CARTILAGE REPAIR BY TISSUE ENGINEERING: MULTI-FUNCTIONAL POLYMERS AS SCAFFOLD MATERIALS

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CARTILAGE REPAIR BY TISSUE ENGINEERING: MULTI-FUNCTIONAL POLYMERS AS SCAFFOLD MATERIALS

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Thesis

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ABSTRACT

Cartilage defects and loss caused by accidents, diseases or degeneration raises serious problems affecting people of all ages. It is difficult for cartilage to heal spontaneously after damage because of the lack of blood vessels in its complex structure and the resultant interruption of nutrition, including growth factors, vitamins, and minerals, to cartilage cells (chondrocytes) to support their development. Many methods, often surgical intervention, are used to restore cartilage, but they are not very successful. Current studies are focusing on a new technique — tissue engineering to repair cartilage defects. Tissue engineering involves biology, biochemistry, materials science, engineering, manufacturing and medicine. Three key aspects of tissue engineering are cell sources, scaffolds and growth factors. Scaffolds, as one of the three important elements, ideally will mimic the natural environment of the cells and tissues in vitro. There are several basic tissue engineering requirements for scaffolds. They should be ideally 1) three-dimensional and porous; 2) biodegradable; 3) biocompatible; and 4) controllable in mechanical properties. Currently, many biomaterials may be used as a scaffold, including natural and synthetic materials and they may be modified by synthesis methods. Several reports indicate the importance of surface charges on cartilage and chondrocytes.
Synthetic approaches that provide interaction between scaffold functional groups and cartilage surfaces could introduce a new promising direction to improve tissue engineering therapy for cartilage impairment. In that regard, the goal of this project is to synthesize multifunctional polymers as scaffolds used for cartilage tissue engineering with the intent that different scaffold functional groups can be utilized successfully to attract and bind chondrocytes. The cell/scaffold constructs would in turn be capable of interacting with cartilage surfaces to assist in augmenting and regenerating the tissue through chondrocyte proliferation and matrix production in situations of cartilage damage and loss. The work in this study has been conducted initially in cell culture with the future prospect of implanting constructs in nude mice to determine their chondrocyte growth and extracellular matrix formation.
DEDICATION

This Master thesis is dedicated to my family, my advisors and all my friends and colleagues who have provided me help and support during the research process.
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INTRODUCTION AND BACKGROUND

1.1 Introduction to cartilage and chondrocytes

1.1.1 Types of cartilage and their components, structures and functions

Cartilage is a flexible, agile, dense, special connective tissue which can be extensively found in the human body and in many other mammals. For example, cartilage comprises joints, knees, ankles and intervertebral discs of mammals and vertebrates. Cartilage generally consists of water, collagens (most are type II collagens although types I and X collagen as in fibrocartilage, for example, may be present as well), polysaccharides and other constituents such as minor protein molecules and cartilage cells, the chondrocytes. Three different types of cartilage exist in the human and animal body: elastic cartilage, hyaline cartilage and fibrocartilage.

Elastic cartilage (or yellow cartilage) is a type of cartilage which can be found in the outer ear, larynx, and epiglottis. This cartilage contains elastic fiber networks, collagen fibers and the major protein, elastin. The elastic cartilage is much like hyaline cartilage in histological analysis under a light microscope, but there is an important difference that elastic cartilage contains amount of yellow elastic fibers, which are tiling on a solid matrix. Because of these fibers, elastic cartilage has a
property of great flexibility so that it is able to withstand bending repeatedly. Chondrocytes of elastic cartilage lie between these yellow elastic fibers.\textsuperscript{3,4}

Figure 1. The position of articular cartilage (Figure used with permission from altunderground.com).\textsuperscript{5}

Hyaline cartilage is second type of cartilage, found widely on joint surfaces (Figure 1). It is pearly bluish in color with firm consistency and considerable amounts of collagen. The structure of hyaline cartilage is relatively simple and there are no nerves or blood vessels can be found in it. This absence of vessels indicates that nutrition and wastes cannot be transported in hyaline cartilage. A fibrous membrane, which is called the perichondrium, covers the hyaline cartilage externally, but not at the articular ends of bones and also where it is found directly under the skin.
as in the ears and nose, for example. The perichondrium contains blood capillaries and other vessels that can provide the tissue with nutrition. Chondrocytes which produce the solid matrix can also be found in hyaline cartilage. The matrix is basically composed of type II collagen and chondroitin sulfate as the typical proteoglycan, and both of them are also parts of elastic cartilage noted above. Fibrocartilage (or white cartilage) consists of a mixture of white fibrous tissue and cartilaginous tissue in different proportions. The flexibility and toughness of fibrocartilage is the result of the mixture of white fibrous tissue, and its elasticity is related to the cartilaginous parts. Fibrocartilage is the only type of cartilage that contains type I collagen in addition to the normal type II collagen.

In this thesis, only hyaline cartilage will be considered for the purpose of discussion for a tissue-engineering approach. As mentioned above, there are no blood vessels in this type of tissue and consequently the synovial fluid contained in the molecular pore space of the extracellular matrix (ECM) transfers almost all the nutrition and waste products for the cartilage. Chondrocytes obtain their nutrients only by diffusion from the synovial fluid. Absence of blood vessels causes the hyaline cartilage to heal very slowly following any type of injury and defect. This fact is one of the reasons why damage or insult to hyaline cartilage is difficult to repair by itself.

The important roles that cartilage plays in animals and humans rely on the functions of cartilage. Different mechanical stresses may affect bone structure, and cartilage acts to facilitate load support and load transfer while translation and rotation
are applied between bones. Compression loading, tension, shear and friction can be major problems for bone movements. In fact, cartilage provides a low friction, wear-resistant surface that can withstand large loads over decades of constant use and movements. Healthy cartilage can withstand many decades of rigorous use without deterioration or failure. The deformation characteristics of articular cartilage can be the principal reasons for its mechanical functionality. The time- and rate-dependent behavior of articular cartilage results from interstitial fluid flow through the solid matrix and is demonstrated by creep, stress relaxation, and energy dissipation or hysteresis.

![Mechanotransduction](Image)

Figure 2. Mechanotransduction can occur by means of pulling, compressing, and shearing the cells. Other stimuli can include streaming potentials (Figure used with permission from *Articular cartilage tissue engineering*).
In cartilage, chondrocytes are the sole resident cell type, and they produce a large amount of ECM composed of collagen fibers to maintain tissue structure and integrity. All chondrocytes have the same traits with respect to gene and protein expression, surface markers and cell metabolism. Those cells lie in the spaces which are called lacunae and there may be up to eight chondrocytes for each lacuna. Chondrocytes are the basic metabolic units in cartilage and are responsible for ECM formation. They are supplied by diffusion and helped by a pumping action which is generated by compression of the articular cartilage or flexion of the elastic cartilage. Disease and injury can alter cartilage physiology as well as tissue turnover, which can accelerate tissue breakdown. As noted above, the problem is the inability of the sparse cell population to repair the cartilage to any extent.

Another problem of chondrocytes is that the cells cultured in monolayers flatten over several days and begin to proliferate and rapidly lose their ability of cell synthesis and characteristic expression, including phenotypic markers. Therefore, the environment and culture time must be very carefully considered. Additionally, the application of mechanical stimuli such as stress, strain, and pressurization can affect the phenotypic expression of chondrocytes through a phenomenon termed mechanotransduction (Figure 2).

