the lung or liver because the aorta contains larger amounts of extracellular matrix and fewer cellular components, rendering them less sensitive to the cytotoxicity \(^{318}\). In addition, Matsuda et al. \(^{328}\) suggested that a lesser inflammatory response would be elicited by the adhesive if the aldehyde groups were immobilized on the surface of the implanted material. This would prevent diffusion deep into the natural tissues, a scenario which could cause a large scale, persistent inflammatory response due to large scale damage and impairment.

It is important to note that it is not clear whether the inflammatory responses to BioGlue have had any long term negative effects on the patient \(^{329}\). Herget et al. \(^{327}\), who repaired bronchial anastomosis in sheep with BioGlue, noted that healing was not complicated by the foreign body reactions, and that the adhesive layer was eventually replaced by connective tissue. However, another group reported tissue healing in glutaraldehyde treated tissues was retarded due to the microenvironment created by the inflammatory responses \(^{325}\).

A number of adhesive systems have been developed specifically for the repair of the damaged intervertebral disc tissues. Repair of a defective annulus can be achieved by applying a curable material, which forms a solid that closes the defect. Slivka et al. \(^{330}\) proposes the delivery of a crosslinking agent into a damaged intervertebral disc in order to stabilize the disc structure, help prevent prolapse of the disc material, and restore or retain biomechanical function. If delivery of the crosslinking agent is targeted at the center of the disc, it would result in crosslinking of the native nucleus pulposus. This
would shift its material properties from a viscous gel to a viscoelastic solid, reducing the risk of herniation. The crosslinking agent can also be used to adhere the nucleus tissues to the surrounding annulus, further reducing its mobility in a degenerated disc. Furthermore, this technology could be used in conjugation with current surgical treatments for lower back pain, such as discectomy, to close the herniated portion of the annulus \(^{330}\).

Slivka et al. proposes using a range of crosslinkers, such as diepoxyl compounds, diisocyanates, bis(sulfosuccinimidyl suberate), or glutaraldehyde. The patent holders note that the crosslinker molecular weight should be over 100 Da, otherwise the agent can too easily diffuse into the tissues surrounding the disc. They also propose modifying the polysaccharides or collagen in the native tissue with aldehyde groups using an oxidizing agent, then allowing spontaneous crosslinking to occur \(^{330}\). A similar method has also been proposed by Liu et al., who development a method for the oxidation of exogenous polysaccharides using periodic acid or periodate \(^{331}\). The aldehyde functionalized polysaccharides will crosslink the collagen molecules in the tissue matrix. Growth factors can be incorporated into the system before or after crosslinking, providing a matrix for the therapeutic repair of the tissue.

Tardy et al. \(^{332}\) put forth a treatment method for collagen that should improve its stability and mechanical characteristics. The tissues are heated via a laser, electrode, magnetic field, or heated aqueous solutions. This shrinks the collagen in the tissue matrix, and then crosslinking is achieved with a solution of periodic acid or periodate. This invention was
intended for the treatment of joint stability problems or the manipulation of skin structure and properties, but it could also be applied to repair of disc tissues. However, heating could produce tissue damage or a non-uniform skin morphology\textsuperscript{330}. 
Figure 2.1 Anterior view of the vertebral column showing the cervical, thoracic, lumbar, and sacral regions.
Figure 2.2 The division of the vertebrae into three regions⁸.
Figure 2.3 (A) Lateral view, (B) superior view, (C) inferior view of the vertebra.

Figure 2.4 The structure of the intervertebral disc. It consists of a nucleus pulposus (NP) surrounded by an annulus fibrosis (AF). Both are sandwiched between two vertebral endplates (VE).
Figure 2.5 A cylindrical interbody fusion cage. A, anterior; P, posterior.\textsuperscript{121}
Figure 2.6 Photograph of the Type III SB Charité.
Figure 2.7 The Charité artificial disc consists of a free-floating biconvex sliding core encased in concave endplates.

146
Figure 2.8 Photograph of the Prodisc-L.
Figure 2.9 Photograph showing the Maverick disc.
Figure 2.10 An implanted Prosthetic Disc Nucleus (PDN) device. 

\[162\]
Figure 2.11 The Newcleus spiral implant.
Figure 2.12 The chemical structure of poly(N-isopropylacrylamide) (PNIPAAm).
Figure 2.13 The chemical structure of poly(ethylene glycol) (PEG).
Figure 2.14 The chemical structure of poly(ethylene imine) (PEI).
Figure 2.15 The chemical structure of glutaraldehyde.
Chapter 3 - RESEARCH GOALS

This work focuses on investigating the properties of a family of \textit{in situ} forming PNIPAAm-based nucleus pulposus replacements. The first objective in this work was to synthesize a class of hydrogels composed of PNIPAAm and PEG. Because the PNIPAAm homopolymer holds little water and shows poor elastic recovery, the swelling and mechanical properties of PNIPAAm gels were tailored by the addition of the PEG. The molecular weight of the PEG chains and the relative proportions of NIPAAm and PEG units were varied.

Then, the range of pertinent material behavior for the PNIPAAm-PEG family of copolymers, and how it varies with polymeric composition was examined. These properties include lower critical solution temperatures, gel swelling, dissolution, stiffness, and elasticity. Evaluation of the swelling and mechanical properties was carried out over long-term \textit{in vitro} studies to determine the stability of the materials. A material candidate from this class of materials was chosen for further evaluation as a synthetic nucleus pulposus replacement. The copolymer formulation was then optimized to minimize water loss following implantation into the intradiscal environment, allowing the implant to remain space filling in the nuclear cavity.

The last objective in this project was to impart bioadhesive properties to the injectable hydrogel system. The PNIPAAm-PEG copolymer was blended with the amine containing polymer poly(ethylene imine) (PEI). After gelation in the disc, the implant is
crosslinked with itself and with the surrounding tissues by the injection of a dialdehyde. This approach can help secure the implant in place, reducing the risk of implant migration or extrusion. The material also has the potential to function as tissue adhesive for the repair of the degenerated intervertebral disc. The specific aims in the project are as follows:

Specific Aim 1: Synthesize a class of polymers composed of PNIPAAm and PEG.

Specific Aim 2: Characterize the gelation, swelling and mechanical properties and long term stability of this family of hydrogels.

Specific Aim 3: Optimize the copolymer formulation to minimize water loss, and subsequent volume loss, due to the de-swelling of the polymer network following injection into the intradiscal environment.

Specific Aim 4: Modify the copolymer chemistry to impart bioadhesive properties to the hydrogel system and evaluate its capacity for tissue adhesion in vitro.
Chapter 4 - SYNTHESIS OF PNIPAAm-PEG BRANCHED COPOLYMERS

4.1 Introduction

The preparation of PNIPAAm-PEG branched copolymers begins with the synthesis of PEG dimethacrylate (PEGDM) from PEG diol and methacryloyl chloride (Figure 4.1). Synthesis was achieved using a modified procedure based on that of Bryant et al. 333.

PNIPAAm-PEG branched copolymers are prepared by polymerizing the monomer NIPAAm in the presence of PEGDM, thereby creating a lightly crosslinked polymer network (Figure 4.2). The PEGDM will be used in low enough quantities for the product to form a flowing solution in water below the LCST of PNIPAAm. Most examples in the literature of crosslinked PNIPAAm systems form hydrated, insoluble polymer networks below the LCST of PNIPAAm 226,227,334.

While PNIPAAm copolymerizations with methacrylate functionalized PEG chains have been reported before, most of these studies used short PEG chains. Bae et al. 215 and Gutowska et al. 226 both prepared PNIPAAm networks that were crosslinked with ethylene glycol dimethacrylate (EGMDA). Another group synthesized microspheres composed of PNIPAAm networks that were crosslinked with a combination of PEG (400 and 1000 g/mol) dimethacrylate. Still, there are some examples where researchers have
explored the use of higher molecular weight PEG chains. PNIPAAm microgels, crosslinked with PEG (8000 g/mol) diacrylate and PNIPAAm copolymers with star-shaped PEGs with arms approximately 2000 g/mol were both studied for drug delivery.

Alava et al. studied the reactivity ratios of NIPAAm and PEG monomethacrylate. The values were determined to be $r_{\text{NIPAAm}}=1.2$ and $r_{\text{PEGM}}=0.13$. This indicates that NIPAAm is more reactive than PEG monomethacrylate to both propagating species. Copolymers of these two species would contain larger amounts of the more reactive monomer, NIPAAm, in random placement.

In this work, a family of copolymers was synthesized by varying the molecular weight of the PEG blocks from 1000 to 10,000 g/mol and the relative amounts of NIPAAm and PEG. Fourier transform infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy were used to characterize the chemical nature of the polymers. Specifically, these analytical tools were used to verify the successful polymerization of NIPAAm, complete removal of residual monomer, and the covalent incorporation of PEG along the polymer backbone.
4.2 Experimental Section

4.2.1 Materials

N-isopropylacrylamide (NIPAAm) monomer (Sigma Aldrich) was recrystallized in n-hexane before use. Polyethylene glycol (PEG) diol ($M_n = 2000, 4600, 8000, \text{ and } 10000$ g/mol) (Sigma Aldrich), initiator 2,2’-Azobisisobutyronitrile (AIBN), 98% (Sigma Aldrich) poly(ethylene glycol) dimethacrylate (PEGDM) (n=1000) (Polysciences), and deuterium oxide (Sigma Aldrich) were all used as received. All solvents and other chemicals were of analytical grade.

4.2.2 Methods

4.2.2.1 Methacrylation of PEG

PEGDM, with molecular weight between 2000 and 10,000 g/mol, was synthesized from PEG diol\textsuperscript{333}. The diol was dissolved in methylene chloride and reacted with an excess of methacryloyl chloride and triethylamine under nitrogen atmosphere for 12 h at 4°C and an additional 24 h at room temperature. The product was then precipitated in ether multiple times to ensure purification and the excess ether was removed in the vacuum oven. The average methacrylation efficiency of the reaction was determined by $^1$H NMR (Varian 300, Palo Alto, CA) in deuterium oxide at 25°C.
4.2.2.2 Copolymer Synthesis

NIPAAm was polymerized in the presence of PEGDM to create PNIPAAm-PEG branched copolymers. Copolymers composed of five different block sizes were synthesized by using PEG 1000, 2000, 4600, 8000, or 10,000 g/mol dimethacrylate. For each PEG molecular weight, a high and low PEG content copolymer was produced by using a molar ratio of NIPAAm monomer units to PEG branches of 700:1 and 1600:1, respectively. The free radical polymerization, initiated by AIBN, took place in methanol at 65°C for 48 hours. The methanol was evaporated from the reaction mixtures after polymerization was complete. The reaction product was purified by treatment with n-hexane at 45°C. The purified polymer was isolated by vacuum filtration and dried under vacuum overnight. It was subsequently ground into powder and analyzed with $^1$H NMR to verify the molar ratio of NIPAAm monomer units to PEG branches.

