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DEVELOPMENT OF PROTEIN-IMPRINTED POLYSILOXANE BIOMATERIALS: PROTEIN SELECTIVITY AND CELLULAR RESPONSES

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ABSTRACT OF THESIS

DEVELOPMENT OF PROTEIN-IMPRINTED POLYSILOXANE BIOMATERIALS: PROTEIN SELECTIVITY AND CELLULAR RESPONSES

Surface modification is an extensively researched approach in order to overcome the limitations, and improve the performance of orthopedic and dental implants. It is at the surface of the implant materials that the initial interactions of tissues or body fluids take place. Therefore, surface properties of biomaterials are the important factors that can control these biological responses. Molecular imprinting is a surface modification technique that creates specific recognition sites on the surface of biomaterials. To develop the recognition sites, a functional monomer is assembled with template biomolecule and then crosslinked. After removal of the template, the surface can rebind the molecules. Therefore, desired reactions can be initiated at the interface between tissue and implants by modifying surfaces to selectively bind certain types of biomolecules, such as proteins. The objective of this project was to observe the potential of molecular imprinting technique for creating biomaterials that can recognize specific biomolecules. Fluorescently labeled lysozyme or RNase A was used as a template biomolecule and the protein-imprinted scaffolds were fabricated by sol-gel processing. To interpret the density of binding sites created, the quantity of surface-accessible protein was determined. The amount of protein available on the surface was proportional to the amount loaded. Protein-imprinted scaffolds were evaluated for their ability to selectively recognize the template biomolecule. Further, for these selectivity studies, a combination of the imprinted protein and a competitor protein were rebound to the polysiloxane scaffolds. The template protein rebound to the surface was measured more than twice as much as competitor. These scaffolds were then tested to understand their interaction with cells. The results of DNA and alkaline phosphatase activities indicate that the scaffolds thus developed support growth and adhesion of osteoblastic cells. These initial selectivity and cytocompatibility studies show the potential of molecular-imprinted polysiloxane scaffolds to be used as tissue engineered materials for stable and controlled interactions at the tissue-implant interface.

KEYWORDS: Molecular imprinting, Polysiloxane, Sol-gel processing, Tissue-implant interface, Cytocompatibility

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DEVELOPMENT OF PROTEIN-IMPRINTED POLYSILOXANE BIOMATERIALS: PROTEIN SELECTIVITY AND CELLULAR RESPONSES

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Kyoungmi Lee

The Graduate School
University of Kentucky
2005

DEVELOPMENT OF PROTEIN-IMPRINTED POLYSILOXANE BIOMATERIALS: PROTEIN SELECTIVITY AND CELLULAR RESPONSES

THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biomedical Engineering
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By

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Lexington, KY

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CHAPTER 1. INTRODUCTION

Metals, ceramics, polymers and composites have been used as hard tissue replacements. Metallic biomaterials are dominant as load-bearing implants for bone. There are two series of factors at the interface of bone tissue and implants. One is the host response to the implants, and the other is the material response to the host [1, 2]. When biomaterials or medical devices are implanted, they contact the body fluids, and then the proteins from blood and the fluids adsorb on the surface of the materials rapidly [3]. During repairing of the gap between implants and tissues, fibrous encapsulation and disruption of the newly forming tissues can occur [2]. Those events mentioned above can result in undesirable local and systemic biological responses, such as aseptic loosening and carcinogenesis, at the interface between tissues and dental or orthopedic implants [4].

Biomaterials for tissue engineering applications have been developed in order to provide a stable interface after implantation of the materials. Tissue engineering is a field that develops biological substitutes with applying the principles of engineering and the life sciences. Hence, biomaterials play an important role in the strategies of tissue engineering [5]. As one of the tissue engineering approaches, the surface modification of such materials has been executed. Shin et al. reviewed materials modified with biomolecules to elicit specific cellular responses and direct tissue formation. The paper showed that surface modification of biomaterials with bioactive molecules has been useful for tissue engineering applications, but several challenges, such as designing adhesion molecules for specific cell type which lead to tissue regeneration, still remain [6]. Molecular imprinting is a technique for preparing substrates for selective binding of particular biomolecules. In case of protein, the technique has met with only limited success, such as Shi et al. who investigated surfaces that could recognize specific proteins [7]. Most studies have used methacrylate polymers to fabricate molecularly imprinted materials [24-27], and a few studies used sol-gel procedure to design the materials [21-13]. Though some studies show the potential molecular selectivity of the imprinted materials using sol-gel processing [22], no research that observes the cell behavior on the molecularly imprinted scaffolds has been shown.

Therefore, the aim of this study was to develop and characterize protein-imprinted biomaterials to enable selective binding of biomolecules using lysozyme and RNase A as model templates. Cellular responses to fabricated materials were also studied for initial cytocompatibility tests.

CHAPTER 2. BACKGROUND AND SIGNIFICANCE

Bones are connective tissues primarily composed of collagen protein matrix (osteoid) that is impregnated with hydroxyapatite-like mineral. Bones have three types of bone cells as well as blood-forming cells in the central marrow cavities [8]. Osteoblasts cells are responsible for bone formation. When these cells are surrounded by calcified matrix, they are differentiated to osteocytes. Osteoclasts are cells that resorb the bone [8]. One of the functions of bone is supporting the human body. However, bones can break or fracture when high loads that they cannot bear are applied to them or when there is repeated force. Thus, in order to repair and fix the defective bones, special therapies of bone grafting, including autografts, allografts, xenografts and synthetic bone materials, are needed, and these therapies have been used in orthopedic surgery [9].

There are three mechanisms of bone generation when bone grafts are used to repair bone defects [10]. The first mechanism is osteogenesis. It involves the provision of cells that direct the production of new bone by osteoblasts. The second mechanism, called osteoconduction, provides a scaffold or substrate that allows bone growth by osteogenic cells. Osteoconductive grafts consist of calcium sulfate, calcium phosphate, hydroxyapatite, and other bioceramic compounds. The third mechanism is osteoinduction, which includes factors that induce the differentiation of stem cells and other undifferentiated cells into osteoblasts. Mesenchymal stem cells and bone morphogenetic proteins are examples of osteoinductive grafts [10].

Bone grafts that provide for bone generation by these mechanisms could be procured from many sources and used for implant operations. There is a surgical procedure called autograft in which tissue is replaced by tissue from another part of patient's body. This procedure offers biological advantages so it does not cause any immune reaction. However, obtaining the grafts needs another surgical procedure and brings up complications such as limitation of donor sites [4]. Allograft is a transplant from a donor of the same species. The graft is able to have the property of osteoconductivity, but there is the possibility of rejection or transmission of diseases [10]. Xenograft is a tissue, such as bovine-derived bone, obtained from a different species [4, 11]. It is also used as a temporary graft for coverage of wounds such as porcine

xenografts [11]. Synthetic materials have been also developed for bone graft substitutes [4, 10]. Calcium phosphates, hydroxyapatite, polymers, demineralized bone and collagen-based materials have been often used as synthetic substitute materials [4]. These materials have been successfully used as scaffolds in orthopedic and dental fields [12].