Chondrocytes originate from mesenchymal stem cells (MSCs). This kind of cell can be found in the bone marrow in mature individuals. During the period of embryogenesis, the MSCs start to differentiate into chondrocytes and secrete a cartilaginous matrix. As cells continue to divide, they pass through various lineage
states. Some of the chondrocytes differentiated from the MSCs stay in a central zone located closely to what will soon become bone at the final step. Other chondrocytes located on the periphery secrete collagen and matrix molecules in the right proportions to produce hyaline cartilage. At last, the mature articular chondrocytes cannot proliferate and are completely encased in matrix.\textsuperscript{18}

In the human body, chondrocytes represent only 1\% of the volume of hyaline cartilage, but they are very important since it is chondrocytes that replace degraded matrix molecules to maintain the proper physical sizes and mechanical properties of the tissue.\textsuperscript{19} Thus, microscopically, the endoplasmic reticulum and Golgi apparatus in chondrocytes are prominent and obvious to observe. In addition, many cells contain lipid and glycogen stores and secretory vesicles for specific functions. All chondrocytes have a primary cilium that extends from the cell into the ECM. The primary cilium is thought to play an important role in sensing the mechanical and chemical environment surrounding the cells and induces the chondrocytes to modify their matrix properties in response to forces and loading.\textsuperscript{20}

Healing of cartilage injuries and defects in the human body poses an important obstacle for current tissue and organ regeneration research and technology. It is difficult for cartilage injuries or defects to heal because of the structure of cartilage and its absence of blood vessels as mentioned previously. Damage and insult can occur to the articular cartilage of a joint on its own as an isolated condition or in conjunction with other bone and tissue injuries and diseases. Anterior cruciate ligament (ACL) injury is commonly associated with the damage to the medial (inner)
and lateral (outer) surfaces of the femur (thigh bone) and tibia (shin bone). This relation results from the fact that one of the functions of the ACL is to prevent the knee from accidental rotation. In the instance of an ACL tear during a twisting movement, the articular cartilage surfaces of the femur and tibia can be damaged.\textsuperscript{21}

1.1.2 Cartilage injuries and defects

There are many types of cartilage injuries in animals or human beings. The main injuries and diseases are as follows: matrix disruption, partial thickness defects and full thickness defects. Matrix disruption is the result of obtuse physical trauma, for example, in cartilage struck against a dashboard or injured by glass in traffic accidents. The ECM could be damaged in this kind of accident. Matrix disruption of this case, however, can actually be healed if the injury is not so extreme. The viable chondrocytes remaining after such injury can be more capable of matrix synthesis and repair of the damaged tissue.

![Figure 3. Partial thickness defects in articular cartilage](image)

Partial thickness defects refer most commonly to the damage on the surface of cartilage of bones (Figure 3). The defects can be caused by heavy use or extreme
movement of the bones. This kind of injury does not go deep to the subchondral tissue and bones. The cells near the injured region begin to proliferate and grow as soon as the damage happens. As observed by some researchers, chondrocytes appear to enter the defects and secrete matrix before the defects are repaired. The reason for this phenomenon is not clear.  

![Figure 4. Full thickness defect that penetrates to the subchondral bone (Figure used with permission from Eur. Cells. Mater.)](#)

Full thickness defects are injuries that affect the entire cartilage of bones and they probably extend deep to the subchondral tissues (Figure 4). These defects create transverse damage through the cartilage and the defects may be filled with some fibrin clots. This type of cartilage injury is different from other injuries mentioned above because it is possible for the existing cells from bone marrow to migrate into the full thickness defects. These cells finally lead to new tissue formation upon the replacement of fibrin clots between hyaline cartilage and fibrocartilage. An important property of the new cartilage tissue is that it is less stiff and more permeable than the original cartilage tissue. These properties of the new
cartilage tissue could cause the degradation of cartilage after a period of time.\textsuperscript{25}

Currently, cartilage injuries can be treated by several different methods (Figure 5). Although favorable outcomes to these methods have been achieved in the past 15 years, there are still some problems and obstacles in the cartilage repair area. For example, approximately 20\% of the patients treated by autologous chondrocyte implantation require revision procedures after a period of time.\textsuperscript{26-29}

\textbf{Figure 5.} Current articular cartilage treatment algorithm.

ACI = autologous chondrocyte implantation, MACI = matrix-assisted autologous chondrocyte implantation, TJR = total joint replacement (Figure used with permission from \textit{Tissue engineering for tissue and organ regeneration}).\textsuperscript{30}
1.1.3 Surface charge of cartilage and chondrocytes

Figure 6. Diagram of articular cartilage layers (Figure used with permission from *Am. J. Sports Med.*).\(^{31}\)

Surface properties of cartilage and chondrocytes are important considerations in repairing cartilage loss, especially with respect to surface defects. Studies of the surface properties of cartilage have been reported for many years and research characterizing the charge properties of cartilage surfaces has been published.\(^{32}\) In this instance, a polycationic derivative of ferritin was used to detect the charges on the uppermost superficial layer of articular cartilage, which is called the lamina splendens (as shown in Figure 6). The irregular distribution of cationic ferritin indicated that the superficial layer of cartilage contained an irregular distribution of fine, granular, electron-dense material. The cationic ferritin was distributed around collagen fibrils and fine fibrillar materials of the cartilage and suggested that the negative charges on the cartilage surface were arranged randomly. Further study demonstrated that the...
negative charges did not derive from the composition of synovial fluid (hyaluronic acid or lubricating glycoprotein) around the cartilage.\textsuperscript{33} The charges rather originated from the property of cartilage surface components, such as collagen fibrils.

Currently, glycosaminoglycans (GAG) are believed to generate these anionic sites on the cartilage surface. Glycosaminoglycans are polyanionic, in which each of their carboxylate and ester sulfate groups carry a single anionic charge. Glycosaminoglycans are always bound to a protein core in cartilage and they generate a high negative charge density in the tissue. As a result, a charge force can be produced between the cartilage surface and other functional charge groups.\textsuperscript{34}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cell_membrane.png}
\caption{Structure and composition of the cell plasma membrane (Figure adapted with permission from \textit{wikipedia.org}).\textsuperscript{35}}
\end{figure}
Chondrocytes are the only cell type in cartilage. They contain plasma membranes that are formed by the self-assembly of lipid bilayers, which have amphipathic properties. As Figure 7 indicates, the hydrophilic heads of the bilayer phospholipids are external in their intra- and extra-cellular membranes and prevent the hydrophobic tails of the phospholipids from exposure to aqueous cytosolic and extracellular environments. A negative membrane potential exists in almost all kinds of cells. Some literature reports show that different charged groups (positive/negative) may affect cell metabolism through the transport of ions from the extracellular environment: “Different surface charge density or combining positive and negative charges on the same substance may allow for graduated cellular uptake, targeting toward specific intracellular organelles.” However, the mechanism of this phenomenon needs further study. The cationic sites of lipoproteins may also be considered in this regard because the interactions between lipoprotein (positively charged amino groups) and polyanion (negatively charged GAG) can affect the binding of proteins during cell metabolism.

1.2 Tissue engineering for cartilage repair

1.2.1 Introduction to tissue engineering

Tissue engineering is a developing technique in organ regeneration and repair and reports show that tissue engineering is a promising technique for cartilage repair. The current definition of tissue engineering was established by Langer and Vacanti as “an interdisciplinary field that applies the principles of engineering and life sciences
toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ.\textsuperscript{39} In another way, tissue engineering has also been defined as “understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use.”\textsuperscript{40}

Tissue engineering is a complicated technology with the combination of many developments in biology, materials science, engineering, manufacturing, medicine and other science areas.\textsuperscript{41} The strategies developed in tissue engineering involve a range of approaches. The main idea of these approaches is the application of biological mechanisms and methods to achieve the repair and healing of damaged and diseased tissues. This basic approach of tissue engineering distinguishes it from the use of other methodology such as, for example, medical devices. The delivery of a natural or synthesized tissue-like substitute makes it different from a general pharmaceutical application. Tissue engineering is trying to solve the problems associated with more involved injuries and diseases resulting in physically damaged tissues and organs in the human body and which cannot be repaired perfectly or fail to repair at all. Generally speaking, “it is the intervention with an engineered tissue that presents the prospect of achieving successful repair where it would not happen, for any condition.”\textsuperscript{42}

1.2.2 Principals of tissue engineering

The most important principal features in tissue engineering are cell sources, scaffolds and growth factors. In tissue engineering, cell sources, which can
determine the general approach of tissue engineering, are very critical determinants of success with an engineering protocol. Different cell types are used for different kinds of tissue engineering. Cells for tissue engineering should have the ability to proliferate and differentiate in vitro and in vivo with the capability that these processes be subject to control. In clinical applications, primary cells harvested from healthy tissues from the patients themselves (autologous cells) are ordinarily the first and best cell source. These cells could prevent many problems of immune rejection. Most of the time, however, it is impossible to harvest cells directly from patients because of limited cell numbers and insufficient human tissue. Under these circumstances, alternate cell sources are used in tissue engineering studies besides autologous cells, and these include utilization of allogeneic cells, xenogeneic cells, syngeneic or isogeneic cells and stem cells.

One of the possibilities and direct intents of tissue engineering is the augmentation, regeneration, and replacement of tissues or organs malformed or malfunctioning, damaged or impaired by accidents, diseases or defects. While cells are key elements in tissue engineering, as just noted, scaffolds are also fundamental to the whole procedure and their design and fabrication must be carefully tuned for their proper function as a biodegradable or permanent cell substrate and support.