Solutions containing 15 wt% polymer were prepared from the ground polymer and deionized water. The polymer was dissolved overnight at 4°C then centrifuged (Kendro Sorvall Legend Mach 1.6/R, Asheville, NC) at 15°C and 10,000 rpm for 45 minutes to remove entrapped air from the viscous solutions.
4.3 Results and Discussion

4.3.1 Methacrylation of PEG

The $^1$H NMR spectrum for the PEGDM can be seen in Figure 4.3. The signals corresponding to the functional groups are shown in the figure. The average methacrylation efficiency of the reaction was tracked over a 12 month period and was found to be 90.8 ± 9.2 %. This was calculated by comparing the area under the integral for the vinyl protons ($\delta=5.7$ ppm, $\delta=6.1$ ppm) to that of the PEG backbone ($\delta=3.5$ ppm).

4.3.2 Synthesis of PNIPAAm-PEG Branched Copolymers

The extent of polymerization of the NIPAAm can be evaluated with FTIR. Shown in Figure 4.4 is the FTIR spectra of the reaction mixture after 24 and 48 hours reaction time at 65ºC. The time point t=0 refers to the FTIR spectrum of the reaction mixture prior to heating. The peaks at 1650cm$^{-1}$, 1543cm$^{-1}$, and 1458cm$^{-1}$ correspond to the Amide I, Amide II, and C-H bending on the NIPAAm and PNIPAAm. In addition, the peaks at 1368cm$^{-1}$ and 1387cm$^{-1}$ correspond to the two isopropyl groups on the N-isopropylacrylamide. Therefore, these peaks are present at all time points. At t=0, the peak at 1620cm$^{-1}$ has been attributed to the C=C stretch on the NIPAAm monomer. In addition, the peaks at 984 cm$^{-1}$ and 912 cm$^{-1}$ correspond to out of plane C-H bending of an alkene with an RCH=CH$_2$ structure, like the NIPAAm monomer. The disappearance of these peaks over the course of the reaction confirms polymerization of the NIPAAm.
It is important to note that peaks for PEG or PEGDM are not seen in the FTIR spectra, likely because the instrument was not sensitive enough to detect the presence of PEG. The ether C-O stretch in PEG is typically seen around 1106 cm\(^{-1}\) and the C-H bending on an alkene with a methacrylate group structure (R\(_2\)C=CH\(_2\)) occurs between 880-900 cm\(^{-1}\) \(^{341}\). Therefore, this FTIR analysis cannot be used to verify the covalent incorporation of PEG along the polymer backbone.

A typical \(^1\)H NMR spectrum for PNIPAAm-PEG branched copolymers is shown in Figure 4.5. The peak assignments have been previously described by Ma et al.\(^{342}\), however the group neglected to assign a peak to the methyl protons on the methacrylate group of the PEG (A), which were incorporated along the polymer backbone during polymerization. The covalent incorporation of PEG along the polymer backbone can be verified by the presence of the characteristic peaks for PEG and PNIPAAm in the spectra, which are designated on the figure, and the absence of the sharp singlets at 5.7 and 6.1 ppm, which represent the vinyl protons on the double bond of the NIPAAm monomer or PEG dimethacrylate. To quantify the relative amounts of NIPAAm and PEG in the copolymer, the area of the peak at \(\delta=3.5\text{ppm}\), characteristic of the protons of the –(CH\(_2\)CH\(_2\)O\(_n\))– segment on the PEG, was compared to the area of the peak at \(\delta=3.6\text{ ppm}\) for the CH protons adjacent to the isopropyl group of the NIPAAm. Based on these calculations, all copolymers had molar ratios satisfactorily close to the targets of 700:1 and 1600:1.
4.4 Conclusions

The successful polymerization of NIPAAm was shown with FTIR. This result was verified with $^1$H NMR. In addition, $^1$H NMR was used to verify the covalent incorporation of PEG along the polymer backbone and quantify the molar ratio of NIPAAm monomer units to PEG blocks in each of the copolymers. A family of copolymers was created by varying the PEG block molecular weight between 1000 and 10,000 g/mol. For each PEG block molecular weight, a high and low PEG content copolymer was synthesized by keeping the molar ratio of NIPAAm monomer units to PEG blocks at either 700:1 or 1600:1. The material properties of this family of copolymers will be examined in subsequent chapters.
Figure 4.1 Schematic for the synthesis of PEGDM.
Figure 4.2 Schematic for the synthesis of PNIPAAm-PEG branched copolymers.
Figure 4.3 $^1$H NMR spectrum for PEGDM in deuterium oxide at 25°C.
Figure 4.4 The changes in spectra over reaction time for PNIPAAm-PEG branched copolymers.
Figure 4.5. NMR Spectra for purified PNIPAAm-PEG branched copolymers in deuterium oxide at 25ºC.
Chapter 5 - CHARACTERIZATION OF PNIPAAm-PEG BRANCHED COPOLYMERS

5.1 Introduction

There have been very few studies investigating PNIPAAm-based materials for orthopedic applications. This may be because the polymer exhibits poor elastic properties and holds little water at physiological temperature due to its hydrophobic nature. Ho et al. \(^{343}\) investigated PNIPAAm-PEG (1000 g/mol) copolymers for soft tissue fixation or repair. The authors found that by engineering a third component into the hydrogel system, trimethacryloxypropyltrimethoxysilane (MPS), the mechanical properties of the network could be tuned. The MPS aided in network formation and increased the mechanical properties of the gels. In addition, the authors note that by using different permutations of PNIPAAm, PEG, and MPS, the modulus of the hydrogels could also be tailored to match that of trabecular bone.

This work focuses on tailoring the swelling and mechanical properties of PNIPAAm network by adding PEG blocks to the network. It is proposed that lightly crosslinking PNIPAAm with a long chain, hydrophilic component will increase its water retention capability and elasticity. PEG was chosen due to its biocompatibility, hydrophilicity, ease of functionalization, and availability in a range of molecular weights. PNIPAAm-PEG branched copolymers were synthesized by polymerizing the monomer NIPAAm in
the presence of PEG dimethacrylate (PEGDM). In the previous chapter, it was shown that a family of copolymers was created by varying the molecular weight of the PEG chains between 1000 and 10,000 g/mol. For each PEG block molecular weight, a high and low PEG content copolymer was synthesized by keeping the molar ratio constant at 700:1 or 1600:1, respectively.

In the following experiments, the structure-property relationship in these copolymers was studied. Specifically, gel water content, stiffness, and elasticity were investigated as a function of PEG content and PEG block molecular weight. It is hypothesized that a material candidate from the class of PNIPAAm-PEG branched copolymers can serve as a synthetic nucleus pulposus replacement exhibiting suitable, stable material properties over long term immersion in the physiological environment.

5.2 Experimental Section

5.2.1 Methods

5.2.1.1 LCST Characterization

The LCST and transition enthalpy of each copolymer was characterized using a DSC Model 2010 (TA Instruments, Wilmington, DE) by placing 7-10mg of solution in a hermetically sealed allografted aluminum pan, and heating from 10 to 60ºC at a rate of 10ºC·min⁻¹. The phase transition of the polymer is detected as an endothermic peak on the DSC thermogram, with the temperature at the peak onset taken as the LCST. The
area of the peak, normalized to the total mass of solution\(^{337}\), was taken as the transition enthalpy.

### 5.2.1.2 Gel swelling and Dissolution

To characterize gel swelling, approximately 2mL of each polymer solution was heated to 37°C within a closed vial for 3 hours. The precipitated gels were then immersed in Dulbecco’s phosphate buffered saline (PBS) at 37°C. A set of gels were evaluated at 0, 7, 14, 30, 60, and 90 days, weighed, and subsequently dried under vacuum and weighed again. Time 0 refers to a set of gels that were weighed immediately after precipitation and never immersed in PBS. The gel water content was calculated according to Equation 5.1, where \( m_{\text{wet,37°C}}(t) \) is the wet gel mass at 37°C at time \( t \) and \( m_{\text{dry}} \) is its dry mass.

\[
\text{Water content(\%)} = \left( 1 - \frac{m_{\text{dry}}(t)}{m_{\text{wet,37°C}}(t)} \right) \times 100
\]

**Equation 5.1**

For the dissolution studies, an identical procedure was used. A set of gels were examined at 30, 60, and 90 days, dried under vacuum, and weighed. The mass retention of the gels at time \( t \) was calculated according to Equation 5.2, with \( m_{\text{wet,25°C}}(t=0) \) being the mass of polymer solution initially placed in the vial at 25°C.

\[
\text{Mass retention (\%)} = \left( \frac{m_{\text{dry}}(t)}{0.15 \times m_{\text{wet,25°C}}(t=0)} \right) \times 100
\]

**Equation 5.2**
5.2.1.3 Mechanical Properties

5.2.1.3.1 Compressive Studies

Unconfined uniaxial compression tests were performed on gel samples after 7, 30, 60, and 90 days immersion in PBS. The hydrogel samples were loaded in an Instron mechanical testing system (Instron Model 3362, Park Ridge, IL) and compressed at a strain rate of 100\%·min\(^{-1}\) while submerged in a 37°C PBS bath. Load and displacement data were recorded with Instron Bluehill 1.2 software and converted to stress and strain values. The compressive moduli at 10, 15, and 20% strain were approximated as the slope of the chord drawn between 5 and 15% strain, 10 and 20% strain, and 15 and 25% strain, respectively.

5.2.1.3.2 Stress Relaxation Studies

The gels’ ability for elastic recovery was evaluated using stress relaxation experiments, performed after 2, 7, 14, and 30 days immersion in PBS. Hydrogel samples were loaded in the Instron mechanical testing system and 20% compressive strain was applied and held for 1 hour while the gel was submerged in a 37°C PBS bath. Load versus time data was recorded with the Instron Series IX software. Based on the Maxwell model, the stress of a polymer under constant strain will decay exponentially\(^ {344} \).

\[
\sigma(t) = \sigma_0 \exp\left(-\frac{t}{\tau}\right)
\]

Equation 5.3
Where $\sigma(t)$ is the stress at some time $t$, and $\tau$ is the relaxation time constant. The relaxation time constant for each copolymer was computed as the time needed to lower the stress from its original value, $\sigma_o$, to $\frac{\sigma_o}{e}$. The hydrogel height and diameter were also measured 0, 30, and 55 minutes after unloading.