Synthetic biomaterials have been used when organs or tissues of the body are damaged or show defects. Early biomaterials, including silicones, polyurethanes, nylon and stainless steel, were not designed for use in human body or organs. Instead, they had been used for machines, such as airplanes or automobiles. In the 1960s, biomaterials began to be designed for use in the body [11]. This naturally resulted in making biomaterials possible to be used in many fields such as vascular, orthopedic and dental materials and controlled drug delivery [13]. Hench et al. classified the biomaterials into three generations. First-generation biomaterials were developed to be bioinert; they had the least effect on toxic response and immune response to the foreign body. Second-generation biomaterials were considered to be resorbable or bioactive materials. Third-generation biomaterials are resorbable and bioactive, so they are being designed to activate and stimulate specific cells and biomolecules [11, 14].

Three-dimensional scaffolds are widely used for synthetic materials [15]. Porous scaffolds can support proliferation and differentiation of cells and enhance bone tissue formation [16]. When scaffolds are fabricated, the characteristics, such as pore size, porosity and surface roughness, should be considered. Pore size of scaffolds should be at least 100 μm, which is large enough to allow cells to grow into the bone structure [17]. For in vivo osteoconduction, optimal pore size of scaffolds is approximately 150 to 500 μm [4, 15]. Interconnectivity of porous scaffolds is one of the crucial factors for cell distribution, cell metabolism and nutrient diffusion. The mechanical stability is also important for tissue formation of new bones [4, 16]. Scaffolds including such properties are necessary as carriers of cells in the tissue engineering area [15].

Tissue engineering is the application of engineering and life sciences in order to develop biological substitutes. Tissue engineering offers the potential to overcome replacement of damaged or lost tissue function [5, 11]. Use of isolated cells or cell substitutes, tissue-inducing substance and cells placed on or within matrices are three general strategies to create new tissue in tissue engineering field [5]. Bone tissue

engineering which typically uses an artificial extracellular matrix (scaffold) such as ceramics or polymers has been researched widely [16].

2.1 Surface Modification

Medical devices for orthopedic and dental applications are required to have good mechanical properties since the function of the bone is for load-bearing. Thus many metallic and polymeric materials have been designed for medical devices. However, undesired reactions at the interface between biological system and the surface of the biomaterial occur in many cases when biomaterials are implanted in the body [4]. Therefore, biomaterial research has high interest in modifying the surface of the materials that come in contact with body fluids [18, 19]. In order to provide desirable characteristics on the biomaterials surface, the process of changing the existing characteristics is required [4]. The mechanical properties of implants can remain without influence when altering the outermost surface composition of biomaterials [3]. The surfaces of biomaterials are mainly modified either in order to prevent the failure of implanted biomaterials or to incorporate a specific functionality onto biomaterials for binding tissues [3, 4]. Therefore, surface modification technique is applied to improve biological, chemical and mechanical properties of implants [20]. Various surface modification technologies are available such as mechanical treatment, thermal spraying, sol-gel, chemical treatment and ion implantation [20]. The following two approaches are examples of surface modification using sol-gel process and molecular imprinting technique. Advincula et al. deposited titanium oxide on the surface of silicon wafers by sol-gel processing. Ultrathin titanium oxide was prepared by reaction of metal alkoxides with a hydroxylated surface. This surface was then modified by self-assembled monolayers (SAMs) of silanes with different functional groups. This study showed the morphology trends of the surface and the properties of protein adhesion on the modified titanium oxide surface [19]. Bures et al. applied a surface modification technique which includes molecular imprinting methods by addition of tethered PEG chains on polyacrylic acid hydrogel. The prepared PEG star polymer gels by gamma-irradiation were used for protein delivery. Thus the materials including micro- or nanoparticulate carriers were

developed for bioadhesive drug delivery systems. This group researched the properties of star polymer hydrogel as proper materials for molecular imprinting [21].

2.2 Molecular Imprinting

Molecular imprinting is a technique for preparing synthetic polymers with selective recognition or binding sites that allow the development of biomimetic compounds as artificial receptors [22]. The technique can manipulate the shape, size and chemical functionality of a polymer matrix depending on template molecules. The molecular imprinting process includes a complex that is an assembly of functional monomer with template molecules in solution, and polymerization with a large quantity of crosslinkers to fix the complex as a stable polymeric material [22, 23]. The complex including functional monomer and template molecules is categorized, non-covalent and covalent approaches [23, 24, 25]. Non-covalent interaction includes hydrogen-bonding, electrostatic and hydrophobic interaction in the complex [25]. In covalent approach, the template molecules are covalently bound to the functional monomer [26]. Removal of the template molecules from the synthesized polymer by a washing procedure affords cavities as binding sites. The cavities have specific shape and chemical complementary to the template molecules [23]. Though most approaches use small molecules [27, 28], macromolecules, such as proteins, are highly interesting as template molecules for molecular imprinting [28]. The cavities, which generated using macromolecules, also have the ability to selectively rebind the template molecules [22]. Figure 1 shows the schematic representation of the creation of recognition sites by molecular imprinting procedure [29]. Molecular imprinting technique seeks to prepare polymers that are able to recognize specific polypeptides and protein [29]. Siloxane based polymer is formed by catalyzing (with acid or base) a series of silane monomer during sol-gel processing (section 2.4). Sol-gel processing can be performed to develop versatile materials, and there are several approaches that involve molecular imprinting polymers related to sol-gel processing [22, 30]. Cummins et al. compared the molecular imprinting of acrylic and sol-gel based polymers. The group expected better selectivity on sol-gel polymerization

system in future work [22]. Therefore, molecular imprinting technique by sol-gel processing deserves more research.