Finally, the third element of tissue engineering is growth factors. Growth factors are defined as substrates that can affect the stimulation of growth, proliferation and differentiation of cells under cell culture condition. Most growth factors are
proteins that can bind to receptors on cell membrane surfaces. Some growth factors are versatile for all types of cells while others are effective for only certain types of cells. For example, epidermal growth factor (EGF) is a growth factor found in the submaxillary gland. It promotes the proliferation of mesenchymal, glial and epithelial cells. Transforming growth factor-α (TGF-α), which can be found in various transformed cells, is associated with EGF in healing wounds or injuries.

1.2.3 Synthetic materials used as scaffolds in tissue engineering

Constructing scaffolds is one of the popular areas in tissue engineering, which involves materials synthesis and manufacture. Requirements for scaffolds used in tissue engineering include their being a three-dimensional and porous structure serving as a template for cell attachment and tissue development, biodegradable, biocompatible, and controllable in mechanical properties. Scaffolds used in tissue engineering can be designed with various combinations of material chemistry (collagen, hydrogels, synthetic polymers), geometry (gels, fibrous meshes, porous sponges), structures factors (porosity, pore size, pore distribution, orientation), mechanical properties (tension, compression, shear resistance, permeability), and rate of degradation.

There are two kinds of materials used as scaffolds for tissue engineering: synthetic polymer materials and natural materials. Many biodegradable polymers such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA) and their copolymers, polypropylene fumarate (PPF), polyhydroxyalkanoates, polyanhydrides and poly
(orthoester) are widely used in clinical applications and biomedical research.\textsuperscript{54} Compared with natural materials (collagen, fibrin, polysaccharides, hyaluronic acid and chitosan, for example) derived from the proteins, synthetic materials have many advantages, including the facts that their properties such as molecular weight, crystallinity, and glass transition temperature can be controlled by synthesis, they are readily available as starting materials, and they are easy to manufacture and process. Currently, a great number of tissue engineering studies focus on modifications of biodegradable synthetic materials to improve their different properties in order to be more effective in tissue or organ regeneration therapy.

1.2.3.1 Synthetic polymer materials

![Polymerization scheme of copolymers from L-lactic acid and D-lactic acid.]

The FDA (U.S. Food and Drug Administration) has approved devices made from several polymers such as PLA, PGA and PCL.\textsuperscript{55} As shown in Figure 8, PLA is
synthesized by poly-condensation of the lactic acid or ring opening polymerization of dilactide.

The linear structure and methyl group side chain of PLA make it amorphous and also hydrophobic if synthesized by racemic lactic acid. PLA can be degraded by homogeneous hydrolysis.\textsuperscript{56} Copolymers of PLA and other polylactones are widely used to modify the degradable properties, such as rate of degradation and the scale of molecular residues, by breaking the process of crystallization and accelerating the degradation of the materials. For the manufacture of PGA scaffolds, solution casting, solvent casting, gel casting, leaching and pressure gas formation are commonly used techniques to generate three-dimensional network structures.

PGA can also be synthesized by poly-condensation of glycolic acid or ring opening polymerization. PGA has high degree of crystallization which makes it insoluble in many organic solvents. The degradation process of PGA involves two steps: first, water diffuses into the amorphous regions of the polymer matrix and cleaves the ester bonds; and second, after the amorphous regions have been eroded, the crystalline portion of the polymer is hydrolyzed.\textsuperscript{57} Processing techniques of PGA can affect the sensitivity of degradation, especially for processing conditions. As mentioned above, the copolymer of PGA and PLA is widely applied in bone tissue engineering because there is fine control of its mechanical properties, biocompatibility and rate of degradation.\textsuperscript{54}

Another important polymer in the aliphatic polyester family is PCL. It can also be synthesized by poly-condensation or ring opening polymerization.\textsuperscript{58}
molecular weight of PCL will affect its crystallinity and other physical properties. Degradation of PCL is similar to that of PLA in which there is random hydrolysis and molecular weight loss. Blends and copolymers of PCL and other biodegradable polymers may be controlled in their physical and biocompatible properties and they produce less harmful byproducts.

PPF is a type of unsaturated linear polyester which can be easily cross-linked to form network structures. The synthesis of PPF involves two steps: the first step is the reaction between diethyl fumarate and excess propylene glycol in the presence of ZnCl$_2$ as a catalyst; the second step is trans-esterification of the bis (hydroxypropyl) fumarate intermediate to the final PPF product.$^{59}$ Crosslinked PPF gives very good mechanical strength as a scaffold, so it is widely used in bone tissue engineering. The agents for crosslinking are various depending on the rate of degradation required for a particular tissue-engineering procedure. The mechanism of degradation refers to the cleavage of ester groups by hydrolytic chain scission. The byproducts of degradation, propylene glycol and fumaric acid, are non-toxic, biocompatible and excreted easily. Some studies have used PPF as an injectable orthopedic implant material in the human body.$^{60}$

1.2.3.2 Natural polymer materials

Natural polymer materials such as collagen, fibrin, hyaluronic acid, chitosan, silk and other materials are used in bone and cartilage tissue engineering applications. The greatest advantage of these natural materials is their excellent biocompatibility as
Natural materials serve as templates for cell growth and
differentiation during regeneration and they are also generally non-immunogenic for
tissues and organs. Although their biocompatibility is extremely important, natural
materials have disadvantages as tissue engineering components as they are typically
low in bulk quantity, costly, and difficult to process in the variety of scaffolds needed
to satisfy clinical applications. To solve these problems to a certain extent,
combinations of two or more natural materials such as silk/chitosan or both synthetic
and natural materials are being investigated to produce scaffolds with improved
properties compared to single materials alone.

Biodegradable materials synthesized with different functional groups of
molecules may be a very promising concept for the design of novel and effective
scaffolds for tissue engineering. Because of the possibility that functional groups
can be developed with many different properties, synthetic materials that are
comprised partially or entirely of multi-functional groups could be the most advanced
choice for clinical applications of tissue and organ regeneration. This study
examines this approach to tissue engineering applied to cartilage repair and
restoration.

1.2.4 General approaches of tissue engineering

The general procedures of tissue engineering are as follows (Figure 9):
1. The initial step is tissue harvesting for the subsequent isolation of cells
appropriate to the tissue or organ intended to be augmented or regenerated by
engineering methods. There are four distinct cartilages in vertebrates, auricular (ear), articular (joint surfaces), costal (rib) and nasoseptal. For tissue-engineering repair of each of these cartilages, optimally the source of material for harvest is the identical autologous cartilage type. Thus a damaged ear would be tissue-engineered with auricular tissue obtained from the patient him/herself, an injured nasoseptal cartilage would be repaired with nasoseptal tissue from another site in the patient and so on.

If no identical cartilage type is available, then cartilage types may be interchanged with some success, such as the use of costal cartilage to substitute for auricular cartilage in an impaired ear. Typically, harvested tissue for human application is obtained from biopsy surgeries but, in certain situations of experimental tissue engineering, as in implantations in immunocompromised nude mice, tissue may be obtained from animal sources and utilized for scientific study without implantation in human body. Following tissue harvest, specific living cells are isolated and cultured with sufficient tissue collected to provide high numbers of cells to grow and expand.

2. After tissue harvest and cell isolation, the cells are cultured under conditions specific to the cell type and intended recipient of engineered tissue. Different kinds of cells require different methods and environments for culture and an early step in tissue engineering is determining the proper match between cell type and culture medium, its supplements, and its culture characteristics. The cells being cultured are allowed to proliferate or differentiate for a period of time for different purposes. Under the appropriate culture environment, cells are cultured to reach confluence or a sufficient or pre-determined cell number for further engineering.
3. Typically for tissue engineering, natural or synthesized polymeric scaffolds are seeded with an appropriate number of cells for further development of tissue constructs in vitro. The scaffolds require appropriate numbers of cells to allow cell growth in three-dimension. Tissue engineering relies on different kinds of scaffolds to mimic the extracellular matrix for cell growth in culture. Growth factors or mechanical stimulus may be added with the cultured constructs to enhance cell proliferation or differentiation of extracellular matrix.

4. After culture periods of sufficient time to ensure satisfactory cell growth and extracellular matrix production, implantation of the resulting cell/scaffold constructs can be conducted. In these instances, the scaffolds or constructs are placed into a
bioreactor to test the efficacy of different tissue-engineering approaches and the potential of different constructs in vivo. The tissue-engineering process starts with certain cells type from animals or the human body and ends with the developed implant being transferred back to its specific recipient to generate new tissue. Further characterization can be considered for the implanted constructs.