5.2.2. Statistical Analysis

The sample sizes for each of the previously described tests were $n=6$. Selected results were compared statistically using one way ANOVA with a 95% confidence level.

5.3 Results

5.3.1 LCST Characterization

The LCSTs in the family of copolymers is shown in Figure 5.1 A. The LCST of the PNIPAAm homopolymer was found to be $31.0 \pm 0.1^\circ C$. Every copolymer synthesized had a significant increase in LCST compared to the homopolymer ($p<0.05$). No significant changes in LCST were made by varying the PEG molecular weight or the molar ratio of NIPAAm monomer units to PEG blocks ($p>0.05$). All of the LCSTs in this family of copolymers were between $31.3^\circ C$ and $32.4^\circ C$, which is in the range necessary for \textit{in situ} gel formation. Similarly, the homopolymer had a transition enthalpy of $4.35\pm0.24$ J/g. Every branched copolymer had a significant increase in transition
enthalpy compared to the homopolymer (p<0.05) (Figure 5.1 B). There were no significant differences in the enthalpy values among the branched copolymers (p>0.05). The low PEG content PNIPAAm-PEG (8000 g/mol) and both the high and low PEG content PNIPAAm-PEG (10,000 g/mol) copolymers were not considered for further study because they only formed semisolid materials above their LCST, disqualifying them as candidate materials for nucleus replacement.

5.3.2 Swelling and dissolution experiments

All of the copolymers exhibited a dry mass retention of 97% or greater over 90 days immersion in vitro. None of the copolymers had a significant change in mass retention between the 30 and 90 day time points except for the low PEG content PNIPAAm-PEG (2000 g/mol), which showed some statistically significant increase to 101.8% (p<0.05). This increase in the dry mass of the polymer could be attributed to ion uptake from the immersion media, but this was not confirmed.

Holding PEG block molecular weight constant and varying the molar ratio of NIPAAm units to PEG blocks between 700:1 and 1600:1 produced no significant differences in equilibrium water content of PNIPAAm-PEG (1000, 2000, and 4600 g/mol) (p>0.05). Equilibrium water contents at 90 days immersion in vitro are shown in Table 5.1.

The data in Figure 5.2 shows the water content for gels composed of block sizes varying between 1000 and 8000 g/mol. The molar ratio of NIPAAm monomer units to PEG
blocks is held constant at approximately 700:1. At the initial time point 0, the
copolymers with PEG block sizes of 2000, 4600, and 8000 g/mol had significantly higher
water contents (p<0.05) than the homopolymer and PNIPAAm-PEG (1000 g/mol).
However, at 90 days immersion, only PNIPAAm-PEG (4600 and 8000 g/mol) had
significantly higher equilibrium water contents (p<0.05) than the homopolymer control.

All of the branched copolymers and the homopolymer had significant decreases in water
content between the 0 and 7 day time points (p<0.05 in all cases). However, none of the
copolymers exhibited significant changes in water content after the 7 day time point
(p>0.05 in all cases).

5.3.3 Compressive mechanical studies

The compressive moduli at 15% for the family of copolymers at 7, 30, 60, and 90 days
immersion in vitro are shown in Figure 5.3. Equilibrium modulus values at 90 days are
shown in Table 5.1. All of the branched copolymers showed increasing trends in
stiffness over 90 days immersion in vitro. All of the copolymers reached an equilibrium
stiffness by 30 days, except for PNIPAAm-PEG (8000 g/mol), which equilibrated by 60
days immersion. Both the high and low PEG content copolymers (Figures 5.3 A and B,
respectively) exhibited decreasing stiffness as the PEG block molecular weight was
increased. However, there were no significant changes in the equilibrium compressive
modulus as the molar ratio of NIPAAm monomer units to PEG blocks was varied
(p>0.05).
5.3.3.1 Network Analysis with Rubber Elasticity Theory

Based on the theory of rubber elasticity, the compressive modulus of the hydrogels, $G$, can be expressed in terms of the compressive stress, $\tau_c$, the polymer volume fraction in the swollen gel $\nu_{2,s}$, and the elongation, $\alpha$. (Equation 5.4) $^{336,346}$ In tensile testing, $\alpha$ is defined as the length of the sample at any time divided by its initial length. Because compressive testing was performed in this case, $\alpha$ was taken as the initial height of the sample divided by the height at any time.

$$\frac{\tau_c}{\alpha - \frac{1}{\alpha^2}} = G \nu_{2,s}^{-1/3}$$

Equation 5.4

The stress-strain behavior of the high PEG content copolymers, normalized to account for differences in swelling behavior, is shown in Figure 5.4. The slope of the curves, or the modulus $G$, decreases with increasing PEG molecular weight. The values for $G$ at 90 days immersion in PBS are illustrated in Figure 5.5.

Using rubber elasticity theory, the effective molecular weight between crosslinks, $M_e$, can also be calculated. For a network crosslinked in the presence of a solvent, the following relationship holds $^{347}$.
Where $\nu_{2,r}$ and $\rho_{2,r}$ are the polymer volume fraction and density in the relaxed state, respectively, and $M_n$ is the number average molecular weight if no crosslinks were introduced. The value for $\nu_{2,r}$ is taken as 1, since the network is in its relaxed state when it is completely dry. The density of the polymer in this state, 0.93 g/mL, was measured with the heptane density determination system. The value of $M_n$ for the PNIPAAm homopolymer was estimated based on the viscosity average molecular weight, $M_v$, (75,000 g/mol), determined previously for the same polymerization scheme by Thomas et al. using Ubbelohode capillary viscometry. For a linear polymer in a theta ($\Theta$) solvent which possesses the most probable molecular weight distribution, $M_v$ can be related to $M_n$ and the weight average molecular weight, $M_w$, through the following expression:

$$M_n : M_v : M_w = 1 : 1.67 : 2$$

Equation 5.6

It has been shown previously that water is a $\Theta$ solvent for PNIPAAm. Using the ratios in Equation 5.6, the estimated value of $M_n$ for the PNIPAAm homopolymer was 44,910 g/mol. The calculated effective molecular weight between crosslinks, $M_e$, was determined for the high PEG content copolymers (Figure 5.6). The values for $M_e$ increased steadily between PEG block molecular weight 2000 and 8000 g/mol.
5.3.4 Stress Relaxation Studies

The relaxation time constants, as determined by Equation 4.3, over 30 days immersion time \textit{in vitro} are shown in Figure 5.7. All of the copolymers showed a significant increase in the value of the time constant compared to the homopolymer control (p<0.05). For the high PEG content copolymers (Figure 5.7 A), there was a significant increase in the time constant between PNIPAAm-PEG (2000 g/mol) and PNIPAAm-PEG (4600 g/mol) (p<0.05). However, varying the PEG block molecular weight did not produce any significant changes in the relaxation time constant for the low PEG content gels (Figure 5.7 B) (p>0.05). All materials recovered between 85 and 98\% of their original height within 55 minutes after unloading.

5.4 Discussion

The PNIPAAm-PEG copolymers had higher LCSTs and transition enthalpies than the PNIPAAm homopolymer due to the presence of the hydrophilic PEG, which hinders the collapse and subsequent dehydration of the PNIPAAm chains. However, there were no significant shifts in LCST or transition enthalpy as the copolymer structure was varied; signifying that the hydrophilic/hydrophobic balance was not impacted significantly by the range of PEG compositions studied.
It was investigated whether the water content of the precipitated phase is related to the weight percent of hydrophilic component in the gel. If this were the case, the high PEG content PNIPAAm-PEG (2000 g/mol) would have the same water content as a low PEG content PNIPAAm-PEG (4600 g/mol) gel, since they contain approximately the same weight percent of PEG. On the contrary, the PNIPAAm-PEG (4600 g/mol) held significantly more water at all time points. This result suggests that overall water content of the precipitated phase is tied to network mesh size. Since the PEG blocks do not collapse like the thermoresponsive PNIPAAm chains after the LCST transition, large spaces in the polymer network are created, allowing the gel to retain more water. The low PEG content PNIPAAm-PEG (8000 g/mol) copolymers, as well as both PNIPAAm-PEG (10000 g/mol) copolymers may have produced semi-solid gels because the high mesh sizes allowed the network to be overwhelmed with free water after the LCST transition. Conversely, PNIPAAm-PEG 1000 g/mol did not hold significantly more water than the homopolymer, likely because of the tighter network structure. Furthermore, the increases in equilibrium gel water content seen experimentally between the high PEG content PNIPAAm-PEG (2000 g/mol) and PNIPAAm-PEG (8000 g/mol) correspond well with the steady increases in \( M_e \) predicted by rubber elasticity theory.

The gels exhibited an overall decreasing trend in compressive modulus as the PEG block molecular weight was increased. This is likely due to the fact that the higher molecular weight PEG blocks caused increases in gel water content. Water in hydrogels acts as a plasticizer, causing increased flexibility of the polymer chains, decreasing the mechanical properties as water content increases \(^{349}\). Additionally, varying the PEG content of the
gels produced no significant changes in equilibrium modulus. This result was also consistent with the swelling results, which indicated that the molar ratio of NIPAAm monomer units to PEG blocks had no effect on gel water content. The increases in gel stiffness seen during the 90 day study is attributed to the gradual de-swelling of the gels, which increases the polymer content, enhancing the mechanical properties. The longer equilibration time for gels in the mechanical study compared to the swelling study is due to the gel geometry. Water in the larger cylindrical samples used in the mechanical study took longer to diffuse out of the gel network than that in the flat discs used in the swelling study.

In addition, Figures 5.4 and 5.5 illustrate the compressive mechanical behavior of the gels normalized to account for differences in swelling behavior. The effect of network crosslinking density on the polymer modulus, $G$, can be determined from this data. Because $G$ decreased with increasing PEG block molecular weight, it can be inferred that high molecular weight PEG blocks result in a looser network structure.

Joshi et al. determined that a polymeric hydrogel implant with an unconfined compressive modulus of 50 kPa at 15% strain could successfully restore intervertebral disc stiffness. The mechanical results presented here can therefore be used as an indication of whether or not there are suitable candidates in this family of copolymers to serve as a nucleus pulposus replacement. The compressive mechanical results demonstrated that, after 7 days immersion in vitro, all of the high PEG content copolymers, except for PNIPAAm-PEG (8000 g/mol), and all of the low PEG content
copolymers, except for PNIPAAm-PEG (4600 g/mol), met this value of stiffness. By 60 days immersion, all of the copolymers met this value of stiffness.

In addition to meeting this minimum stiffness, the material must be designed so that its elastic recovery is maximized. In this analysis, the relaxation time constant is used as a measure of material elasticity. More elastic gels will exhibit a slower decrease in stress over time and hence have higher relaxation times.