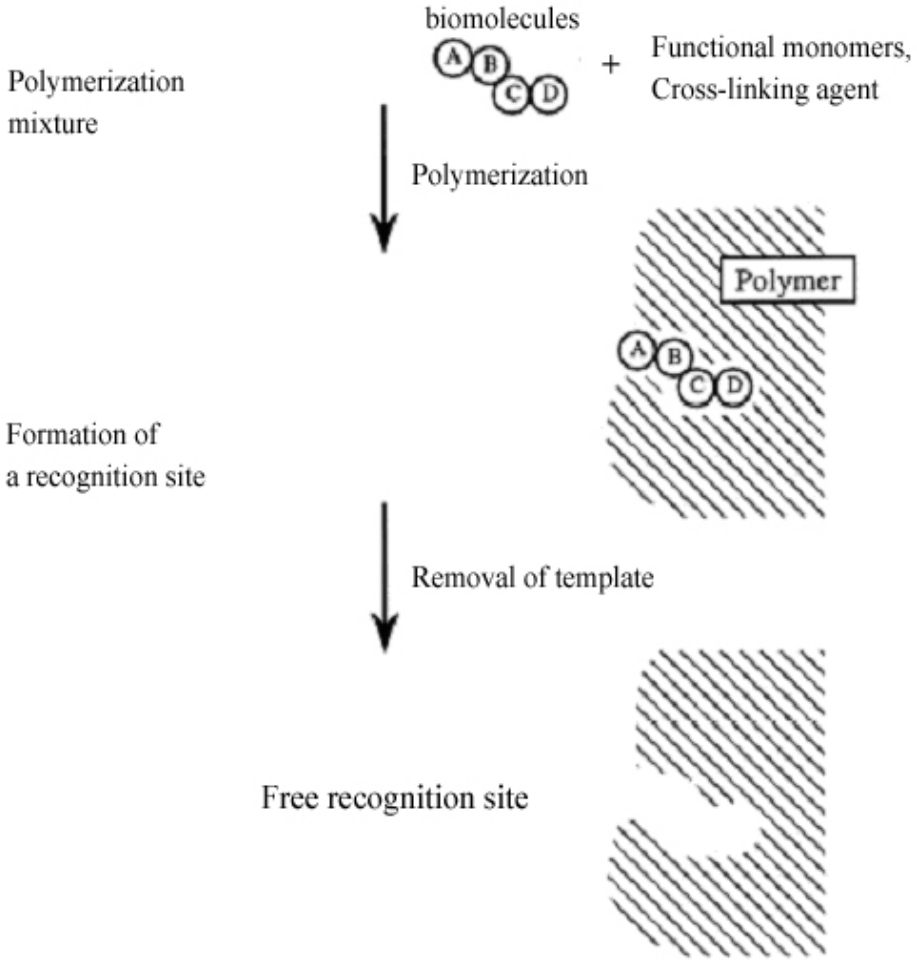

Figure 1. Schematic diagram of the molecular imprinting [29].

2.3 Protein Adsorption

Proteins are biological macromolecules composed of carbon, hydrogen, oxygen, nitrogen and small amounts of other elements. The proteins are formed by the linking of a large number of small subunits, 20 common amino acids including amino group, carboxyl group and α carbon, to form a long chain [8]. There are four levels of protein structure. The primary structure is a linear sequence of amino acids. The secondary structure consists of bending chain of α-helix and β-sheet due to hydrogen bonding between near amino acids in the same chain. The tertiary structure, twisting and folding

of the chain, results from ionic interaction, salt bridge, hydrophobic interactions, hydrogen bonding and covalent disulfide bonds between more distant amino acids. The quaternary structure is similar to tertiary structure but composed of two or more polypeptides chains. Figure 2 shows a structure of lysozyme which includes some of the protein structure levels [8, 31, 32].

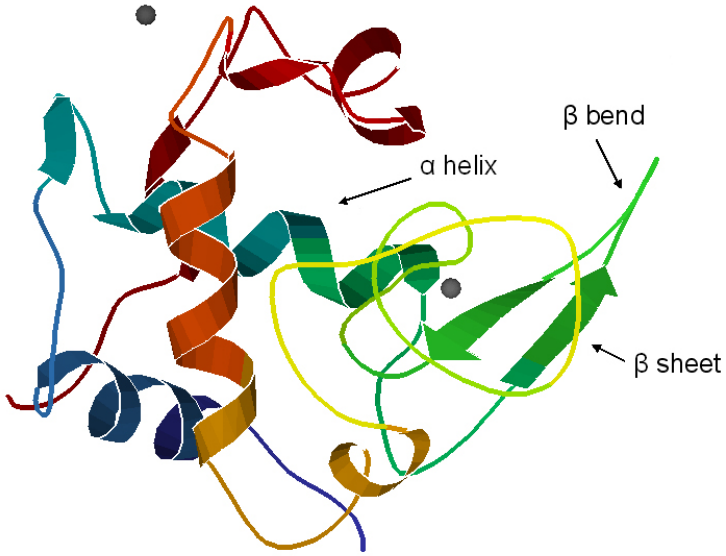

Figure 2. The structure of lysozyme showing α helix, β sheet and β bend from protein data bank (PDB ID: 193L).

The molecular weight of molecules plays an important role in adsorption. Molecular weight is the sum of the atomic weights of all the atoms in a protein, and reflects the size of a protein [8, 31]. Adsorption is a characteristic of solid surface that the molecules tend to stick on the surface at the solid-liquid interface (Figure 3). At the interface of biological systems with the solid surface of biomaterials, protein adsorption quickly occurs after implantation [33]. Several domains, which maybe hydrophobic, charged and polar, exist at the surface of protein. These are the basis of protein adsorption with a similar domain-like character at a solid surface. Protein adsorption can be affected by physicochemical factors, such as pH and ionic strength [31]. In biological fluids, soluble proteins in blood plasma are primarily involved in protein adsorption to implanted biomaterials. The physicochemical character of biomaterials also influences the orientation and conformation of protein adsorption [34]. Desired cellular responses can be induced by control of nonspecific protein adsorption. Therefore, biomaterials can

stimulate responses of osteoblast cells at the tissue-implant interface if the materials have selectivity for particular biomolecules such as osteotropic molecules [2].

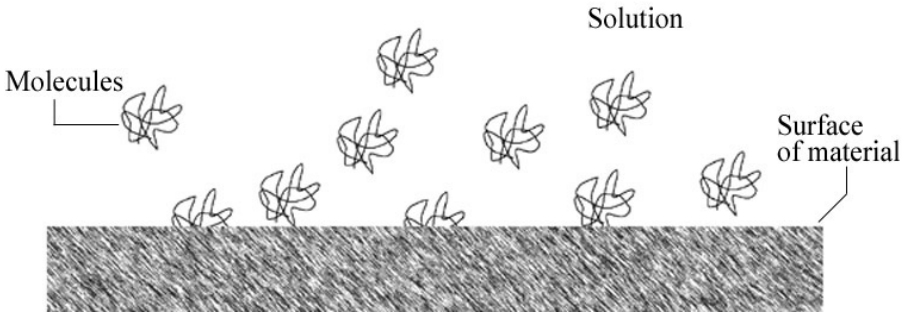

Figure 3. Surface reaction of molecular adsorption during which molecules stick on the surface.

2.4 Sol-gel processing

The best merit of sol-gel processing is the easy and convenient method of making functionalized inorganic materials at room temperature [35]. Sol-gel chemistry has been mainly used to prepare conventional glasses, ceramics fibers and many other new systems since its discovery in the 1800s [36, 37]. As the name 'sol-gel' denotes, 'sol' (solution) refers to a dispersion formation of colloidal suspension, and 'gel' refers to assembly of the sol to form an interconnected polymeric network [36]. Alkoxysilanes, such as tetramethoxysilane (TMOS) and tetraethoxysilane (TEOS), are the most widely used metal alkoxides as crosslinkers (precursors) for sol-gel processing [38]. TMOS is more expensive than TEOS, sometimes generates dangerous fumes, and causes blindness. Hence, TEOS is shown to be the preferred one for sol-gel processing in many publications [39].

Sol-gel process usually involves three reactions. These are hydrolysis, alcohol condensation and water condensation, as shown in Figure 4 [36, 38]. Through the hydrolysis reaction, alkoxide groups (OR) are replaced with hydroxyl groups (OH). Silanol groups (Si-OH) produce siloxane bonds (Si-O-Si) and water or alcohol through water and alcohol condensation, respectively [38]. The hydrolysis and condensation reactions that lead to a gel are affected by pH. Hydrolysis can be rapid when acids or ammonia are used. For condensation, the approximate pH domains were divided into

three, pH<2, pH 2-7 and pH>7. Gel time decreases steadily between pH 2 and ca. pH 6, and the condensation rate is proportional to [OH^-]. The particles of silicas are positively charged at low pH and negatively charged at high pH. Above pH 7, highly condensed particles are formed in a few minutes. The polymerization rate is proportional to [H^+] below pH 2 [35].