1.3 Chondrocyte behavior in tissue engineering

The growth of chondrocytes must be tested and analyzed in two different principal aspects: the quantity and quality of the cells. The quantity of chondrocytes refers to the number of viable cells in culture flasks or residing on a scaffold after being cultured for the appropriate period of time. Because of many factors such as the structure and material properties of the scaffold, the culture environment or character of a particular cell type, not all cells initially inoculated into flasks or seeded to the scaffold will attach to the substrate and grow. Unattached cells die and viable cells may be counted by various methods.\(^6^2\)

The quality of chondrocytes refers to the metabolism and character of the cells after being cultured.\(^6^3\) Quality involves many cell properties such as their morphology, gene expression and protein synthesis and secretion, membrane function and ability to proliferate and differentiate.\(^6^4\) Such properties determine if the cells behave normally and express and maintain functions and biosynthesized products compatible with those of normal chondrocytes.
1.4 Hypothesis for multi-functional synthetic materials used for cartilage repair

The major work of this project is to use of multi-functional biodegradable polymers as substrates or scaffolds on which to grow chondrocytes isolated from human articular cartilage. The specific aim of the study is to improve the tissue engineering repair of cartilage defects. Hypotheses for this investigation are that different kinds and percentages of functional groups designed for tissue engineering scaffolds can be used to induce and enhance the nature of chondrocyte attachment, proliferation and differentiation by the mechanism of charge interaction. An ultimate goal of the work is to synthesize different functional groups to the same polymer backbone for simultaneous interactions with both chondrocytes and cartilage, with the long-term hope of chondrocyte extracellular matrix secretion for repair of cartilage surface defects.

Functional groups used for this project include those with negative charges (carboxyl group, COOH) and positive charges (amine group, NH₂) and those that control the mechanical properties of the polymeric material, for instance, ethyl group (Et) and phenyl group (ph). For principal studies in this thesis, 2-D culture substrates are considered appropriate for investigating possible cytotoxicity of cells and testing chondrocyte behavior on each multifunctional polymer designed and developed for potential use.
CHAPTER II

EXPERIMENTAL PROCEDURE

2.1 Materials, reagents and equipment

**Biological part:**

**Materials:** Fresh tissue from human donors was obtained Chicago (obtained from the Gift of Hope Tissue & Organ Donor Network, Elmhurst, IL, through Rush University Medical Center, Rush University, Chicago, IL, USA); Dulbecco’s phosphate-buffered saline (DPBS, Mediatech, Manassas, VA); Ham’s F12, 1X (modified, Mediatech, Manassas, VA); Dulbecco’s modified Eagle’s medium (DMEM, Mediatech, Manassas, VA); primocin (50 mg/ml, InvivoGen, San Diego, CA); penicillin-streptomycin (antibiotic/antimycotic) solution (Mediatech, Manassas, VA); Type II collagenase (300 u/mg, Worthington Biochemical Co., Freehold, NJ); 10% fetal bovine serum (FBS, HyClone, Thermo, Logan, UT); trypsin-EDTA, 1X (0.05% trypsin, 53 mM EDTA in Hank’s Balanced Salt Solution, Mediatech, Manassas, VA); ascorbic acid (EMD Chemicals Inc., Darmstadt, Germany) and trypan blue (0.4%, BioWhittake, Inc, Walkersville, MD); gentamicin sulfate (MP Biochemicals, LLC., Solon, Ohio, USA).

**Equipment/Instrumentation:** Cell culture flasks (T-75, BD falcon, Bedford, MD); pipettes (1-50 ml, Van Waters and Rogers Company, Coraopolis, PA); sterilized
centrifuge tubes (15 ml, 50 ml, Van Waters and Rogers Company, Coraopolis, PA) hemocytometer with cover-slip; centrifuge (Centrifuge 5810 R, 15 amp version, NY); sterilized nylon mesh funnels (150 μm); water bath, biological hood (Class II type A/B3); micro-pipette (10 μl-1 L, Van Waters and Rogers Company, Coraopolis, PA), bio-incubator (37 °C, 5% CO₂, Forma Scientific, Marietta, OH); sterile syringe filter (Van Waters and Rogers Company, Coraopolis, PA); orbital shaker (Forma Scientific, Marietta, OH); sterilized scissors, tweezers, gauzes and scalpels (No. 10, BD Bard-Parker, Franklin Lakes, NJ); electronic balance (Sartorius, Mettler Toledo, Columbus, OH) and optical microscope (Olympus Co., Cleveland, OH); 12-well cell culture cluster plate (Corning Incorporated, Corning, NY); glass cover slips (diameter = 22 mm, Deckglaser, Germany).

Synthetic part:

Reagents:  Diethanol amine (99%, ACROS ORGANICS, Morris Plains, NJ), ethyl propionate (99%, Alfa Aesar, Ward Hill, MA), hydrocinnamic acid (99%, Alfa Aesar, Ward Hill, MA), thionyl chloride (99%, Fluka Analytical, Sigma Aldrich, St Louis, MO), imidazole (Oakwood Products Inc., West Columbia, SC), TBDMSCl (tert-butyldichlorodimethylsilane, 97%, Sigma Aldrich, St Louis, MO), succinic anhydride (99%, ACROS ORGANICS, Morris Plains, NJ), NHS (N-Hydroxysuccinimide, 98%, Sigma Aldrich, St Louis, MO), DMAP (4-Dimethylaminopyridine, 99%, Sigma Aldrich, St Louis, MO), tBuOH (tert-Butyl alcohol, Certified, Fisher Scientific, Pittsburgh, PA), EDC

**Equipment/Instrumentation:** Polymer molecular weights were analyzed on TOSOH EcoSec HLC-8320 GPC, with two PSS Gram Analytical GPC Columns in series, using 10 mM LiBr solution of DMF as eluent. The column temperature and detector temperature was 50 °C. The flow rate of the eluent is 0.8 mL/min. NMR spectra of the monomers and polymers were obtained on Varian MERCURY 300 spectrometers. Thermal transitions were analyzed using TA differential calorimeter Q2000 with liquid N$_2$ cooling unit at cooling cycle of 10 °C/ min and heating rate also at 10 °C/ min. TA Q500 thermogravimetric analyzer was used to collect 5% decomposition temperature data in a N$_2$ atmosphere. Spin-coating machine (Model WS-400B-6NPP/LITE, Laurell Co., North Wales, PA).
2.2 Synthesis of multifunctional polymers

a) Synthesis of diols with ethyl groups

\[
\text{Diethanol amine (1 equivalent) and ethyl propionate (1 equivalent) was taken in the RB flask and heated at 70°C for 10 hours and refluxed. The crude product was purified by column chromatography with methanol-dichloromethane as the gradient solvent system. Yield = 14.3 g (90%).}
\]

b) Synthesis of diols with phenol groups

\[
\text{Hydrocinnamic acid (1.501 g, 10 mmol) was taken in the RB flask and kept in ice (0°C). Thionyl chloride (0.726 ml, 10 mmol) was added in flask by dropwise addition. Dry methanol (20 ml, 500 mmol) was added to the flask. The reaction was started at room temperature and carried out overnight. After completion of the reaction, methanol was removed under reduced pressure and the compound was extracted in 50 ml EtOAc and 50 ml water. The resulting organic layer was washed with 10% NaHCO\textsubscript{3} solution and brine and dried over Na\textsubscript{2}SO\textsubscript{4}. The product was dried in a vacuum oven. Yield = 1.54 g (93%).}
\]
Diethanol amine (1.15 g, 10 mmol) and 2a (1.80 g, 10 mmol) were taken in the RB flask and heated at 70°C for 24 hours and refluxed. After the reaction, the flask was cooled to room temperature. The crude product was purified by column chromatography with methanol-dichloromethane as the gradient solvent system. Yield = 0.628 g (26%).

c) Synthesis of diols with protected carboxyl groups

Diethanol amine (1.638 g, 15 mmol) and imidazole (4.08 g, 60 mmol) were taken in the RB flask and dissolved in dry DMF (10 ml). The mixture was cooled (0°C). TBDMSCl (tert-butylchlorodimethylsilane, 5.66 g, 36 mmol) was added to the flask. The mixture became solidified. After 60 min, the mixture was extracted with dichloromethane (50 ml) and H₂O (50 ml) and then washed with brine (50 ml). The crude product was purified by column chromatography with methanol-dichloromethane. Yield = 4.93 g (98%).
Succinic anhydride (5 g, 50 mmol), NHS (N-Hydroxysuccinimide, 1.73 g, 15 mmol) and DMAP (4-Dimethylaminopyridine, 0.61 g, 5 mmol) were taken in the two-neck of an RB flask and dissolved in toluene (80 ml). A drying pipe and water cooling pipe were used for the next step. tBuOH (tert-Butyl alcohol, 88 mmol) and Et₃N (triethylamine, 2.1 ml, 15 mmol) were added to the flask. Refluxing followed for 24 hours at room temperature. After the reaction, the mixture was extracted with EtOAc (50 ml) and washed with 10% citric acid solution and brine, then dried over Na₂SO₄. The crude product was purified by column chromatography with hexane-EtOAc. Yield = 6.75 g (77%).