The elastic behavior of macromolecules can be linked to how their monomer units interact with one another and with surrounding solvent molecules. Above the LCST of PNIPAAm, its polymer chains collapse into densely packed globular conformations due to the positive attractions between monomer units and between chains. In other words, the energy of interaction between monomer-monomer molecules is lower than that of monomer-solvent molecules. Compressing a PNIPAAm gel increases the frequency of monomer-monomer contacts. Since this is the lowest free energy configuration for the chains, they do not have a tendency to return to a more stretched out conformation after the external force is released. On the contrary, PEG molecules swell in water because the monomer units prefer to be surrounded by the solvent molecules. When the PEG chains are uniaxially compressed, the repulsive interactions between monomer units cause the free energy of confinement for PEG chains to be high. This is manifested in a high elastic force compared to PNIPAAm chains. For this reason, all PNIPAAm-PEG branched copolymers in this study had significantly higher relaxation time constants than the homopolymer.
The end-to-end distance, $R$, of a polymer chain increases with molecular weight. The free energy of confining a real linear chain in a good solvent into a slit of spacing $D$ or a cylinder with diameter $D$ is proportional to $R^{3/5}$. Consequently, high molecular weight PEG chains exhibit high free energies of confinement. This property accounts for the increase in elasticity between the high PEG content PNIPAAm-PEG (2000 g/mol) and PNIPAAm-PEG (4600 g/mol). It is likely that the low PEG content copolymers showed no changes in time constant as the PEG block molecular weight was increased because there were not enough PEG chains in the system to impact the elasticity of the gel.

5.5. Conclusions

All of the copolymers had LCSTs in the range necessary for injectability and exhibited satisfactory mass retention over 90 days immersion in vitro. PNIPAAm-PEG (4600 and 8000 g/mol) had significantly higher mass swelling ratios than the PNIPAAm homopolymer, with the latter having the highest water content of all the copolymers. After 7 days immersion in vitro, the high PEG content PNIPAAm-PEG (1000, 2000, and 4600 g/mol) and the low PEG content PNIPAAm-PEG (1000 and 2000 g/mol) met the minimum stiffness of 50 kPa for the restoration of intervertebral disc stiffness. After 60 days in vitro, all of the copolymers met the minimum stiffness. The branched copolymers showed improved elasticity over the PNIPAAm homopolymer, but PNIPAAm-PEG (4600 and 8000 g/mol) had the highest relaxation time constants, indicating maximum elasticity. Because of its high water content and suitable
mechanical properties, the high PEG content PNIPAAm-PEG (4600 g/mol) will be further assessed as a material candidate for nucleus pulposus replacement.
Table 5.1 Equilibrium water contents and compressive moduli for PNIPAAm-PEG after 90 days immersion in vitro.

<table>
<thead>
<tr>
<th>PEG molecular weight (g/mol)</th>
<th>PEG content</th>
<th>Water content</th>
<th>Compressive modulus at 15% strain at 90 days immersion in vitro (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>Low</td>
<td>0.35 ± 0.02</td>
<td>205 ± 54</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>0.35 ± 0.03</td>
<td>127 ± 64</td>
</tr>
<tr>
<td>2000</td>
<td>Low</td>
<td>0.47 ± 0.11</td>
<td>115 ± 40</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>0.41 ± 0.01</td>
<td>119 ± 38</td>
</tr>
<tr>
<td>4600</td>
<td>Low</td>
<td>0.51 ± 0.01</td>
<td>141 ± 36</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>0.52 ± 0.02</td>
<td>90.3 ± 9.3</td>
</tr>
<tr>
<td>8000</td>
<td>High</td>
<td>0.71 ± 0.00</td>
<td>52.3 ± 8.9</td>
</tr>
</tbody>
</table>
Figure 5.1 LCST and enthalpy of transition as a function of weight percent PEG in PNIPAAm-PEG branched copolymers.
Figure 5.2 Effect of PEG block molecular weight on gel water content.
Figure 5.3 Effect of PEG block size on the equilibrium compressive modulus of PNIPAAm-PEG branched copolymers.
Figure 5.4 Stress-strain behavior of PNIPAAm-PEG branched copolymers at 90 days immersion in PBS, normalized to account for the swelling of the gel.
Figure 5.5 Modulus, $G$, for the high PEG content PNIPAAm-PEG branched copolymers at 90 days immersion in PBS, normalized to account for differences in the swelling behavior of the gels.
Figure 5.6 The effective molecular weight between crosslinks, $M_e$, for the high PEG content PNIPAAm-PEG branched copolymers at 90 days immersion in PBS.
Figure 5.7 Effect of immersion time *in vitro* on the compressive modulus at 15% strain of high PEG content (a) and low PEG content (b) PNIPAAm-PEG branched copolymers.
Chapter 6 - OPTIMIZATION STUDIES TO PREVENT WATER LOSS IN VIVO

6.1 Introduction

This chapter describes a two part study that was conducted to better understand the dehydration behavior of the PNIPAAm-PEG (4600 g/mol) gels. The in vitro characterization studies in Chapter 5 showed that the de-swelling behavior of the gels upon heating to 37ºC is characterized by an initial gradual water loss, followed by an equilibrium condition. To predict the implant behavior in vivo, it is necessary to understand how the de-swelling rate and equilibrium water content change with osmotic pressure of the surrounding medium. The disc tissues possess a high osmotic pressure due to the flux of ions into the tissues generated by the fixed negative charges on the proteoglycans. J.P. Urban’s group performed studies on the osmotic pressure of lumbar disc tissues and reported the osmotic pressure of the healthy inner annulus and nucleus pulposus to be approximately the same, at 0.2 MPa. The first part of the study will focus on analyzing the de-swelling behavior of PNIPAAm-PEG (4600 g/mol) gels over a range of physiologically pertinent osmotic pressures.

PNIPAAm gels also exhibit a decrease in volume as a result of dehydration. Significant volume loss in vivo will prevent the implant from having intimate contact with the inner annulus. It has been shown experimentally and through finite element
modeling that total nucleus replacements which are oversized, allowing for intimate contact with the inner annulus, were most effective at restoring the compressive stiffness of the intervertebral disc \(^{171}\). The second part of this dehydration study will focus on optimizing the copolymer formulation to minimize water loss and subsequent volume loss upon injection into the intradiscal environment. Ideally, the volume of injected solution would equal that of the gel formed, allowing the implant to remain space filling after the thermal transition.

### 6.2 Experimental Section

#### 6.2.1 Materials

PEG diol (20,000 g/mol) (Serva), dialysis tubing (Spectrum Spectra/Por Biotech cellulose ester, 500 Dalton MWCO), and 55 mm dialysis clips (Fisher Scientific) were all used as received.

#### 6.2.2 Methods

Gel swelling under a range osmotic pressures was evaluated using a modified version of the procedure developed by J.P. Urban \(^{24,26}\). Varied concentrations of aqueous solutions of PEG (20,000 g/mol) were prepared. The osmotic pressure of a macromolecular solution is given by Equation 6.1, where \( \Pi \) is osmotic pressure, \( R \) is the gas constant, \( T \) is absolute temperature, \( M_2 \) is the molecular weight of the macromolecule, \( B \) and \( C \) are
the second and third virial coefficients, and \( c_2 \) is the concentration of the macromolecule in solution \(^{351}\).

\[
\Pi = RT \left( \frac{C_2}{M^2} + Bc^2 + Cc^3 + \ldots \right) \tag{Equation 6.1}
\]

The values used for the second and third virial coefficients were 2.59e10\(^{-3}\) and 1.35e10\(^{-2}\), respectively \(^{353}\). Approximately 2mL of 10, 15, or 25 wt% aqueous PNIPAAm-PEG (4600g/mol) solution were injected into dialysis tubing and sealed with dialysis clips. The dialysis bags were then immersed in aqueous PEG solution at 37ºC. The volume of PEG solution used was approximately 100 times the volume of PNIPAAm-PEG solution. After 1, 2, 7, 14, and 30 days, the gels were removed from the dialysis bags, weighed, dried under vacuum, and weighed again. Time 0 refers to solutions that were not immersed in PEG solution, but immediately weighed, dried under vacuum, and weighed again to determine water content prior to swelling. The water content was calculated at each time point according to Equation 4.1.

### 6.2.3 Statistical Analysis

The sample sizes for each of the previously described tests were \( n=3 \). Selected results were compared statistically with one way ANOVA and a 95% confidence level.
6.3 Results and Discussion

6.3.1 Osmotic Swelling Studies

The water content of PNIPAAm-PEG (4600 g/mol) gels over 30 days immersion in 37°C aqueous PEG (20,000g/mol) solution, with an osmotic pressure of 0.2 MPa, is shown in Figure 6.1. As a comparison, a set of gels were also swollen in 0.4 MPa and pure water. Between the 0 and 2 day time points, the gels swollen in 0.4 MPa dehydrated faster than those swollen in 0.2 MPa osmotic pressure and water. By 7 days immersion, there were no significant differences between the water contents of the gels swollen in 0.2 and 0.4 MPa (p>0.05). By 30 days, there were no significant differences in the water contents of all three sets of gels (p>0.05).

These results indicate that the equilibrium gel water content for PNIPAAm-PEG (4600 g/mol) is not significantly affected by varying osmotic pressures in the range studied. Osmotic pressure measurements are based on the use of a semi-permeable membrane through which solvent molecules pass freely and polymer molecules cannot pass\textsuperscript{354}. It is possible that higher osmotic pressures did not appreciably affect equilibrium water contents of the gels because the remaining water in the gel is sufficiently associated with the polymer through hydrogen bonding such that the osmotic pressures studied did not generate enough thermodynamic drive to cause further dehydration.

To ensure the implant remains space filling, it is necessary to eliminate the drastic water loss occurring between the 0 and 2 day time points in Figure 6.1. The first avenue
explored was analyzing the relationship between the water content of the polymer solution at room temperature and that of the gel at equilibrium. Figure 6.2 shows the water content of gels swelled for 30 days at 37°C in aqueous solutions of PEG (20,000 g/mol) with an osmotic pressure of 0.2 MPa. The gels were formed from aqueous 10, 15, or 25 wt% PNIPAAm-PEG solutions at room temperature. Initially, the gels swelled under the highest osmotic pressure dehydrated at the quickest rate. However, by 30 days immersion, the water content of all three sets of gels was very similar, between 0.4 and 0.5.

Logically, it follows, that in order to minimize the magnitude of water loss after gelation, the solution from which the gels are formed must be more concentrated. However, solutions above 25 wt% polymer cannot be prepared directly by combining dry polymer and water because they will not form uniformly.