Hydrolysis reaction

$$\equiv Si\text{-}OR + H_2O \rightarrow \equiv Si\text{-}OH + ROH$$

Alcohol condensation

$$\equiv Si\text{-}OH + RO\text{-}Si \rightarrow Si\text{-}O\text{-}Si \equiv + ROH$$

Water condensation

$$\equiv Si\text{-}OH + HO\text{-}Si \rightarrow Si\text{-}O\text{-}Si \equiv + H_2O$$

Figure 4. Three reactions of alkoxysilanes [38].

During sol-gel processing, the structure and properties of gels change with aging. The chemical reactions continue after gelation, thus causing strengthening, stiffening, and shrinkage of the network during aging. The process of drying also leads to shrinkage of materials as a volume of liquid is evaporated from the body of the materials [38].

2.5 Biocompatibility

Biomaterials and medical devices, such as synthetic bone grafts and artificial blood vessels, are required to establish biocompatibility because they contact tissues of human body directly or indirectly [40]. There are several definitions, and emphases of biocompatibility have changed in past years. For example, in 1987, Williams defined biocompatibility as "the ability of a material to perform with and appropriate host response in a specific application." [11]. Later, Ratner defined biocompatibility as "the exploitation by materials of the proteins and cells of the body to meet a specific performance goal." [41]. Biocompatibility involves two principal areas. The first principle is biosafety, and the other principle is biofunctionality. Biosafety includes the elimination of harmful effects of biomaterials on the biological system. Biofunctionality means the ability to elicit proper host response [40]. In order to evaluate the

biocompatibility of the synthetic biomaterials, both *in vivo* and *in vitro* tests have been performed [42, 43, 44]. *In vitro* biocompatibility tests are widely used in the laboratory to predict *in vivo* responses. Various types of cell culture methods are usually applied for *in vitro* biocompatibility (cytocompatibility) tests [45]. Therefore, in the initial step of cytocompatibility testing of biomaterials, proper types of cells are cultured for the evaluation [46]. According to cytotoxicity, cell morphology, adhesion and viability tests, cytocompatibility of biomaterials can be assessed [45]. Cytocompatibility can also be evaluated by the morphology of cell lines and observation of the cell interactions on the surface of biomaterials using scanning electron microscopy (SEM) [44]. International Standardization Organization (ISO) and American Society for Testing and Materials (ASTM) define the method of tests for some biological evaluations of medical and dental devices [47]. Hence, ISO categorizes the evaluation of biocompatibility including morphological assessment of cell damage, measurement of cell damage, cell growth and specific aspect of cellular metabolism. Cytocompatibility is generally performed by cell adhesion, cell spreading, cell proliferation and cell biosynthetic function tests [40]. For the specific function of a medical device, cells relevant to the function of the device should be properly chosen for the test. Thus, osteoblastic cells would be the logical choice for biofunctionality testing of bone grafts or bone biomaterials [42].

2.6 Significance

Tissue engineering is becoming more critical in biomaterials research. Biomaterials for tissue engineering have been widely studied. Ideally, biomaterials should have ability to provide specific biological responses when they are implanted into the body. In reality, however, trivial to significant problems remain at the interface between the surface of materials and tissue of the body. Surface modification on bone implants may produce controlled interaction on orthopedic and dental applications. By using molecular imprinting, which is one of the surface modification techniques, on the bone biomaterials, we may design the biomaterials that support specific and desired cellular responses at the interface. As shown in Figure 5, the materials would mostly bind the round (☻) molecules, which are used as template molecules, in a solution where

several different types of molecules exist. Hence, if cell surface molecules, such as osteotropic protein receptors, are used as templates, the material would be selective for the specific cell type.

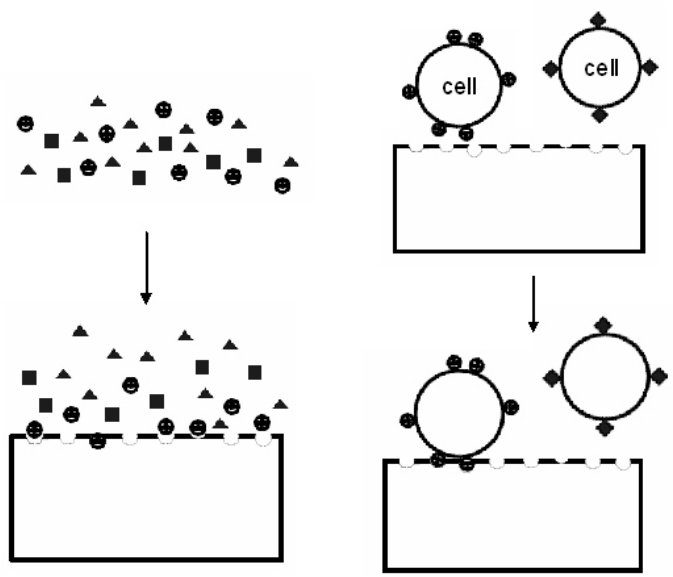

Figure 5. Binding of specific molecules (left) and cells (right) on molecularly imprinted biomaterials.

Therefore, the objective of this research was to develop molecularly imprinted polysiloxane biomaterials that assemble crosslinker (TEOS) and functional monomer (APS) with template biomolecules (proteins) using sol-gel processing. The biomaterials were designed to have ability to discriminate between template proteins and competitor proteins. Cytocompatibility of the biomaterials was then evaluated.

CHAPTER 3. MATERIALS AND METHODS

3.1 Fabrication of polysiloxane scaffolds

The polysiloxane scaffolds, which could be imprinted with template biomolecules and have selectivity for the biomolecules, were developed after trying various mixtures of tetraethoxysilane (TEOS) and γ-aminopropyltriethoxysilane (APS). The best composition of scaffolds was determined at TEOS (Fluka; Milwaukee, WI) to APS (Sigma; St. Louis, MO) volume ratio of 4:1 since the composition was useful for protein loading, selectivity and cytocompatibility tests. Proteins, such as lysozyme (Sigma) and ribonuclease A (RNase A; Sigma), were used as template molecules.

3.1.1 Polysiloxane scaffolds

Figure 6 shows the diagram of the process used to fabricate the scaffolds. Polysiloxane scaffolds were fabricated by a two-step sol-gel procedure. Solution I, which included 36.5% of TEOS, 6.5% of deionized water, 9.1% of 0.1 M HCl, and 11.1% of absolute ethanol in a cylindrical plastic vial, was mixed on an orbital shaker at room temperature for 24 hours. In this step, TEOS, as crosslinker, was hydrolyzed. In the second step, 9.1% of APS and 27.7% of sodiumdocecylsulfate (SDS; Sigma) were prepared to create solution II, which then was added to solution I after 30 minutes. APS was used as a functional monomer, and SDS was used in this step as a foaming agent due to the need for macroporosity for cell ingrowth. Mixtures of solution I and II which were containing volume ratio of 4:1 at TEOS to APS formed gels in 15 seconds. After covering the vials, the gels were aged at room temperature for 24 hours and then dried in an oven at 40˚C for 48 hours. When scaffolds were dried, a glass-like surface covered the samples. This caused different properties between inside and outside surfaces of the scaffolds. In order to remove the glass-like surface, the upper and bottom parts of the scaffolds were ground off using ECOMET 3 variable speed grinder-polisher (Buehler) using 600 grit silicon carbide papers.