3b (1.04 g, 6 mmol) and EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, 1.31 g, 6.5 mmol) were taken in the RB flask and dissolved in DMF (Dimethylformamide, 10 ml). The mixture was cooled to 0°C for 10 min. 3a (1.69 g, 5 mmol) was dissolved in DMF (10 ml) and added in the RB flask dropwise addition. The reaction was carried out overnight at room temperature. After the reaction, DMF was removed under reduced pressure and the mixture was extracted
with EtOAc (50 ml×2). The crude product was purified by column chromatography with hexane-EtOAc. Yield = 1.5 g (61%).

Adipic acid (7.307 g, 50 mmol), benzyl alcohol (7.76 ml, 75 mmol) and PTSA (p-Toluenesulfonic acid, 1.3 mg, 0.5 mmol) were dissolved in toluene (40 ml) in an RB flask. A Dean-Staric trap was used and the mixture was heated until reflux began. The reflux was carried out overnight. After the reaction, H₂O (40 ml) was added and the pH value of the mixture was adjusted to 8.0 with NaOH. The resulting aqueous layer was washed with ether (2×30 ml) and the pH was adjusted to 2.0 with HCl. The organic layer was dried with Na₂SO₄. Yield = 5.3 g (44%).

3d (1.45 g, 6 mmol) and EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, 1.31 g, 6.5 mmol) were taken in the RB flask and dissolved by DMF (Dimethylformamide, 10 ml). The mixture was cooled to 0°C for 10 min. 3a (1.69 g, 5 mmol) was dissolved in DMF (10 ml) and added in the RB flask by dropwise
addition. The reaction was carried out overnight at room temperature. After the reaction, DMF was removed under reduced pressure and the mixture was extracted with EtOAc (50 ml×2). The crude product was purified by column chromatography with hexane-EtOAc. Yield = 1.24g (44%).

3c (4.2 g, 8 mmol) and iodine (0.84 g, 20% of 3c) were dissolved in methanol and reacted overnight at room temperature. After the reaction, methanol was removed under reduced pressure. The crude product was extracted with EtOAc and purified by column chromatography with methanol-dichloromethane.

3e (4.2 g, 8 mmol) and iodine (0.84 g, 20% of 3e) were dissolved in methanol and reacted overnight at room temperature. After the reaction, methanol was removed under reduced pressure. The crude product was extracted with EtOAc and purified by column chromatography with methanol-dichloromethane.
purified by column chromatography with methanol-dichloromethane. Yield = 1.1 g (61%).

d) Synthesis of diols with protected amine groups

\[ \text{H}_2\text{N}\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OH} + \text{SOCl}_2 \xrightarrow{\text{MeOH}} \text{H}_2\text{N}\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OCl} + \text{MeCl} \]

6-aminohexanoic acid (3.49 g, 26 mmol) was taken in an RB flask and dissolved in methanol (20 ml). Thionyl chloride (2.18 ml, 40 mmol) was added by dropwise addition. The reaction was carried out overnight at room temperature. After the reaction, methanol was removed under reduced pressure. Yield = 3.7 g (80%).

\[ \text{H}_2\text{N}\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OCl} + \text{(Boc)}\text{O} (\text{di-tert-butyl dicarbonate, 4.837 g, 22 mmol}) \xrightarrow{\text{et}_{3}\text{N}} \text{H}_2\text{O, 1,4-dioxane}} \text{BocHNCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{OMe} \]

4a (3.38 g, 19 mmol) was dissolved in a mixture of solvent water: 1,4-dioxane = 1:1. (Boc)\text{O} (di-tert-butyl dicarbonate, 4.837 g, 22 mmol) was added in the RB flask. The system was cooled to 0°C and Et\text{3}N (triethylamine, 6.27 ml, 45 mmol) was added by dropwise addition. The reaction was carried out overnight at room temperature. After the reaction, the mixture was extracted by EtOAc and the crude product was purified by column chromatography with hexane-EtOAc. Yield = 3.2 g (68%).
Diethanol amine (2.55 g, 24 mmol) and 4d (4 g, 16 mmol) were taken in the RB flask and refluxed overnight at 80°C. After the reaction, the crude product was purified by column chromatography with methanol-dichloromethane. Yield = 2.7 g (53%).

e) Synthesis of 90% Et - 10% NH₂ polymer

4c (0.127 g, 0.4 mmol), 1 (0.584 g, 3.6 mmol) and succinic acid (0.473 g, 4 mmol) were taken in the RB flask. DPTS (4-(Dimethylamino) pyridinium 4-toluenesulfonate, 0.467 g, 1.6 mmol) was added in the flask. Dichloromethane (3 ml) was added to the system as a solvent. The mixture was cooled and DIC
(N,N’-Diisopropylcarbodiimide, 1.586 ml, 12 mmol) was added by dropwise addition. The mixture was reacted at room temperature for 48 hours and then the polymer was precipitated from cold iso-propanol and dried.

\[ 5a \] (0.5 g) was dissolved in dichloromethane (2 ml) and the mixture was cooled to 0°C. Triisopropylsilane (2 equivalent of the total protected groups) and TFA (trifluoroacetic acid, 2 ml) was added by dropwise addition. The reaction was carried out for 2 hours and then the polymer was precipitated from cold iso-propanol and dried.

f) Synthesis of 80% Et – 20% NH\(_2\) polymer

The procedure is similar to the synthesis of e).

Product: 6a (protected) and 6b (de-protected)
g) Synthesis of 70% Et – 30% NH$_2$ polymer

The procedure is similar to the synthesis of e).

Product: $7a$ (protected) and $7b$ (de-protected).

h) Synthesis of 50% ph – 50% COOH polymer

4c (0.243 g, 1 mmol), 1 (0.223 g, 1 mmol) and succinic acid (0.23 g, 2 mmol) were taken in the RB flask. DPTS (4-(Dimethylamino) pyridinium 4-toluensulfonate, 0.117 g, 0.4 mmol) was added in the flask. Dichloromethane (3 ml) was added to the system as a solvent. The mixture was cooled and DIC (N,N'-Diisopropylcarbodiimide, 0.879 ml, 3 mmol) was added by dropwise addition. The mixture was reacted at room temperature for 48 hours and then the polymer was precipitated from cold iso-propanol and dried.
8a (0.5 g) was dissolved in dichloromethane (2 ml) and the mixture was cooled to 0°C. Triisopropylsilane (2 equivalent of the total protected groups) and TFA (trifluoroacetic acid, 2 ml) was added by dropwise addition. The reaction was carried out for 2 hours and then the polymer was precipitated from cold iso-propanol and dried.

2.3 Spin coating of multifunctional polymers

Before coating, all glass slides were washed with base bath solution and distilled water and then dried overnight. All polymer samples are made to a concentration of 10 mg/ml. For polymers functionalized with amine groups, methanol was used as solvent. For polymers functionalized with carboxyl groups, chloroform was used as solvent. The speed for spin coating was 2600 rpm. The coating time was 60 seconds for each sample.
After coating, all the samples were dried in a vacuum oven for 48 hours and carefully transferred to 12-well plates. Before seeding chondrocytes, all the samples including blank glass controls were sterilized by exposure to UV light for 60 min in a biological hood.

2.4 Cell isolation from the human knee and chondrocyte culture

- Chondrocyte isolation:

  Human cadaveric knees were transferred to the laboratory and kept on ice during transport. Before harvest of cells, serum-free medium (F12, DMEM, primocin, antibiotic/antimycotic) was prepared and kept at 4°C. A cell culture hood was cleaned and sterilized with ultraviolet light for at least 15 min. Sterilized scissors, sterilized tweezers, sterilized gauze and scalpels were also prepared.

  Fresh tissue was coated with butadiene to prevent bacterial contamination during dissection. The surrounding muscle and connective tissue on the knee were removed with a scalpel in order to collect clean cartilage. Dissection of small pieces of cartilage was accomplished with sterilized scissors, tweezers, gauze and scalpel blades under wet conditions (PBS applied liberally) to keep the tissue active. After removing all the muscles and tissue, use scalpel to remove cartilage. The cartilage pieces were shaped to approximately 5×5×1 mm in dimensions by carving the exposed cartilage surface into small rectangles, which were then removed from the whole donor specimen. Cartilage samples were transferred to serum-free medium in a 50 ml centrifuge tube. During the dissection process, cartilage or other useful
tissue was kept wet with PBS to maintain tissue activity, in case of other experiment may be needed.