6.3.2 Thermal Cycling Studies

Water loss upon gelation was minimized by thermal cycling. Solutions composed of 15 wt% PNIPAAm-PEG (4600 g/mol) were injected into dialysis bags at room temperature and allowed to equilibrate at 37°C and 0.4 MPa for 48 hours. When these gels were subsequently cooled to room temperature they formed concentrated, homogenous polymer solutions and the water content was measured. The water content of the solutions before and after this 2 day cycle is indicated in Figure 6.3 as thermal cycles 0 and 1. As expected, there was significant water loss during this period (p<0.05), which
was large in magnitude. These concentrated polymer solutions were subsequently injected into dialysis bags again at room temperature and re-immersed in the same 37°C osmotic environment for an additional 48 hours. The water content of the solutions after this second thermal cycle is shown in Figure 6.3 as thermal cycle 2. There were no significant changes in water content (p>0.05) compared to cycle 1. The cycle was performed an additional 3 times to ensure repeatability. There were no significant changes in water content between cycles 1 and 3 (p>0.05). However, there were slight decreases in water content between cycles 1 and 4 and 1 and 5, which were statistically significant (p<0.05), though small in magnitude. During the initial 48 hours immersion, the solutions only retained approximately 25% of their initial volume. However, there were no more significant changes in solution volume thereafter (p>0.05).

While homogenous polymer solutions cannot be prepared from dry powder at these high concentrations, these results indicate that reversibility of the PNIPAAm’s phase transition can be exploited in order to form them. Dilute polymer solutions can be thermally cycled by equilibration in a 37°C osmotic environment. These gels can then be cooled to room temperature, forming more concentrated solutions that should show minimal water loss once injected into the intradiscal environment.

6.4 Conclusions

The osmotic swelling studies revealed that while osmotic pressure influenced the rate of PNIPAAm-PEG (4600 g/mol) gel dehydration, the equilibrium water content was not
dependent on osmotic pressure of the immersion media. Furthermore, equilibrium water content of the gels is not appreciably affected by the concentration of the polymer solution from which they were formed. Significant implant water loss upon gelation can be prevented by equilibrating dilute polymer solutions in a 37 ºC environment. Upon cooling to room temperature, the polymers will form uniform, concentrated solutions which should show minimal water loss when gelled again.
Figure 6.1 Effect of immersion media osmotic pressure on the water content of PNIPAAm-PEG (4600 g/mol).
Figure 6.2 Effect of solution concentration at room temperature on equilibrium water content of PNIPAAm-PEG (4600 g/mol) gels.
Figure 6.3 Effect of thermally cycling the polymer solution by equilibrating in a 37 ºC osmotic environment, followed by cooling to room temperature.
Chapter 7 - SYNTHESIS AND CHARACTERIZATION OF BIOADHESIVE PNIPAAM-PEG/PEI BLENDED COPOLYMERS

7.1 Introduction

Imparting bioadhesive properties to this hydrogel system will reduce the risk of implant migration or expulsion out of the annular defect created by injection of the nucleus replacement. Additionally, bioadhesive properties will increase the potential for using this material as nucleus replacement in discs which are in the later stages of degeneration, which possess tears, cracks, and fissures that would normally increase the risk of implant migration or expulsion.

Because of its injectable nature, this bioadhesive could be used as a closure material for the repair of a damaged annulus. The technology could be used in conjunction with a discectomy, to close the herniated disc, or with a total nucleus replacement, to close the defect created by injection of the material and help anchor the implant in the center of the disc. The bioadhesive could also repair annular tears associated with degeneration. Using a hydrogel material to close these annular defects would help stabilize the disc structure, prevent prolapse of disc material, and restore biomechanical function. This strategy can also be used to address pain produced by cytokines and inflammatory mediators that can migrate through annular fissures and produce pain by stimulating the
nociceptors in the outer annulus. Sealing annular fissures will prevent the diffusion of these molecules to the nerve roots in the outer annulus.

The plan for imparting bioadhesive properties to the hydrogel system begins with the incorporation of a third component in the hydrogel system, poly(ethylene imine) (PEI). PEI is a highly branched polyamine. The pKa value of the primary amine is around 9, the secondary amine around 8 and the tertiary amine around 6–7. At or above a pH of 10, the amine groups are uncharged. In the pH range from 10 to 4, approximately 60% of the total amount of amino groups are protonated.

PEI will be incorporated in the PNIPAAm-PEG (4600 g/mol) network by physical blending. The blends will be prepared by co-dissolving PNIPAAm-PEG and PEI in water at room temperature. PEI will comprise approximately 4.25 wt% of the aqueous solution, while the PNIPAAm-PEG copolymer content will be fixed at 15 wt%. This weight percent of PEI was chosen because it is approaching the maximum amount of PEI that can be co-dissolved with 15 wt% PNIPAAm-PEG. Above the LCST of PNIPAAm, a collapsed network will form, physically entrapping the PEI chains within it.

The three component system will be injected into the disc and allowed to thermally transition, forming a gel. This will be followed by the injection of aqueous glutaraldehyde solution into the gel core. The aldehyde groups of the glutaraldehyde will become covalently bonded with the primary amines of PEI, crosslinking PEI with itself. This reaction between amine and aldehyde functionality is known as a Schiff’s base.
reaction\textsuperscript{356} (Equation 7.1) and has been studied with Fourier Transform Infrared (FTIR) spectroscopy. For this reason, FTIR will be used to verify the crosslinking chemistry in the PNIPAAm-PEG/PEI blends.

\[ \text{NH}_2 + \text{O=CH} \rightarrow \text{N=CH} + \text{H}_2\text{O} \quad \text{Equation 7.1} \]

In the disc, after the glutaraldehyde crosslinks the PEI, unreacted glutaraldehyde will continue to diffuse through the gel and crosslink it to the tissues in contact with the implant by reacting with the amine functions present in the proteins of the tissue extracellular matrix. Because only small amounts of the reacting dial will be injected directly into the gel, reaction with tissues not in immediate proximity to the gel should not occur.

It is postulated that this system is feasible for use in the intervertebral disc despite the potential cytotoxicity of PEI\textsuperscript{280,283,290,291,357} and glutaraldehyde\textsuperscript{318,325,327,328,358}. Because the disc tissues contain large amounts of extracellular matrix proteins and fewer cellular components\textsuperscript{19,318}, they are less sensitive to the cytotoxicity, and the inflammatory response from the body can be minimized with a controlled release profile of glutaraldehyde.

Preliminary work for this project has indicated that injecting larger volumes of dilute glutaraldehyde solutions results in a more uniform crosslinking than small volumes of highly concentrated solutions. Therefore, the swelling, mechanical, and adhesive
properties of the hydrogels will be evaluated based on the injection of 0.62 mL of a 5 wt% glutaraldehyde solution in water into the gel core. As a comparison, the properties of the system were also evaluated using 10 and 20 wt% glutaraldehyde solutions.

The overall objective of these studies is to successfully impart bioadhesive properties to the injectable PNIPAAm-PEG hydrogel system. The basic synthesis and characterization techniques will be established, laying the foundation for future optimization of the adhesion system.

### 7.2 Experimental Section

#### 7.2.1 Materials

Poly(ethylene imine) (PEI) \((M_n=10,000\ \text{g/mol, water-free})\), Copper(II) sulfate pentahydrate, Iron(III) chloride, 3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH), and deuterium oxide \((D_2O)\) were obtained from Sigma Aldrich and used as received. PEI \((M_n=60,000\ \text{g/mol, 50 wt\% in water})\) and glutaraldehyde \((70\ \text{wt\% in water})\) were also obtained from Sigma Aldrich and diluted before use to the concentrations specified in the method descriptions. Fresh porcine skin was obtained from a butcher.
7.2.2 Methods

7.2.2.1 PNIPAAm-PEG/PEI Blend Preparation and Characterization

A polymer solution was prepared at ambient temperatures composed of 15 wt% PNIPAAm-PEG (4600 g/mol), 4.25wt% PEI (Mₙ= 60,000 g/mol), and the balance water. The solutions were heated to 37°C for gelation to occur. The precipitated gels were lyophilized overnight and re-dissolved in D₂O. They were analyzed with ¹H NMR (Varian 300, Palo Alto, CA) to verify and quantify the incorporation of PEI into the gel network.

The chemical stability of the blends was evaluated by comparing the amount of PEI released from the network upon gelation to the amount dissolved in the room temperature solution. Approximately 2 mL of the PNIPAAm-PEG/PEI (Mₙ=10,000) or (60,000 g/mol) solutions were heated inside a closed vial for 10 minutes. Then, 10 mL of deionized water, preheated to 37°C, was added to each vial. The vials were agitated at 40 rpm for 2 minutes. Then 500 μL of each immersion media was added to 500 μL of 0.4 wt% aqueous copper(II) sulfate pentahydrate. Upon the addition of copper (II) ions, PEI forms a blue cuprammonium complex, allowing the PEI to be quantified spectrophotometrically. The absorbance of these solutions was measured at 550 nm with a plate reader (BIO-TEK, model ELx800, Winooski, VT). The concentration of PEI in the gel immersion media was determined using a standard curve of known PEI concentrations. A linear relationship (r²=0.999 for PEI Mₙ=60,000 g/mol and r²=0.993 for PEI Mₙ=10,000 g/mol) was found between the absorbance at 550 nm and the amount
of PEI in concentration range tested, which was 0.001 to 6.67 mg/mL. Each optical
density measurement was performed in triplicate and the sample size was n=3.

### 7.2.2.2 PNIPAAm-PEG/PEI Crosslinking with Glutaraldehyde

For this and all subsequent characterization studies, uniformly sized cylindrical gel
samples were formed by injecting aqueous solutions of 15 wt% PNIPAAm-PEG (4600
g/mol) and 4.25 wt% PEI (Mₙ= 60,000 g/mol) into molds and heating to 37°C for 10
minutes. The precipitated gels were removed from the molds and 0.62 mL of 5 wt%
aqueous glutaraldehyde was injected into the gel core with a 26 gauge needle at rate of 40
cc/hr, using a syringe pump (Autosyringe, model 5A, Hooksett, NH). The gels were then
lyophilized overnight and analyzed with FTIR (Nicolet Magna IR 560, Madison, WI) to
verify the covalent crosslinking of the PEI with the glutaraldehyde.