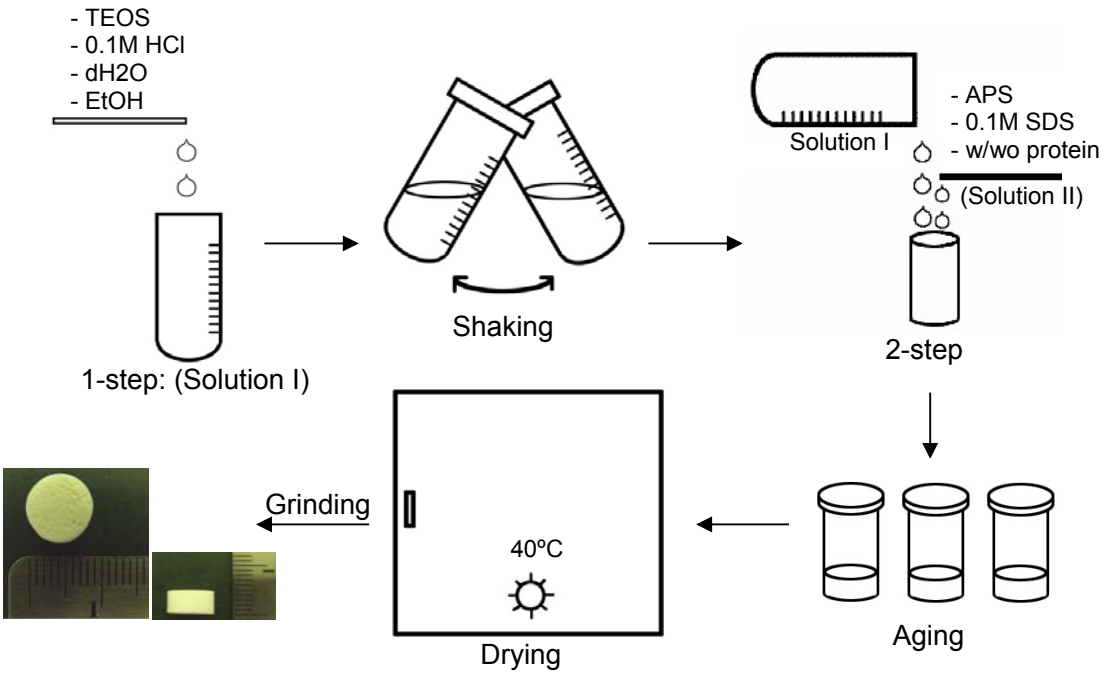

Figure 6. The fabrication of polysiloxane scaffolds.

3.1.2 Imprinted scaffolds

Protein-imprinted samples were also fabricated when blank polysiloxane scaffolds were made. The various amounts of lysozyme and RNase A that were added to the scaffolds ranged from 0.05 to 6 mg per sample. The protein was added to solution II right before mixing with solution I. After the mixture gelled, it was aged at room temperature for 24 hours and dried for 48 hours. For the protein loading and selectivity tests, labeled proteins (section 3.2) were needed. In this case, the gels were stored in a dark place because the labeling dye compounds were light sensitive. It was not necessary for the scaffolds to be imprinted with labeled protein for the cytocompatibility tests. Then the aged gels were dried and ground. All samples were washed with phosphate buffered saline (PBS), pH 7.4, after grinding.

3.2 Protein labeling

In order to determine the amount of protein available on the surface, the proteins were fluorescently labeled with Alexa Fluor 350 (Molecular Probes; Eugene, OR) before

imprinting. Molecular weight of lysozyme is about 14.3 kD, and RNase A is about 13.7 kD. These two proteins were chosen due to the similarity in size and the cost effectiveness.

3.2.1 Lysozyme labeling

The concentration of the protein in reaction buffer was 20 mg/mL. Thus 20 mg of lysozyme was dissolved in 1mL of 0.1M carb-bicarbonate buffer, pH 8.5. The reactive dye solution was prepared by dissolving amine-reactive compound, Alexa Fluor 350, in dimethyl sulfoxide (DMSO) at 1mg/100µl. While the lysozyme solution was being stirred, 100 µl of the reactive dye solution was gradually added. Then the solution was incubated for an hour with continuous stirring at room temperature. Unreacted labeling reagent needed to be separated from the protein solution. Hence, the solution was centrifuged with an Amicon Ultra-15 centrifugal filter device (Millipore; Bedford, MA), which has 10,000 molecular weight cut off (MWCO), in a Marathon 21 K/R centrifuge (Fisher Scientific, Pittsburgh, PA) at 3000 rpm (revolution per minutes) and 25˚C for 6 hours. The solution was washed with the carb-bicarbonate buffer three times during centrifugation. The protein solution needed to be generally stored under the same conditions (-10˚C in this study) used for the parent protein [48]. The concentration of the protein solution was determined using Bicinchoninic Acid (BCA) protein assay reagent (Pierce, Rockford, IL). This working reagent was used by mixing of 50 parts reagent A with 1 part reagent B. Then a stock bovine serum albumin (BSA; Pierce) solution was serially diluted two-fold in order to prepare a set of protein standards. A volume of 10 µl of each standard and each protein solution sample was added to a 96-well plate (Costar; Cambridge, MA), and then a volume of 200 µl of prepared working reagents was added each well. The well plate was covered and incubated at 37˚C for 30 minutes. The plate was read absorbance at 570 nm by a Dynatech MR5000 spectrophotometer (Chantilly, VA).

3.2.2 RNase A labeling

Using methods similar to those for labeling lysozyme, 20 mg RNase A was dissolved in 1 ml of 0.1M carb-bicarbonate buffer, pH 8.5, and 1 mg of Alexa Fluor 350

in 100 µl of DMSO was added to the RNase A solution and kept stirring for an hour. The protein solution was also centrifuged with an Amicon Ultra-15 centrifugal filter device in a Marathon 21 K/R to filter the Alexa Fluor 350 label that did not bind to the protein. RNase A dissolved into the buffer very easily so the solution was very clear and the filtering time was shorter than the filtering time of lysozyme. The concentration of the RNase A solution was then determined by BCA protein assay. The solution was stored in the freezer when not used right after the preparation.

3.3 Protein loading

Labeled template molecules, lysozyme and RNase A, with Alexa Fluor 350 were added to the scaffolds in increasing amounts (section 3.1.2). The amount of protein available on the surface of scaffolds after loading was measured by fluorometry. The imprinted scaffolds were immersed in 0.4 mg protease (Sigma) in 1 ml of 0.1M carb-bicarbonate buffer, pH 8.5, for 24 hours. The amount of protein released from the scaffold surfaces was observed after 3 hours and 24 hours. In order to calculate the amount of protein exposed to the surface of materials, another set of protein standards was obtained. The concentration of labeled protein solution was measured by BCA assay and used for imprinting scaffolds in section 3.1 and 3.2. The labeled protein solution was serially diluted two-fold for the standard. Fluorescence of the standard was measured using a fluorometric plate reader (Spectra MAX Gemini XS; Sunnyvale, CA) at excitation wavelength of 346 nm and emission wavelength of 442 nm. Then the fluorescence of protein released from each scaffold was also measured by the fluorometric plate reader.