After the dissection of cartilage, the pieces were washed with serum-free medium several times before placing them in an enzymatic solution to digest. Digestion in a sterilized pronase solution (2 mg/ml) was carried out for 1 hour in an orbital shaker (37°C) and then in the sterilized type II collagenase solution overnight in the orbital shaker (37°C).

- Chondrocyte seeding and culture:

After digestion, the tube with cartilage was removed from the orbital shaker and visually inspected. The solution in the tube should be very cloudy, showing that the cartilage pieces were digested by the collagenase. The cartilage suspension was then passed through a sterilized nylon mesh funnel (100 nm) to remove tissue residue and release chondrocytes. The funnel was washed before and after filtering with complete medium and the cell suspension was collected in a 50 ml centrifuge tube. The cell suspension was next centrifuged at 2410 rpm for 10 min at 4°C. A pellet should be present in the tube bottom. Old medium was carefully removed by aspiration and new complete medium was added to the tube. The procedure was repeated three times with the pellet resuspended each time in complete medium. Cells at this time point are identified as “passage zero” cells.

The number of isolated chondrocytes was determined by cell counting with a hemocytometer, and the viability of chondrocytes was determined by trypan blue exclusion. Twenty ml cell suspension was mixed with 20 ml trypan blue solution to
yield a dilution factor of 2 for calculation. As Figure 10 shows, only the central area of the hemocytometer (5×5) was use for counting cells under a light microscope. The number of cells was calculated according to the following equation:

\[
n (\text{cell number})
\]

\[
= \text{average cell counting number} \times \text{dilution factor} \times \text{volum} \times 10^4
\]

Figure 10. Hemocytometer used for cell viability. Small dots represented the live cells that were stained by trypan blue. Only the cells in the central 5×5 area were counted and calculated by using a dilution factor of 2 in the equation to calculate total cell number.

Cells and medium were next placed into T75 culture flasks. The number of viable cells and the volume of medium in a T75 culture flask was 10-15 ml total (cell
suspension + complete medium). Generally speaking, to control the time of cell confluence, the number of cells seeded in culture flasks was between $0.5 \times 10^6$~$1.0 \times 10^6$ cells per T75 flask (depending on different time lines of cell confluence and passage). The appropriate volume of cell suspension was delivered into the T75 flasks with a pipette, and complete medium was added to the flask. Fresh ascorbic acid (25 μl of a 5 mg/ml solution) was added to cells from the lip opening of the culture flasks every other day. All culture flasks were placed in an incubator at $37^\circ C$ with 5% CO$_2$. Culture medium was replaced every other day together with fresh ascorbic acid.

- Chondrocyte passage:

  Chondrocytes need to be passaged before they reach 100% confluence to keep cells growing in a healthy manner. The best time point for cell passage is when they reach 80% confluence. At this time point, the cell growth rate reaches its highest activity and then begins to decrease. Passaging is a method to maintain live cells that grow for extended periods of time under the same culture conditions. After removing the old medium from flasks and washing the cells with PBS, 5 ml trypsin-EDTA, 1X (0.05% trypsin, 53 mM EDTA in HBSS, Mediatech, Manassas, VA) were added to each culture flask to detach cells from the bottom of culture flasks. Flasks were placed back in the incubator ($37^\circ C$, 5% CO$_2$) for 3 to 4 min allowing the trypsin to act. Under a light microscope, cells were observed to determine whether they were all detached from the bottom of flasks. The detached cell suspension was transferred to a 50 ml tube. Flasks were washed with medium to ensure all the cells
were transferred to the centrifuge tube. All tubes were centrifuged at 2410 rpm for 10 min at 4°C. The cell number was measured by cell counting using a hemocytometer as illustrated above, and then they were reseeded into T75 culture flasks. At this generation, cells were identified as “passage one.”

Cell passage may be continued several times during an entire culture procedure. However, passaging too many times is not advisable since the passaged cells may not maintain their initial cell properties and may dedifferentiate into other cell types.

For primary culture, chondrocytes in this study were limited to three passages.

2.5 Chondrocyte seeding on biomaterial substrates

Synthetic materials were spin-coated on glass cover slips and completely dried. Blank cover slips (no spin-coated materials) were used as control samples. All the samples were sterilized by ultraviolet light in a biological hood for 1 hour. All the equipment used in the experiment was sterilized. Polymer-coated and blank cover slips were placed into individual wells of the 12-well culture dishes with no gap between the cover slips and the sides of the wells. Chondrocytes were detached from culture flasks using trypin-EDTA, and cell number was measured using a hemocytometer as described above. For each well, the total volume of culture medium was 2 ml. Based on literature publication, the optimal cell number for each well of the 12-well culture plates was $0.1 \times 10^6$ cells (the surface area of each well is $401 \text{ mm}^2$). A micropipette was used to transfer the cell suspension to the polymer-coated or uncoated cover slips and then the culture plates were placed back
into the incubator for 30 to 60 min to allow the chondrocytes to attach to the cover slip surface. Complete culture medium (2 ml) was added to each well and plates were cultured in the incubator at 37°C, 5% CO₂.

Cell numbers of each well were measured with a hemocytometer at specific time points of days 1, 3 and 5. The protocols were the same as described above. The reason to follow cell number in each well is to test if the difference of substrates can affect cell growth and cytotoxicity of the synthetic polymer substrates. Cell number was documented and plotted as graphs with error bars given as the standard error of mean values. Analysis of the rate of cell growth is presented below in the Results and Discussion sections of this study. Light microscopic images of the cells were recorded each day under 10X magnification. Images were documented and white-balanced.
CHAPTER III

RESULTS AND DISCUSSION

3.1 NMR spectra for material structure identification

NMR spectra of the monomers and polymers were obtained on Varian MERCURY 300 spectrometers and analyzed by ACDLABS-1 D NMR software.

\(^1\)H NMR of 1 in CDCl\(_3\)
$^1$H NMR of 2a in CDCl$_3$

$^1$H NMR of 2b in CDCl$_3$
$^1$H NMR of $3a$ in CDCl$_3$

$^1$H NMR of $3b$ in CDCl$_3$
$^1$H NMR of 3d in CDCl$_3$

![Graph of H NMR of 3d in CDCl$_3$]

$^1$H NMR of 3e in CDCl$_3$

![Graph of H NMR of 3e in CDCl$_3$]
$^1$H NMR of $3f$ in CDCl$_3$

$^1$H NMR of $3g$ in CDCl$_3$
$^1$H NMR of $4a$ in DMSO

$^1$H NMR of $4b$ in CDCl$_3$
$^1$H NMR of 4c in CDCl$_3$

$^1$H NMR of 5a in CDCl$_3$
\textsuperscript{1}H NMR of 5b in CDCl\textsubscript{3}

\textsuperscript{1}H NMR of 6a in CDCl\textsubscript{3}
$^1$H NMR of 6b in CDCl$_3$

$^1$H NMR of 7a in CDCl$_3$
$^1$H NMR of 7b in CDCl$_3$

$^1$H NMR of 8a in CDCl$_3$
**1H NMR of 8b in CDCl₃**

![1H NMR of 8b in CDCl₃](image)

**Diol 1:** Yield = 90%, ¹H NMR: (300 MHz, CDCl₃) δ 1.16 (dd, 3H, J₁= 9 Hz, J₂= 12 Hz), 2.41-2.48 (dd, 2H, J₁= 6 Hz, J₂= 9 Hz), 3.50-3.60 (m, 4H), 3.80-3.91 (m, 4H)

**2a:** Yield = 93%, ¹H NMR: (300 MHz, CDCl₃) δ 2.62-2.67 (t, 2H, J₁= 7.5 Hz), 2.94-2.99 (t, 2H, J₁= 7.5 Hz), 3.62-3.75 (t, 3H, J₁= 19.5 Hz), 7.20-7.34 (m, 5H)

**Diol 2b:** Yield = 26%, ¹H NMR: (300 MHz, CDCl₃) δ 2.69-2.78 (dd, J₁= 9 Hz, J₂= 12 Hz, 2H), 2.97-3.02 (t, 2H, J= 7.5 Hz), 3.43 (t, 2H, J= 6 Hz), 3.57-3.59 (d, 2H, J= 9 Hz), 3.71-3.73 (d, 2H, J= 6 Hz), 3.85-3.88 (t, 2H, J= 4.5 Hz), 7.19-7.34 (m, 5H)