### 7.2.2.3 Free Glutaraldehyde Release

Uniformly sized gel samples formed from aqueous solutions composed of 15 wt%
PNIPAAm-PEG and 4.25 wt% PEI (Mₙ=60,000 g/mol) were injected with either 5, 10, or
20 wt% glutaraldehyde. Once again, 0.62 mL of glutaraldehyde solution was injected
into the gel core with a 26 gauge needle at a rate of 40 cc/hr. This process was carried
out while the gels were fully immersed in 150 mL of deionized water. The gels were
swelled in this same immersion media throughout the study. The amount of free
glutaraldehyde in the immersion media over time was then quantified with the MBTH method \(^{360}\). At t=10 mins, 3, 24, 48, and 96 hours, 300 µL of immersion media was combined with 300 µL of 0.4 wt% aqueous MBTH and 0.2 wt% Iron (III) chloride. The reaction was allowed to stand for approximately 10 minutes, and then 2 mL of acetone was added. The optical density of the solution at 405 nm was measured with the plate reader. The concentration of glutaraldehyde in the immersion media was determined using the standard curve of known glutaraldehyde concentrations. A linear relationship \((r^2=0.951)\) was found between the absorbance and the amount of glutaraldehyde in concentration range 0.0002 to 0.10 mg/mL. The amount of glutaraldehyde released at each time point was compared to the total amount injected into the gel core. Each optical density measurement was performed in triplicate and the sample size was n=3.

### 7.2.2.4 PNIPAAm-PEG/PEI Gel Swelling

To characterize the swelling properties of the blends, uniformly sized cylindrical gel samples composed of PNIPAAm-PEG/PEI \((M_n=60,000 \text{ g/mol})\) were prepared using molds and immersed in PBS at 37°C. A set of gels were pulled at 0, 1, 7, 14, and 30 days, weighed, and subsequently dried under vacuum and weighed again. Time 0 refers to solutions that were not heated to 37°C or immersed in PBS, but weighed, dried under vacuum, and weighed again to determine water content prior to gel formation and swelling. This procedure was repeated 3 additional times with a set of gels which were injected with 5, 10, or 20 wt% glutaraldehyde solutions, according to the procedure
described above prior to immersion in PBS. The gel water content was calculated according to Equation 4.1.

7.2.2.5 PNIPAAm-PEG/PEI Compressive Mechanical Properties

Unconfined uniaxial compression tests were performed on gel samples injected with 5, 10, or 20 wt% glutaraldehyde after 7 days immersion in PBS. As a comparison, a set of gel samples which were not injected with glutaraldehyde were also tested. The hydrogels were loaded in an Instron mechanical testing system (Instron Model 4442, Park Ridge, IL) and compressed to 50% strain at a rate of $100\%\cdot\text{min}^{-1}$ while submerged in a 37°C PBS bath. Load and displacement data were recorded with the Instron BlueHill 1.2 software and converted to stress and strain values. The compressive moduli at 10, 15, and 20% strain were approximated as the slope of the chord drawn between 5 and 15% strain, 10 and 20% strain, and 15 and 20% strain, respectively.

7.2.2.6 Bioadhesive Force Studies

The bioadhesive capacity of PNIPAAm-PEG/PEI ($M_n=60,000$ g/mol) blends was determined using an Instron mechanical testing system (Instron Model 4442, Park Ridge, IL). Uniformly sized cylindrical gels were fixed to the upper support with cyanoacrylate adhesive. Sections of fresh porcine skin were cut and cleaned to remove excess fat. The substrate was fixed to the bottom support with cyanoacrylate adhesive. The gel was
lowered slowly until full contact with the skin was achieved. Subsequently, the gels were injected with 0.62 mL of 5, 10, or 20wt% glutaraldehyde at 40 cc/hr using a 26 gauge needle. The gels were kept in contact with the substrate for 10 minutes. The upper fixture was then withdrawn at a rate of 2mm·min$^{-1}$ and the load-displacement data was recorded by the Instron BlueHill 1.2 software until the polymer became completely detached from the skin. During the test, the skin and gel were submerged in a 37°C PBS bath. The sample size was n=3. A fresh piece of tissue was used for each repeat.

The load versus displacement data was converted to stress strain values and the maximum force required to detach the gels from the biological samples was read directly from this the curve. In addition, the work of adhesion was calculated by fitting the stress strain curve for each sample to a sixth order polynomial, and calculating the area under the curve between the strain values of 0 and that after which complete detachment occurred.

7.2.2.7 Statistical Analysis

The sample sizes for each of the previously described tests were n=3. Selected results were compared statistically with one way ANOVA and a 95% confidence level.
7.3 Results

7.3.1 PNIPAAm-PEG/PEI Blend Characterization

The successful physical incorporation of PEI (M<sub>n</sub>=60,000 g/mol) in the PNIPAAm-PEG (4600 g/mol) network was verified with the <sup>1</sup>H NMR. The NMR spectrum for the blends is shown in Figure 7.1. The incorporation of PEI was verified by the presence of the peak for the CH<sub>2</sub> protons on the PEI at δ=2.2 ppm. Additionally it was determined that approximately 10.3±4.2% of PEI (M<sub>n</sub>=60,000 g/mol) that was dissolved in the solution at room temperature was released from the network upon gelation (Figure 7.2). As a comparison, the same measurement was performed on blends composed of PEI (M<sub>n</sub>=10,000 g/mol) and it was found that 56.7±4.0% was released.

7.3.2 Gel Crosslinking with Glutaraldehyde

The FTIR spectra for a dry PNIPAAm-PEG/PEI (M<sub>n</sub>=60,000 g/mol) gel, glutaraldehyde/PEI mixture, and a gel injected with glutaraldehyde are shown in Figures 7.3 A, B, and C, respectively. As can be seen in 7.3 A, the spectrum for the blend is completely dominated by PNIPAAm, the characteristic peaks being the Amide I and II bands at 1649 and 1543 cm<sup>-1</sup>. The peaks for PEI are not seen in this spectrum, likely because the FTIR is not sensitive enough to detect its presence. The peak for free NH<sub>2</sub> amines on the PEI should occur at 1596 cm<sup>-1</sup>, the C-H bending at 1456 cm<sup>-1</sup>, and C-C vibration and C-N stretch between 1300 and 1000 cm<sup>-1</sup>. 
Illustrated in 7.3 B is the spectrum for a dried 1:1 mixture of PEI ($M_n=60,000$ g/mol) and 5 wt% glutaraldehyde. The spectrum is shown simply to indicate that the peak at 1661 cm$^{-1}$ represents the $-C=\text{N}$- covalent bond formed as a crosslink between the NH$_2$ on PEI and the HC=O group on the glutaraldehyde $^{316,363}$. In addition, the amide II band ($HN=\text{C}$-) on the crosslinked PEI occurs at 1560 cm$^{-1}$ $^{316}$. These peaks could not be detected in the spectrum of the PNIPAAm-PEG/PEI ($M_n=10,000$ g/mol) blend that was injected with glutaraldehyde (7.3 C) because PNIPAAm once again dominates the spectrum.

When this overlap occurs, the spectrum of one component can be subtracted from the combined system in order to reveal the peaks of interest $^{364}$. In this case, the spectrum for the PNIPAAm-PEG/PEI blend was subtracted from that of the same blend injected with 5% glutaraldehyde. The results of the spectral subtraction are shown in Figure 7.4. Peaks in the characteristic range for the $-C=\text{N}$- and $HN=\text{C}$- bonds occur at 1668 cm$^{-1}$ and 1540 cm$^{-1}$. The results of the FTIR difference spectroscopy indicated that covalent crosslinking of the PEI occurring in this system can be described as a Schiff’s base reaction.

### 7.3.3 Free Glutaraldehyde Release

Shown in Figure 7.5 are the release profiles of free glutaraldehyde from the gels. Initially, 10, 21, and 16% of the glutaraldehyde injected was released from the gels injected with 5, 10, and 20% glutaraldehyde, respectively. This corresponds to a total of $3.0\pm0.4$, $13.3\pm7.4$, and $20.2\pm10.0$ mg of glutaraldehyde. After 96 hours, 47, 83, and 59%
of the glutaraldehyde was released from the gels injected with 5, 10, and 20% glutaraldehyde, respectively. This corresponds to a cumulative total of 14.5±3.9, 51.5±3.4, 73.2±14.8 mg of glutaraldehyde, respectively.

7.3.4 PNIPAAm-PEG/PEI Gel Swelling

The water content of PNIPAAm-PEG/PEI (Mₙ=60,000 g/mol) blends with and without glutaraldehyde injection over a 30 day immersion in PBS is shown in Figure 7.6. All of the PEI-containing gels, including those that were crosslinked with glutaraldehyde, held significantly more water than the PNIPAAm-PEG copolymer alone (p<0.05). At 30 days immersion, only the gels crosslinked with 20% glutaraldehyde underwent a significant decrease in water content compared to the polymer solution from which it was formed (p<0.05). Furthermore, the PNIPAAm-PEG/PEI blend alone exhibited a significant increase in water content between 0 and 30 days, from 78.2±0.0 to 83.7±1.2 % (p<0.05). Though varying the concentration of glutaraldehyde solution injected made no significant changes in gel water content (p>0.05), all of the crosslinked gels exhibited decreasing trends in water content compared to the PNIPAAm-PEG/PEI blend. At 30 days immersion, the gels injected with 10 and 20% glutaraldehyde held significantly less water than the PNIPAAm-PEG/PEI alone (p<0.05).
7.3.5 PNIPAAm-PEG/PEI Compressive Mechanical Properties

Shown in Figure 7.7 are changes in the compressive mechanical properties of PNIPAAm-PEG/PEI as a result of glutaraldehyde injection. The compressive modulus of the PNIPAAm-PEG/PEI blend at 15% strain was 6.3±2.6 kPa. All of the blends exhibited significant increases in compressive modulus (p<0.05) with glutaraldehyde injection. However, there were no significant changes in the modulus value as the glutaraldehyde concentration was varied from 5 to 20 wt% (p>0.05).

7.3.6 Bioadhesive Force Studies

Figure 7.8 illustrates the typical stress-strain curve for the *in vitro* bioadhesive force studies. The curves shown in shades of blue represent PNIPAAm-PEG and PNIPAAm-PEG/PEI hydrogels which were never injected with glutaraldehyde, but fully contacted with the porcine skin and withdrawn at 2mm·min⁻¹. The curves for these samples show a gradual increase in stress followed by a plateau region. The initial increase in load is likely caused by two factors. First, as the gel loses contact with the skin, the upper fixture supports an increasingly greater portion of the weight of the gel itself. Secondly, as the fixture moves up, it encounters resistance as it moves through water, contributing to the load. The plateau region represents full loss of gel contact with the substrate.