3.4. Characterization of the scaffolds

3.4.1 Scanning Electron Microscopy (SEM)

Polysiloxane scaffolds with (imprinted) or without (blank) protein, were examined using SEM. A blank scaffold, 1 mg and 10 mg lysozyme-imprinted scaffolds were mounted on SEM stubs with colloidal graphite. Scaffolds on the stubs were sputter-

coated with gold in argon gas using the Emscope sc 400. Then samples were examined with Hitachi S-3200 (Tokyo, Japan) at an accelerating voltage of 20 kV.

3.4.2 Surface Area

The surface areas of scaffolds were measured by nitrogen gas adsorption. Scaffolds (blank, 0.1, 1 and 6 mg of both lysozyme- and RNase A-imprinted) were placed in an oven at 40 ºC for three days and then under vacuum for 24 hours. The weight of each scaffold was measured after desiccation in a sample tube at 120 ºC for 24 hours. The surface area was measured with TriStar 3000 (Micromeritics; Norcross, GA) and calculated using the BET (Brunauer-Emmett-Teller) Method.

3.4.3 Porosity

The porosity measurements of scaffolds were made by Autopore IV 9500 mercury intrusion porosimeter (Micromeritics). Blank, 0.05 mg of lysozyme- and RNase A-imprinted scaffolds were completely dried at 40 ºC for three days, and then tested. Three samples were place in a 5cc penetrometer (07-0649). The porosity of a material was characterized by applying various levels of pressure with intruding mercury into the pores of the sample. As raising the pressure of the penetrometer, the mercury intrudes from the largest pores to the smallest pores. Then the mercury porosimetry can determine total intrusion volume, average pore diameter, density and porosity of the sample.

3.5 Protein Selectivity

In order to examine the specificity and selectivity of the imprinted scaffolds, rebinding solutions were prepared. The rebinding solutions contained labeled template protein and labeled competitor protein. The proteins for the solutions were labeled with other dyes that have different excitation and emission wavelengths than the labeling dye using for protein loading. Similar to the method described in section 3.2.1, 20 mg of lysozyme was labeled with orange Alexa Fluor 488 dye (Molecular Probes), and 20 mg of RNase A was labeled with purple Alexa Fluor 594 (Molecular Probes). The excitation

and emission wavelengths of Alexa Fluor 488 were 495 nm and 519 nm. The wavelengths of Alexa Fluor 594 were 590 nm and 617 nm.

For the lysozyme-imprinted scaffolds, labeled lysozyme was used as template protein and labeled RNase A was used as competitor protein in the rebinding solution. The concentration of both proteins in rebinding solutions was kept constant at the maximum amount of protein released from each samples. The ratios of template to competitor protein were 1:0, 1:1 and 0:1. In order to check the selectivity of scaffolds for another protein, RNase A-imprinted scaffolds were used for another selectivity test. This time the rebinding solutions including template protein (RNase A) and competitor protein (lysozyme) were prepared at template to competitor protein ratios of 1:0, 3:1, 1:1, 1:3 and 0:1. The prepared scaffolds for each selectivity test were immersed in the rebinding solutions for 24 hours on an orbital shaker. Then the solutions were aspirated and scaffolds were rinsed three times with PBS, pH 7.4. Like the protein loading test, 0.4 mg protease in 1 mL of 0.1 M carb-bicarbonate buffer, pH 8.5, was added to digest proteins that rebound to the scaffolds. The amount of protein released from scaffolds into the protease solution was measured after 3 and 24 hours using the fluorometric plate reader.

3.6 Cytocompatibility

Basic cytocompatibility tests were conducted first to verify the ability of the scaffolds to support cell growth and activity. SaOS-2 osteoblastic cells (ATCC HTB-85; Manassas, VA) and C3H/10T1/2 cells (ATCC CCL-226) were prepared for the tests.

3.6.1 Preparation

SaOS-2 osteoblastic cells and C3H/10T1/2 (C3H) cells were cultured and passaged on T-75 cell culture flasks (Corning; Corning, NY). McCoy's 5A medium with 10% fetal bovine serum (FBS) (Gibco; Grand Island, NY) was used for SaOS-2, and Basal Eagle Medium (Gibco), which included 2 mM of L-glutamine and 10% heat-inactivated FBS (FBS-HI) to destroy heat-labile complement proteins, was added for C3H cells. Several steps of scaffold preparation were taken before seeding cells on them. Because the pH of scaffolds was highly alkaline, the first step was to control the pH to be

more neutral. Samples needed to be in a very acidic environment in the beginning and then sterilized. Therefore, samples were placed in a 24-well tissue culture plate (Costar) with 2 ml PBS, and then 15 µl of 25% HCl was added to neutralize the scaffolds. The plate was shaken for 24 hours, and then the samples were rinsed three times with PBS, pH 7.4. Scaffolds were sterilized in the second step. The scaffolds were placed into a beaker with 20 ml of PBS and then autoclaved with slow exhaust using a Napco Model 8000-DSE Autoclave. After the scaffolds in PBS were cooled down, the samples were immersed in cell culture medium for 48 hours. The medium was changed at least twice before the cells were seeded.

The cultured cells in T-75 flasks were rinsed twice with PBS and detached from the surface of the flask with an enzymatic solution that contained 0.02 M Hepes, 0.15 M NaCl, 0.26 mM ethylene glycol-bis (β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), 0.5% w/v of polyvinyl-pyrrolidone (PVP-10) and 0.05% w/v of trypsin (Sigma), pH 7.6. The detached cells were added to a conical tube with medium and centrifuged at 3000 rpm for 3 minutes to inactivate and remove the trypsin. Then, the cells were counted using a hemocytometer. The amount of 50,000 cells/well was seeded on plates and scaffolds. The seeded cells were cultured for 1, 3 and 7 days, and medium was changed every other day.

3.6.2 DNA Assay

Hoechst 33258 assay was used to determine DNA contents for the *in vitro* cytocompatibility experiments. The plates and scaffolds on which cells were seeded were carefully washed twice with warm (37 ºC) PBS, pH 7.4. Then 1 ml of high salt buffer, pH 7.4, containing 0.05 M NaH_2PO_4, 2 M NaCl and 2 mM EDTA, was added to each well. Each well including cells and scaffolds was sonicated and stored in the freezer. All plates were run through three freeze-thaw cycles to lyse more cells after sonication. The cell lysates were centrifuged to precipitate the particles of the scaffolds using an Eppendorf 5415 centrifuge. Serial two-fold dilutions of a standard DNA solution were added to 96-well plates (Costar). Each supernatant of the samples was diluted two-fold with the high salt buffer, and added to the 96-wll plates. Then, a volume of 5 µg Hoechst 33258 per 1 ml of high salt buffer was added to the each well. The plate was shaken gently and placed

at room temperature in the dark for 10 minutes. Fluorescence was measured at λ_{ex}=356 nm and λ_{em}=458 nm using a fluorometric plate reader.