**3a:** Yield = 98%, ¹H NMR: (300 MHz, CDCl₃) δ 0.07 (t, 2H, J₁= 7.5 Hz), 2.94-2.99 (t, 2H, J₁= 7.5 Hz), 3.62-3.75 (t, 3H, J₁= 19.5 Hz), 7.20-7.34 (m, 5H)

**3b:** Yield = 93%, ¹H NMR: (300 MHz, CDCl₃) δ 1.24-1.52 (m, 9H), 2.55-2.69 (m, 4H)
**3d**: Yield = 44%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.67-1.78 (m, 4H), 2.32-2.43 (m, 4H), 5.13 (d, 2H, $J$= 18 Hz), 7.27-7.43 (m, 5H)

**3e**: Yield = 44%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$0.04-0.05 (d, 12H, $J$= 3 Hz), 0.88 (s, 18H), 1.68-1.70 (t, 4H, $J$= 3 Hz), 2.40 (s, 4H), 3.45-3.53 (m, 4H), 3.69-3.76 (m, 4H), 5.12 (s, 2H), 7.36 (s, 5H)

**3f**: Yield = 76%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.37-1.51 (t, 9H, $J$= 21 Hz), 2.65 (s, 4H), 3.55-3.59 (m, 4H), 3.82-3.87 (m, 4H)

**3g**: Yield = 61%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.71-1.73 (t, 4H, $J$= 3 Hz), 2.41-2.43 (d, 4H, $J$= 6 Hz), 3.47-3.49 (d, 2H, $J$= 6 Hz), 3.56-3.58 (d, 2H, $J$= 6 Hz), 3.86 (s, 2H), 3.77 (s, 2H), 5.12 (s, 2H), 3.36-3.37 (d, 5H, $J$= 3 Hz)

**4a**: Yield = 80%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.28-1.36 (m, 2H), 1.51-1.60 (m, 4H), 2.28-2.33 (t, 2H, $J$= 7.5 Hz), 2.73-2.77 (t, 2H, $J$= 6 Hz), 3.59 (s, 3H), 7.99 (s, 3H)

**4b**: Yield = 68%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.26-1.39 (m, 2H), 1.44 (m, 9H), 1.53 (s, 2H), 1.65-1.67 (d, 2H, $J$= 6 Hz), 2.29-2.34 (t, 2H, $J$= 7.5 Hz), 3.08-3.14 (m, 2H), 3.67-3.70 (d, 3H, $J$= 9 Hz)

**4c**: Yield = 53%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.37-1.40 (d, 2H, $J$= 9 Hz), 1.44 (s, 9H), 1.51 (s, 2H), 1.67-1.72 (m, 2H), 2.40-2.45 (t, 2H, $J$= 7.5 Hz), 3.07-3.12 (m, 2H), 3.49-3.51 (m, 2H), 3.57-3.59 (m, 2H), 3.78-3.80 (m, 2H), 3.86-3.89 (t, 2H, $J$= 4.5 Hz)

**5a**: m:n = 0.1:0.9, Yield = 43.5%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.15-1.22 (m, 13H), $\delta$1.44-1.63 (m, 4H), $\delta$2.38-2.40 (m, 4H), $\delta$2.62 (t, 8H), $\delta$3.62-3.69 (m, 8H), $\delta$4.24 (m, 8H)
5b: m:n = 0.1:0.9, Yield = 83%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.13-1.15 (m, 3H), $\delta$1.21-1.23 (m, 4H), $\delta$2.36-2.43 (m, 4H), $\delta$2.62 (t, 8H), $\delta$3.63-3.70 (m, 8H), $\delta$4.23-4.25 (m, 8H)

6a: m:n = 0.2:0.8, Yield = 65%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.12-1.16 (m, 13H), $\delta$1.58-1.65 (m, 4H), $\delta$2.36-2.43 (m, 4H), $\delta$2.62 (t, 8H), $\delta$3.61-3.63 (m, 8H), $\delta$4.23-4.24 (m, 8H)

6b: m:n = 0.2:0.8, Yield = 80.6%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.14 (m, 3H), $\delta$1.21-1.24 (m, 4H), $\delta$2.38-2.41 (m, 4H), $\delta$2.62 (s, 8H), $\delta$3.61-3.70 (m, 8H), $\delta$4.23 (s, 8H)

7a: m:n = 0.3:0.7, Yield = 78.35%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.14-1.24 (m, 13H), $\delta$1.45 (m, 4H), $\delta$2.37-2.41 (m, 4H), $\delta$2.64 (t, 8H), $\delta$3.64-3.71 (m, 8H), $\delta$4.26 (m, 8H)

7b: m:n = 0.3:0.7, Yield = 85.2%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.14-1.22 (m, 3H), $\delta$1.24-1.27 (m, 4H), $\delta$2.36-2.43 (m, 4H), $\delta$2.62 (t, 8H), $\delta$3.61-3.69 (m, 8H), $\delta$4.24 (m, 8H)

8a: m:n = 0.5:0.5, Yield = 54.2%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.44 (m, 9H), $\delta$2.52-2.69 (m, 14H), $\delta$2.95-3.00 (m, 2H), $\delta$3.54-3.65 (m, 8H), $\delta$4.14-4.23 (m, 8H), $\delta$7.21-7.30 (m, 5H)

8b: m:n = 0.5:0.5, Yield = 74.5%, $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$1.82 (m, 3H), $\delta$2.59-2.67 (m, 14H), $\delta$2.95 (m, 2H), $\delta$3.60 (m, 8H), $\delta$4.22 (m, 8H), $\delta$7.20-7.34 (m, 5H)
3.2 Degradation temperatures and glass transition temperatures

Table 1. Degradation temperatures and glass transition temperatures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Td</th>
<th>Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% NHBoc-90% Et</td>
<td>223.81°C</td>
<td>-27.73°C</td>
</tr>
<tr>
<td>20% NHBoc-80% Et</td>
<td>116.02°C</td>
<td>-16.45°C</td>
</tr>
<tr>
<td>30% NHBoc-70% Et</td>
<td>146.51°C</td>
<td>-16.54°C</td>
</tr>
<tr>
<td>50% COOtBu-50% ph</td>
<td>222.67°C</td>
<td>15.76°C</td>
</tr>
<tr>
<td>10% NH₂-90% Et</td>
<td>185.85°C</td>
<td>0.99°C</td>
</tr>
<tr>
<td>20% NH₂-80% Et</td>
<td>176.05°C</td>
<td>-48.36°C</td>
</tr>
<tr>
<td>30% NH₂-70% Et</td>
<td>175.91°C</td>
<td>6.65°C</td>
</tr>
<tr>
<td>50% COOH-50% ph</td>
<td>186.54°C</td>
<td>21.56°C</td>
</tr>
</tbody>
</table>

Degradation temperatures and glass transition temperatures of all polymers are listed. The physical property of polymer can be indicated from the data. All polymers examined are sticky and stiff can be reshaped with tweezers.

3.3 Molecular weight of multifunctional polymers

Table 2. Molecular weight and PDI

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mₙ</th>
<th>Mₘ</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% NHBoc-90% Et</td>
<td>16558</td>
<td>26740</td>
<td>1.615</td>
</tr>
<tr>
<td>20% NHBoc-80% Et</td>
<td>24605</td>
<td>36036</td>
<td>1.465</td>
</tr>
<tr>
<td>30% NHBoc-70% Et</td>
<td>10029</td>
<td>13283</td>
<td>1.324</td>
</tr>
<tr>
<td>50% COOtBu-50% ph</td>
<td>82824</td>
<td>137001</td>
<td>1.654</td>
</tr>
</tbody>
</table>

Molecular weight was determined by gel permeation chromatography (GPC) and calculated with software. All the results yield a molecular weight for each
polymer with a narrow PDI (poly-distribution index). Protected polymers were used to test the molecular weights since amine or carboxyl groups may be easily absorbed to the column in the GPC machine.