The curves shown in red, orange, and pink represent samples that were injected with glutaraldehyde while in contact with the porcine skin and subsequently withdrawn.
These samples also exhibited an initial increase in load but they continue to a higher maximum value in the stress. This is indicative of greater adherence to the substrate. The stress field caused by the withdrawal of the upper fixture will seek the most vulnerable point in the adhesive bond and failure is initiated there. This initial failure is represented on the stress-strain curve as the first drop in stress. The subsequent drops in stress represent the failure propagating. Again, complete separation from the substrate is represented by the plateau region. It is worth noting that failure can occur at any of the adhesive interfaces or by the fracture of the gel sample. In each of these cases, the separation occurred at the interface between the gel and the substrate.

The mean maximum force required to detach PNIPAAm-PEG and PNIPAAm-PEG/PEI gels from the porcine skin was taken as the maximum in the stress-strain curve before complete detachment occurred. These values are shown in Table 7.1. The mean maximum force of detachment for PNIPAAm-PEG and PNIPAAm-PEG/PEI without glutaraldehyde injection was 0.64±0.14 kPa and 0.71±0.24 kPa, respectively. There were no significant differences between these two values (p>0.05). All of the PNIPAAm-PEG/PEI gels injected with glutaraldehyde exhibited significant increases in the mean maximum force of detachment (p<0.05). Among the gels injected with glutaraldehyde, varying the concentration between 5 and 20% increased the adhesion strength by a factor of 1.6. There were no significant differences in the mean maximum force of detachment for the gels injected with 10 and 20% glutaraldehyde (p>0.05), but both values were significantly higher than that of the gels injected with 5% glutaraldehyde (p<0.05). The values for work of adhesion are shown also shown in Table 7.1. While there were no
significant differences in work of adhesion for the PEI blends injected with glutaraldehyde (\(p>0.05\)), all of them had significantly higher values that the PNIPAAm-PEG/PEI blend and the PNIPAAm-PEG copolymer alone (\(p<0.05\)).

### 7.3 Discussion

A chemically stable hydrogel blend will be defined in this context as one in which all of the PEI dissolved in the room temperature solution becomes physically entrapped in the gel network during the collapse of the PNIPAAm chains. The blends composed of PEI (\(M_n=10,000\) g/mol) released 5 times as much PEI as the blends composed of PEI (\(M_n=60,000\) g/mol) immediately after gelation. In other words, the blends prepared from the higher molecular PEI were more chemically stable. It is likely that the higher molecular weight PEI allows for a greater degree of physical entanglements with the PNIPAAm-PEG upon network collapse, reducing the free PEI released. A minimal release of free PEI is considered a positive scenario since PEI with molecular weight higher than 2000 Da has been shown to be cytotoxic\(^{290,291}\). It is for this reason that the PNIPAAm-PEG/PEI (\(M_n=60,000\) g/mol) gels were further characterized in these studies.

It is likely that the chemical stability of these blended networks will be further enhanced by the injection of the glutaraldehyde, which will crosslink the PEI to itself. The amount of PEI released from the network post-injection of the glutaraldehyde cannot be quantified with the current method because the free glutaraldehyde and PEI in the immersion media will readily react before they can be quantified. If histological studies
indicate a necessary decrease in the release of free PEI, a higher molecular weight PEI can be utilized in the blend. Another option is to incorporate the PEI along the polymer backbone as grafts. PEI chains can be functionalized with methacrylate groups and then polymerized in the presence of NIPAAm and PEG dimethacrylate. It should be considered, however, that an increase in free PEI release could potentially increase the adhesive strength of the gels. A thick coating of PEI on the gel surface could play an important role in stabilizing the adhesive bond between the material and the surrounding tissue.

The incorporation of PEI into the PNIPAAm-PEG network increased the swelling capacity of the network appreciably. The equilibrium water content of PNIPAAm-PEG increased significantly with the incorporation of PEI from 63.5±1.2 to 83.7±1.2 %. In addition, the PNIPAAm-PEG/PEI blend exhibited a significant increase in water content (p<0.05) between the 0 and 30 day time points. At the pH of the PBS, approximately 7.4, several of the primary and secondary amine groups on the PEI are ionized. In salt solutions, the charges on the PEI attract an influx of counterions into the polymer network. The osmotic pressure created by these ions causes the polymer to swell. The network expansion could also be due to the ionic repulsions between the positively charged PEI chains. Overall, these results are consistent with the findings of Qui et al., who showed that incorporation of ionizable groups into PNIPAAm hydrogels can improve the swelling of the network more effectively than non-electrolyte hydrophilic components alone.
This increase in water content is responsible for decreasing the mechanical properties of the PNIPAAm-PEG. While the previous characterization studies have shown the modulus of this copolymer after 7 days immersion in PBS to be over 50 kPa, the modulus of the PEI blend at this same strain level was 6.3±2.6 kPa. The glutaraldehyde crosslinking did, however, cause an 8 to 12-fold increase in the compressive modulus of the gels, putting the moduli back above 50 kPa, indicating the potential of the material to function as a total nucleus replacement by restoring the compressive stiffness of intervertebral disc stiffness.

Varying the concentration of glutaraldehyde injected did not produce significant changes in gel water content. In addition, while the crosslinked gels exhibited a small increasing trend in stiffness with increased glutaraldehyde concentration, the differences were not significant (p>0.05). These results indicate that the crosslinking density of the gels did not vary greatly with increasing glutaraldehyde concentration. Furthermore, increasing glutaraldehyde concentration only produced modest gains in adhesive strength. The adhesion tests were conducted 10 minutes after glutaraldehyde injection, corresponding to approximately 3.0±0.4, 13.3±7.4, and 20.2±10.0 mg of free glutaraldehyde release for the 5, 10, and 20% glutaraldehyde solutions, respectively. The adhesive strength only increased by a factor of 1.6 in this range.

The above results can be better understood by analyzing the release profile of free glutaraldehyde shown in Figure 7.6. A sustained release of unreacted glutaraldehyde from the gel network occurred for 48 hours. It is apparent that a large portion of the
glutaraldehyde diffused out of the network unreacted, and only a small portion of the glutaraldehyde is contributing to the crosslinking of the PEI and the adhesion to the tissue.

Ideally, a much smaller portion of the glutaraldehyde should be released from the network, though the target amount will be a balance between the desired adhesive strength and minimization of large scale tissue damage. A higher concentration of PEI inside the gel is one way to minimize the long term release of glutaraldehyde. This would increase the likelihood of the amine-aldehyde reaction, so it could also potentially increase the crosslink density of the network, lower the water content and increase the stiffness. Employing more dilute solutions of glutaraldehyde and a slower injection rate could also minimize the long term release of glutaraldehyde.

Slivka et al. conjectures that the crosslinker molecular weight should be at least 100 Da, otherwise diffusion into the environment surrounding the disc would occur too easily. Using a high molecular weight dialdehyde, such as PEG dialdehyde, would slow the diffusion of the molecules through the network, also making reaction more likely. Perhaps the best option in terms of minimizing the diffusion of aldehydes into the tissue would be to immobilize the aldehyde groups on the surface of the implanted material. Perhaps the best option in terms of minimizing the diffusion of aldehydes into the tissue would be to immobilize the aldehyde groups on the surface of the implanted material. This could be achieved by conjugating an aldehyde group to one end of a PEG chain that possesses a methacrylate group on the other end. The methacrylate group can be reacted in the presence of NIPAAm monomer to form a copolymer.
7.4 Conclusions

In these studies, an injectable, bioadhesive hydrogel system was developed which has the potential to serve as a synthetic replacement for the nucleus pulposus of the intervertebral disc or as an annulus closure material. Gel samples were formed at 37°C from aqueous solutions at room temperature composed of 15 wt% PNIPAAm-PEG (4600 g/mol) and 4.25 wt% PEI (M_n=60,000 g/mol). \(^1\)H NMR results indicated the successful physical incorporation of PEI into the PNIPAAm-PEG network by blending, though 10.3±4.2 % of the PEI that was dissolved in the room temperature solution was released from the network immediately upon gelation. This value increases with decreasing PEI molecular weight. The covalent crosslinking between the amine functionalities on the PEI and the aldehyde functionalities on the glutaraldehyde was verified using FTIR difference spectroscopy. Mechanical characterization of these blends showed a significant increase in compressive modulus following glutaraldehyde injection, also an indication that crosslinking occurred. Post-injection of the glutaraldehyde, all of the gels attained a modulus value of at least 50 kPa at 15% strain, indicating their potential for restoring the compressive intervertebral disc stiffness as a total nucleus replacement \(^1\)71. Varying the amount of glutaraldehyde injected into the gels at 40 cc/hr did not have an appreciable effect on the water content or mechanical stiffness of the gels.

The bioadhesive force studies show a significant increase in the mean maximum force to the break the adhesive bond between the PNIPAAm-PEG and PNIPAAm-PEG/PEI gels and the porcine skin when 5, 10, or 20 wt% glutaraldehyde was injected into the gel core. The bioadhesive strength of the gels injected with 10 and 20 wt% glutaraldehyde was
significantly higher than that of the gels injected with 5 wt% glutaraldehyde (p<0.05). The results from this study indicate that the reactivity between amines and aldehyde functionalities can be exploited in order to impart bioadhesive properties to PNIPAAm-PEG/PEI copolymers.
Table 7.1 The mean maximum force and work of adhesion required to detach the PNIPAAm-PEG and PNIPAAm-PEG/PEI gels from the biological substrates.

<table>
<thead>
<tr>
<th></th>
<th>Mean Maximum Force of Detachment (kPa)</th>
<th>Work of Adhesion (kPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% glutaraldehyde</td>
<td>0.7±0.3</td>
<td>1.5±0.7</td>
</tr>
<tr>
<td>10% glutaraldehyde</td>
<td>0.6±0.1</td>
<td>1.6±.08</td>
</tr>
<tr>
<td>20% glutaraldehyde</td>
<td>1.4±0.0</td>
<td>1.7±1.1</td>
</tr>
<tr>
<td>PNIPAAm-PEG/PEI</td>
<td>2.5±0.2</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>PNIPAAm-PEG</td>
<td>2.2±0.1</td>
<td>0.0±0.0</td>
</tr>
</tbody>
</table>
Figure 7.1 The $^1$H NMR spectrum for PNIPAAm-PEG/PEI ($M_n=60,000$ g/mol) blend in deuterium oxide at 25°C.
Figure 7.2 The chemical stability of PNIPAAm-PEG/PEI ($M_n$=10,000) and PNIPAAm-PEG/PEI (60,000 g/mol) blends.
Figure 7.3 The FTIR spectra for a dry (A) PNIPAAm-PEG/PEI gel, (B) glutaraldehyde/PEI mixture, (C) PNIPAAm-PEG/PEI gel injected with glutaraldehyde.
Figure 7.4 FTIR difference spectroscopy for the analysis of PNIPAAm-PEG/PEI crosslinked gels.
Figure 7.5 Release profile of free glutaraldehyde from the PNIPAAm-PEG/PEI network.
Figure 7.6 The water content over a 30 day immersion in PBS for PNIPAAm-PEG/PEI blends with and without glutaraldehyde injection.
Figure 7.7 The compressive modulus of PNIPAAm-PEG/PEI gels at 15% strain with and without glutaraldehyde crosslinking at 7 days immersion in PBS.
Figure 7.8 Typical stress strain curves for the *in vitro* bioadhesive force studies.
Chapter 8 - CONCLUSIONS AND FUTURE RECOMMENDATIONS

8.1 Conclusions

The long term objective of this project was to develop an *in situ* forming nucleus pulposus replacement, which upon implantation, could prevent or postpone the annular degeneration process by restoring the healthy biomechanics of the intervertebral disc. A class of PNIPAAm-PEG branched copolymers was characterized. From this class, a material candidate which exhibited suitable, stable material properties over long term immersion *in vitro* was further optimized to minimize water loss upon gelation, therefore remaining space-filling *in vivo*. Bioadhesive properties were then imparted to the hydrogel system, increasing its potential for use as a total nucleus replacement or tissue adhesive in discs with annular degeneration.