3.6.3 Alkaline Phosphatase (AP) Assay

The osteoblastic activity of the cells was measured using the alkaline phosphatase assay. The supernatant used for the Hoechst assay was also used for AP. A volume of 10 µl of sample supernatant was added to wells of a 96-well plate. p-Nitrophenyl phosphate (pNPP) was added to 0.6 M 2-amino-2-methyl-1-propanol buffer (AMP), pH 10, to make a 10 mM working reagent, and then 0.01 M $MgCl_2 \cdot 6H_2O$ was added. A volume of 50 µl of working reagent was added to each well of the 96-well plate and then incubated at 37˚C (Forma Scientific). The color of the solution changed from clear to yellow, and absorbance was measured with a Dynatech MR5000 spectrophotometer at 410 nm after 24 hours of incubation. The values of the absorbance were converted to nmoles and then expressed in units as nmoles of substrate degraded per µg DNA.

3.6.4 Scanning Electron Microscopy (SEM)

A special preparation was needed for the scaffolds on which cells were seeded. Samples were rinsed three times with warm (37 ºC) PBS, pH 7.4, and then immersed in warm (37 ºC) 2.5% glutaraldehyde (Sigma) in 1 M cacodylate buffer, pH 7.2, for an hour to fix the cells on the scaffolds. The samples were rinsed with 0.1 M cacodylate buffer three times at room temperature and then dehydrated using an ethanol series, 50%, 70%, 90%, 95% and 100% of ethanol (10 minutes each step). Finally, samples were immersed in hexamethyldisilazane (HMDS, Polysciences, INC.; Warrington, PA) for ten minutes, and the samples completely air dried. Samples were placed on SEM stubs with colloidal graphite, sputter-coated with gold, and observed with Hitachi S-3200.

3.7 Statistical Analysis

One-way analysis of variance (ANOVA) was done using the computer application InStat (Graphpad Software, San Diego, CA). The Tukey-Kramer test was performed when the p-value was significant.

CHAPTER 4. RESULTS

4.1 Fabrication of scaffolds

4.1.1 Polysiloxane scaffolds

As shown in the last photo in Figure 6, the fabricated polysiloxane scaffolds were white in color with a cylindrical geometry of 4.3 mm in height and 9 mm in diameter. Initially we found that the APS was highly alkaline, and hence the scaffolds were highly alkaline. Consequently, 0.25 M HCl was added to solution I instead of 0.1 M HCl in order to control pH during the fabrication of scaffolds. The mixture of solutions I and II for these scaffolds was about pH 7.8. The whole scaffolds were transparent, like a glass, and had non-interconnected pores on the surface (Figure 7). When the scaffolds were immersed in a liquid (dH_2O or PBS), they were fractured into little particles. Some preliminary experiments with the particles showed that the particles could not release enough protein for quantification test, so the amount of protein rebound was also extremely small.

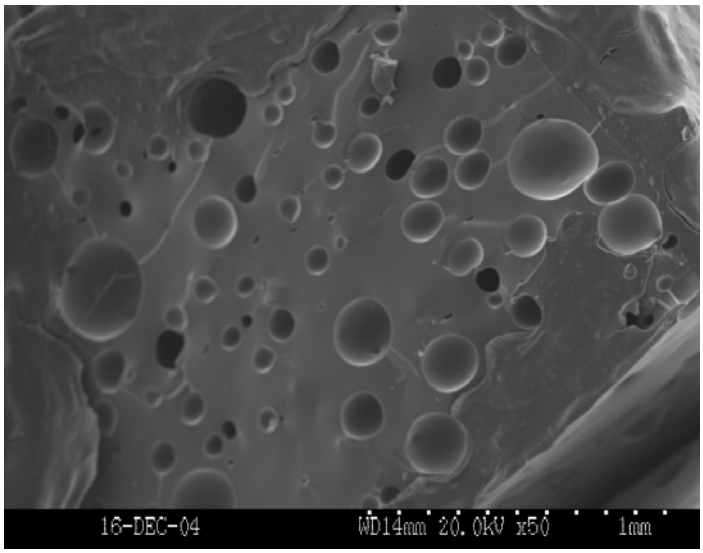

Figure 7. SEM image showing morphology of glass-like scaffolds when the mixture of solutions I and II was pH 7.8.

Figure 8 shows the scaffolds fabricated with 0.1 M HCl in solution I, which were white and covered with a glass-like surface. The mixture solutions I and II for this scaffold was pH 9.5. The glassy covering affected the amount of protein that could be

released from the materials (section 4.2). Attempts to cut off the glass-like surface with razor blades were unsuccessful. Thus we ground the surface with a polisher. The surface of initial scaffolds had ruffled textures and did not show any pores. On the other hand, the scaffolds after grinding were highly textured and included micro-, meso- and macro-pores (Figure 9). Porosity of the scaffolds was approximately 40%. The scaffolds could stay in high temperature (150 ºC), and 80% of the fabricated scaffolds could be soaked in a liquid for a long time (around 4 weeks) without changing their shape, such as cracking.

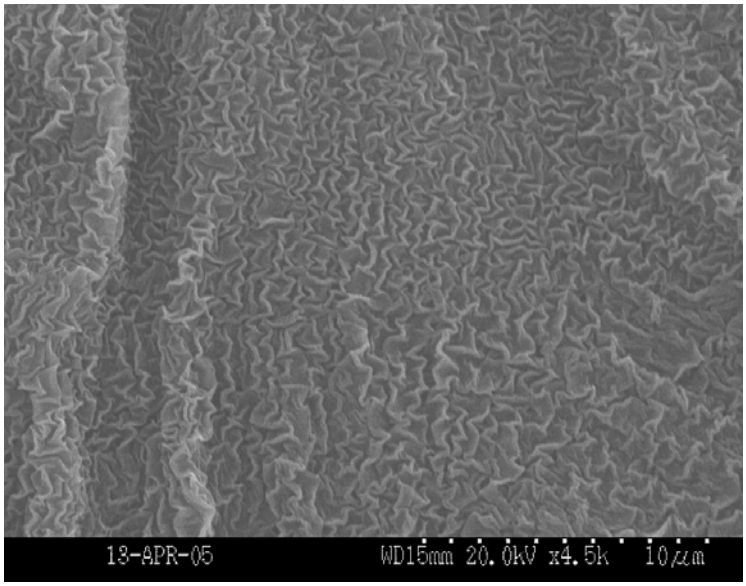

Figure 8. SEM image showing morphology of polysiloxane scaffolds before grinding.