3.4 Chondrocyte numbers at specific culture time points

Table 3. Cell number at day 1

<table>
<thead>
<tr>
<th>DAY 1</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% NH₂-Ethyl</td>
<td>1.6 × 10⁵</td>
<td>1.36 × 10⁵</td>
<td>0.92 × 10⁵</td>
<td>1.29 × 10⁵</td>
<td>0.2816 × 10⁵</td>
</tr>
<tr>
<td>20% NH₂-Ethyl</td>
<td>1.12 × 10⁵</td>
<td>0.68 × 10⁵</td>
<td>1.28 × 10⁵</td>
<td>1.03 × 10⁵</td>
<td>0.2537 × 10⁵</td>
</tr>
<tr>
<td>30% NH₂-Ethyl</td>
<td>1.56 × 10⁵</td>
<td>1.56 × 10⁵</td>
<td>1.4 × 10⁵</td>
<td>1.51 × 10⁵</td>
<td>0.0755 × 10⁵</td>
</tr>
<tr>
<td>COOH-ph</td>
<td>0.92 × 10⁵</td>
<td>1.16 × 10⁵</td>
<td>1.04 × 10⁵</td>
<td>1.04 × 10⁵</td>
<td>0.0980 × 10⁵</td>
</tr>
<tr>
<td>Blank</td>
<td>1.8 × 10⁵</td>
<td>2.7 × 10⁵</td>
<td>2.2 × 10⁵</td>
<td>2.23 × 10⁵</td>
<td>0.3682 × 10⁵</td>
</tr>
</tbody>
</table>

Table 4. Cell number at day 3

<table>
<thead>
<tr>
<th>DAY 3</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% NH₂-Ethyl</td>
<td>1.84 × 10⁵</td>
<td>1.56 × 10⁵</td>
<td>1.84 × 10⁵</td>
<td>1.74 × 10⁵</td>
<td>0.1321 × 10⁵</td>
</tr>
<tr>
<td>20% NH₂-Ethyl</td>
<td>1.36 × 10⁵</td>
<td>1.76 × 10⁵</td>
<td>1.44 × 10⁵</td>
<td>1.52 × 10⁵</td>
<td>0.1728 × 10⁵</td>
</tr>
<tr>
<td>30% NH₂-Ethyl</td>
<td>1.96 × 10⁵</td>
<td>1.84 × 10⁵</td>
<td>1.72 × 10⁵</td>
<td>1.84 × 10⁵</td>
<td>0.0979 × 10⁵</td>
</tr>
<tr>
<td>COOH-ph</td>
<td>1.32 × 10⁵</td>
<td>1.92 × 10⁵</td>
<td>2.24 × 10⁵</td>
<td>1.82 × 10⁵</td>
<td>0.3814 × 10⁵</td>
</tr>
<tr>
<td>Blank</td>
<td>2.28 × 10⁵</td>
<td>2.28 × 10⁵</td>
<td>2.32 × 10⁵</td>
<td>2.29 × 10⁵</td>
<td>0.0191 × 10⁵</td>
</tr>
</tbody>
</table>

Table 5. Cell number at day 5

<table>
<thead>
<tr>
<th>DAY 5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% NH₂-Ethyl</td>
<td>2.2 × 10⁵</td>
<td>2.0 × 10⁵</td>
<td>2.04 × 10⁵</td>
<td>2.08 × 10⁵</td>
<td>0.0864 × 10⁵</td>
</tr>
<tr>
<td>20% NH₂-Ethyl</td>
<td>1.84 × 10⁵</td>
<td>3.32 × 10⁵</td>
<td>2.92 × 10⁵</td>
<td>2.69 × 10⁵</td>
<td>0.6251 × 10⁵</td>
</tr>
<tr>
<td>30% NH₂-Ethyl</td>
<td>1.24 × 10⁵</td>
<td>2.92 × 10⁵</td>
<td>4.2 × 10⁵</td>
<td>2.78 × 10⁵</td>
<td>1.2121 × 10⁵</td>
</tr>
<tr>
<td>COOH-ph</td>
<td>2.4 × 10⁵</td>
<td>2.68 × 10⁵</td>
<td>2.96 × 10⁵</td>
<td>2.68 × 10⁵</td>
<td>0.2286 × 10⁵</td>
</tr>
<tr>
<td>Blank</td>
<td>8 × 10⁵</td>
<td>8.2 × 10⁵</td>
<td>8 × 10⁵</td>
<td>8.06 × 10⁵</td>
<td>0.0945 × 10⁵</td>
</tr>
</tbody>
</table>
Errors are calculated for each cell counting result and the errors are in a small range. The average values are plotted as a bar graph and a line graph below.

Figure 11. Cell number vs. culture time (bar graph)

Figure 12. Cell number vs. culture time (line graph)
From these two graphs, several conclusions can be made. All the spin-coated polymer samples are non-toxic to chondrocytes as the cells grew and followed their normal growth trends. From cell numbers, the blank control sample (no spin-coated polymer) is optimal for cell growth. For all the polymer-coated substrates, the cell number increases with an increase in the polymer functionalized with the amine groups. Substrates with carboxyl groups yield lower cell numbers at the day 5 culture end point comparing polymers functionalized with 30% amine groups with those functionalized with 70% ethyl groups. However, one set of cell testing has been followed here and that is not sufficient to provide conclusive data. Additional cell counting is needed to establish statistical data.

3.5 Microscopic images of chondrocytes on polymer-coated or glass substrates

Images of cells on substrates over days 1 to 6 of culture were taken by optical microscopy (Olympus) under 10X magnification. All images were white-balanced to compare images equivalently in contrast and brightness. The scale bar for each image is 200 μm given in the lower right corner of each image. As the images show, chondrocytes harvested from a cadaveric human knee can attach and grow on all the substrates, a result indicating that the polymers are non-toxic to the cells. Comparing different samples at the same time point (for example, day 3), chondrocytes grew optimally on the control sample (blank glass cover slip), consistent with cell counting shown in Figures 11 and 12.
Blank control:

Figure 13. Chondrocytes on blank control glass substrates.
10% NH₂-90% Et polymer:

Figure 14. Chondrocytes on 10% NH₂-90% Et polymer substrates.
20% NH$_2$-80% Et polymer:

Figure 15. Chondrocytes on 20% NH$_2$-80% Et polymer substrates.
30% NH$_2$-70% Et polymer:

Figure 16. Chondrocytes on 30% NH$_2$-70% Et polymer substrates.
50% COOH-50% ph polymer:

Figure 17. Chondrocytes on 50% COOH-50% ph polymer substrates.
For all the polymer-coated substrates, increasingly higher percentages of amine in the polymer produced increasing chondrocyte growth. These results indicate that polymers functionalized with amine groups play an important role in attracting and growing chondrocytes on these substrates. Polymers with carboxyl groups showed poorer chondrocyte viability than the 30% amine/70% ethyl polymer. As a possible explanation to the latter result, the similarly negatively charged carboxyl groups and chondrocyte cell surfaces may be repulsive so that cells cannot adhere and proliferate on these types of substrates.

**Day 6 cell culture behavior of 50% COOH-50% ph polymer:**

![Image of chondrocytes on 50% COOH-50% ph polymer substrates at day 6.](image)

Figure 18. Chondrocytes on 50% COOH-50% ph polymer substrates at day 6.
However, chondrocytes grown on the substrates containing carboxyl groups show another interesting phenomenon. Figure 13 illustrates that there are some interconnecting structures between and about the cells. A possibility is that these connections between these chondrocytes are the extracellular matrix generated by the cells. As described in the Introduction to this study, ECM is the precursor for the generation of connective tissue. Chondrocytes on 50% COOH/50% phenyl polymer substrates may be activated by the carboxyl groups and the growth, proliferation and ECM production of chondrocytes were enhanced. Of course, further evidence from histological and protein analysis will be needed to demonstrate that this possible cell product is the ECM component such as proteoglycan or type II collagen characteristic of cartilage.
As mentioned above, cell viability experiments need to be repeated several times. Only one cycle of cell counting as followed in this study is insufficient for demonstrating and establishing a complete set of cell growth data and any differences in cell number for each sample substrate. Moreover, longer culture times could be investigated to allow additional chondrocyte exploration on the various polymer substrates. Culture times could be expended to eight or more days or until the cells become confluent on the substrates. Statistical analysis for all cell counts is also necessary to compare different time point samples.

In further studies, histology can be used to determine ECM products during cell culture. The major ECM products that may be present in these chondrocyte cultures are proteoglycan and type II collagen. Safranin-O staining may be used to identify proteoglycan and Picrosirius red staining (with and without polarization optics) may be applied to identify type II collagen.

Additionally, substrates made by spin coating should be considered for some basic characterization, for instance, surface roughness and film sickness. Atomic force microscopy can be used to measure the surface roughness of the film. For the film sickness of the coated substrates, ellipsometer can be used to measure the value
for general purpose. Finally for other analysis of cartilage development, multifunctional polymers can be produced as 3-D scaffolds with controllable structure, porosity and additional properties. Electrospining is a reliable method to manufacture 3-D polymer scaffolds or sheets to allow cell attachment inside the scaffold and the polymer scaffold may induce chondrocyte attachment and growth. Fabrication and characterization of the 3-D polymer scaffold can be studied in the future.
REFERENCES


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