First, the successful polymerization of NIPAAm was shown with FTIR. This result was verified with \(^1\)H NMR. In addition, \(^1\)H NMR was used to verify the covalent incorporation of PEG along the polymer backbone and quantify the molar ratio of NIPAAm monomer units to PEG blocks in each of the copolymers. A family of copolymers was created by varying the PEG block molecular weight between 1000 and 10,000 g/mol. For each PEG block molecular weight, a high and low PEG content copolymer was synthesized by keeping the molar ratio of NIPAAm monomer units to PEG blocks at either 700:1 or 1600:1.
All of the copolymers had LCSTs in the range necessary for injectability and exhibited satisfactory mass retention over 90 days immersion in vitro. PNIPAAm-PEG (4600 g/mol) and PNIPAAm-PEG (8000 g/mol) had significantly higher water content than the PNIPAAm homopolymer, with the latter having the highest water content of all the copolymers. After 7 days immersion in vitro, the high PEG content PNIPAAm-PEG (1000, 2000, and 4600 g/mol) and the low PEG content PNIPAAm-PEG (1000 and 2000 g/mol) met the stiffness value of 50 kPa, indicating potential for the restoration of intervertebral disc stiffness. After 60 days in vitro, all of the copolymers met this value of stiffness. The branched copolymers showed improved elasticity over the PNIPAAm homopolymer, but PNIPAAm-PEG (4600) and PNIPAAm-PEG (8000 g/mol) had the highest relaxation time constants, indicating maximum elasticity. Because of its high water content and suitable mechanical properties, the high PEG content PNIPAAm-PEG (4600 g/mol) was further assessed as a material candidate for nucleus pulposus replacement.

The osmotic swelling studies revealed that while osmotic pressure influenced the rate of PNIPAAm-PEG (4600 g/mol) gel dehydration, the equilibrium water content was not dependent on osmotic pressure of the immersion media in the range tested. Furthermore, equilibrium water content of the gels is not appreciably affected by the concentration of the polymer solution from which they were formed. Significant implant water loss due to de-swelling can be prevented by equilibrating 15 wt% polymer solutions in a 37 °C
environment. Upon cooling to room temperature, the polymers will form uniform, more concentrated solutions which should show minimal water loss when gelled again.

In the last portion of this project, the hydrogel system was made bioadhesive. Gel samples were formed at 37°C from aqueous solutions composed of 15 wt% PNIPAAm-PEG (4600 g/mol) and 4.25 wt% PEI (M_n=60,000 g/mol). ¹H NMR results indicated the successful physical incorporation of PEI into the PNIPAAm-PEG network by blending. The chemical stability of the blended network decreased with decreasing PEI molecular weight. The covalent crosslinking between the amine functionalities on the PEI and the aldehyde functionalities on the glutaraldehyde was verified using FTIR difference spectroscopy and can be described by Schiff’s base chemistry. The swelling characterization revealed that PNIPAAm-PEG/PEI blends exhibit significantly better water retention than PNIPAAm-PEG alone (p<0.05) in PBS. Mechanical characterization of these blends showed significant increases in compressive modulus following the injection of 5, 10, or 20 wt% glutaraldehyde. Post-injection of the glutaraldehyde, all of the gels attained a modulus value of at least 50 kPa at 15% strain, indicating their potential for restoring the compressive intervertebral disc stiffness as a total nucleus replacement. Varying the concentration of glutaraldehyde injected into the gels at 40 cc/hr did not have an appreciable effect on the water content or mechanical stiffness of the gels. The results from the bioadhesive force study indicate that the reactivity between amines and aldehyde functionalities can be exploited in order to impart bioadhesive properties to PNIPAAm-PEG/PEI copolymer blends. A significant increase (p<0.05) was observed in the mean maximum force to break the adhesive bond.
between the PNIPAAm-PEG and PNIPAAm-PEG/PEI gels and the porcine skin when 5, 10, or 20 wt% glutaraldehyde was injected into the gel core. The bioadhesive strength of the gels injected with 10 and 20 wt% glutaraldehyde was significantly higher than that of the gels injected with 5 wt% glutaraldehyde (p<0.05).

8.2 Recommendations for Future Work

More in-depth mechanical and biomechanical studies on the PNIPAAm-PEG (4600 g/mol) base formulation and PNIPAAm-PEG/PEI adhesive formulation are warranted. Joshi et al. \(^{350}\) evaluated solid polymer hydrogels formed from blends of poly(vinyl alcohol) and poly(vinyl pyrrolidone) for nucleus replacement. The hydrogels were loaded dynamically at 10 million cycles for compression fatigue. Fatigue testing has also been performed on a number of other material candidates being investigated for nucleus pulposus replacement \(^{175,163,182}\). Joshi et al. \(^{350}\) also implanted the hydrogels in cadaveric specimens to evaluate the compressive behavior of the implanted discs. The authors also recommended testing the implant under complex loading conditions.

With the addition of PEI to the network, the swelling behavior of the copolymers in PBS improved dramatically. The swelling behavior of PEI containing gels should also be evaluated within a range of physiologically pertinent osmotic pressures. Furthermore, the need for thermal cycling to minimize water loss upon gelation needs to be re-evaluated. It is likely that the presence of PEI will increase the swelling capacity of the network such that minimal volume loss upon gelation will be achieved with more dilute polymer
solutions at room temperature. The chemical stability of the blends post-glutaraldehyde injection should also be characterized over long term immersion \textit{in vitro} and under repetitive loading conditions.

A target adhesive strength needs to be established for the injectable, bioadhesive hydrogel system. In order to do so, gels of varying adhesive capacities (determined by \textit{in vitro} bioadhesive force studies) should be implanted into the discs of cadaveric specimens, and subjected to static, cyclic, bending, and torsional loading to seek signs of implant migration or expulsion. An understanding can be gained about the minimum bioadhesive strength that is suitable for the application. In addition, if the material were also used as an adhesive for the repair of annular defects, mechanical tests are warranted to determine if the requisite mechanical properties differ from that of a nucleus replacement.

The work in the current studies has led to valuable insight into the parameters that can be varied in order to modify the properties of the material. Slight increases in adhesive strength were seen with increasing free glutaraldehyde release into the porcine tissue. The release of free glutaraldehyde from the network can likely be tailored by using varied injection flow rates, glutaraldehyde solution volumes, or glutaraldehyde solution concentrations. It is also recommended that other dialdehyde molecules such as PEG dialdehyde be studied. Larger crosslinkers would likely diffuse through the network slower, making them more likely to react, and decreasing their release into the surrounding medium. If the release profile indicates a prolonged dialdehyde release,
adhesion tests should be performed at time points following extended contact between the material and tissue. To eliminate diffusion of aldehydes into the tissues all together, aldehyde groups can be immobilized on the surface of the implanted material. This could be achieved by conjugating an aldehyde group to one end of a PEG chain that possesses a methacrylate group on the other end. The methacrylate group can be reacted in the presence of NIPAAm monomer to form a copolymer.

It is also proposed that blending PNIPAAm-PEG with lower molecular weight PEI will increase the adhesive capacity of the material. The loss of chemical stability of the gel network, probably due to a lower frequency of molecular entanglements, causes a thick coating of PEI to form on the surface of newly formed gels which can enhance adhesion to the tissue. Therefore, the adhesive capacity of blends composed of PEI (M_n=10,000 and 800 g/mol) should be investigated. The blend needs to be chemically stable enough, however, to retain enough PEI in the network for uniform crosslinking to occur following glutaraldehyde injection.

It is likely that the adhesive capacity of the gels will have to be balanced with the cytotoxic effects of PEI and glutaraldehyde. Therefore, histological studies will be warranted to determine the in vivo inflammatory response to the system. The inflammatory response to the optimized system should be minor and small in duration. It is also recommended that the use of less cytotoxic crosslinkers, such as genipin, be evaluated. Genipin is a naturally occurring compound, derived from
fruits of *Gardenia jasminoides Ellis*\textsuperscript{367}. Genipin crosslinks molecules containing primary amines\textsuperscript{368}. 
References


114. Thomsen, Christensen FB, Eiskjaer SP, Hansen ES, Fruensgaard S, Bunger CE. The effect of pedicle screw instrumentation on functional outcome and fusion


149. Mirza S. Point of view: Commentary on the research reports that led to Food and Drug Administration approval of an artificial disc. Spine 2005;30:1561-1564.


Vita

Jennifer Vernengo was born on February 8, 1980 in Puebla, Mexico. She graduated from Highland High School in Blackwood, NJ in 1998 and enrolled in Drexel University in Philadelphia, PA. At Drexel, Jennifer pursued a Bachelors of Science in chemical engineering. In 1999, she worked as a co-op at Dupont and assisted in the operation and analytical support of their wastewater treatment plant. In 2000, Jennifer worked as a co-op at Dupont Fluoroproducts, where she supervised the production of purified Zyron© C-318 gas via an extractive distillation system. In 2001, Jennifer completed a final co-op cycle at the Dupont Experimental Station, where she assisted in the development of a lab reactor system for the production of oxetane and gained valuable experience with the design, testing, and optimization of chemical processes. In 2003, Jennifer completed her undergraduate degree and began to pursue a Ph.D. in chemical engineering under the guidance of Dr. Anthony Lowman. Her research focused on the design and development of injectable, bioadhesive hydrogels for nucleus pulposus replacement and the repair of the damaged intervertebral disc. During her time at Drexel, Jennifer has coauthored publications, presented her research at national and regional conferences, and received numerous awards and honors. Jennifer defended her Ph.D. thesis on January 31, 2007.