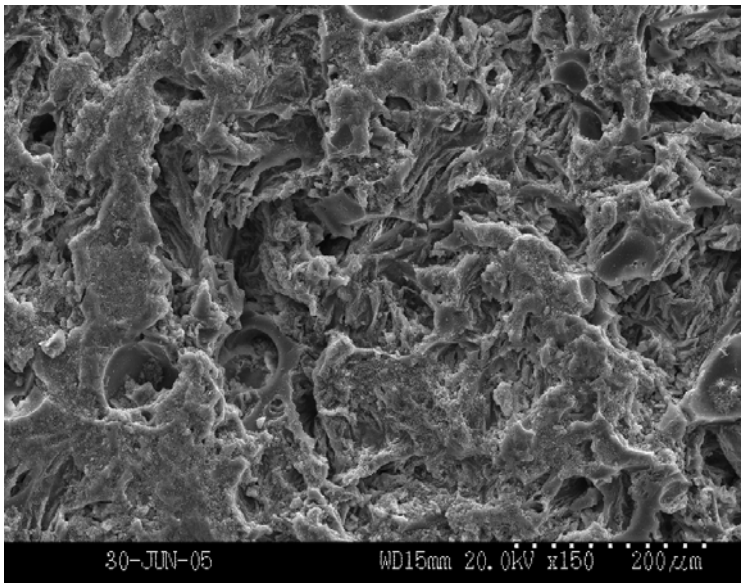

Figure 9. SEM image showing morphology of polysiloxane scaffolds after grinding.

4.1.2 Imprinted Polysiloxane Scaffolds.

Protein (lysozyme) was added to the scaffolds during the sol-gel process. Figure 10 shows protein embedded on the surface of a scaffold, and Figure 11 shows the surface after the embedded protein was exposed to the protease solution. Submicron-sized pores were revealed after digestion of surface-exposed protein with the enzyme. The porosity of imprinted scaffold was approximately 40%, similar to blank materials.

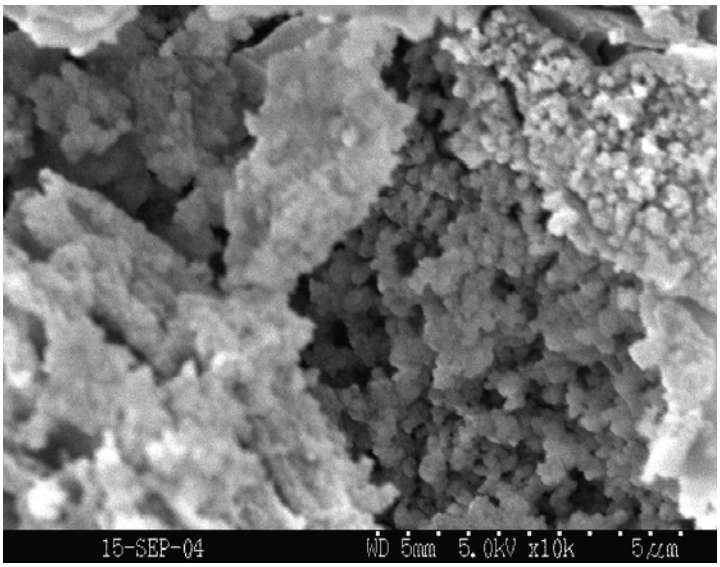

Figure 10. SEM image of protein (lysozyme)-imprinted scaffolds before exposure to protease.

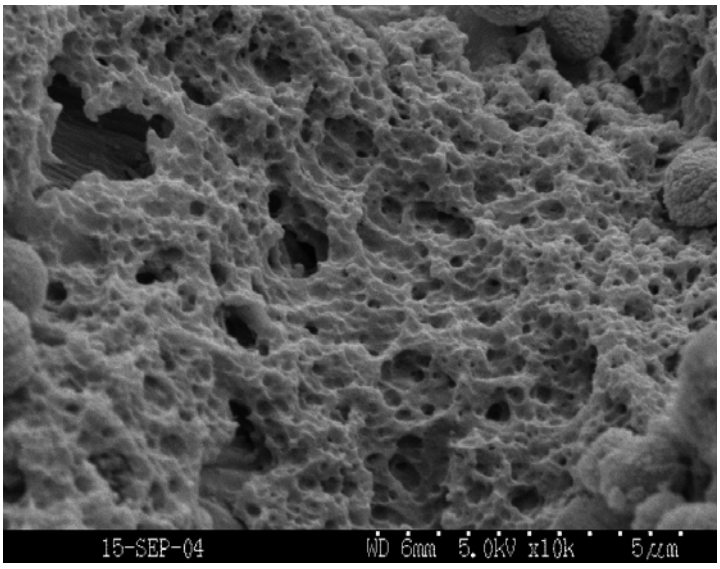

Figure 11. SEM image of protein (lysozyme)-imprinted scaffolds after digestion of protein.

4.2 Protein loading

4.2.1 Lysozyme-imprinted scaffolds

Protein was loaded with increasing amount of lysozyme (0.1, 0.4, 1, 3 and 6 mg) in order to determine the effect of loading on the surface of the scaffolds. Then the scaffolds were digested in protease solution for 3 hours and 24 hours. Figures 12 and 13 show that the amount of protein released into the protease solution depended on the amount of protein added to the mixture of solutions I and II. First we tried to release protein for only 3 hours due to the method obtained from [49], but then we found digestion was not complete at this time. Approximately 15% of lysozyme loaded to the sample was available on the surface after 3 hours. Therefore protein digestion was measured at both 3 and 24 hours. Figure 12 shows the amount of protein released after 3, and Figure 13 shows the amount released after 24 hours. The amount of protein exposed to the surface of scaffolds increased with the increasing the amount of protein loaded on the surface. There was no statistically significant difference between the releasing after 3 hours and 24 hours when less than 1 mg of lysozyme was added to the scaffolds. However, when more than 1 mg of lysozyme was loaded, the amount released after 24 hours was approximately five times greater than the amount after 3 hours ($p<0.001$). Most of the lysozyme (80%) that was added to the scaffolds was released from the materials after 24 hours.

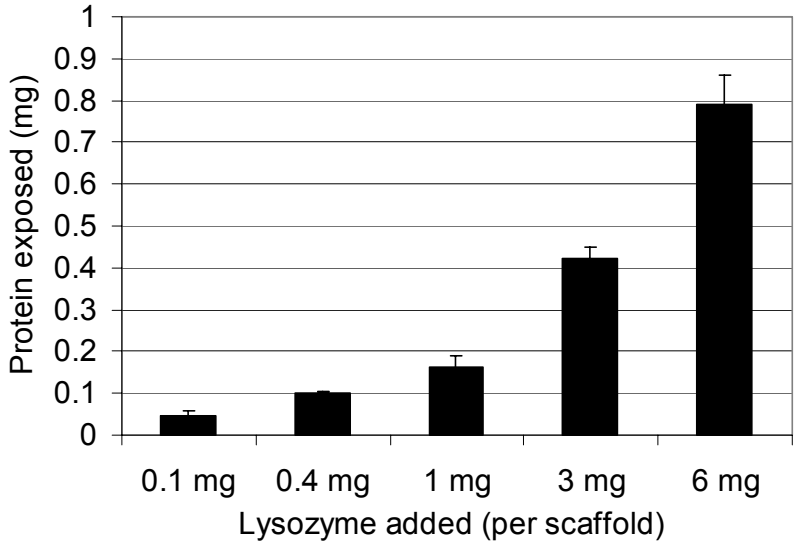

Figure 12. Amount of lysozyme released from the surface of scaffolds after 3 hours of digestion.

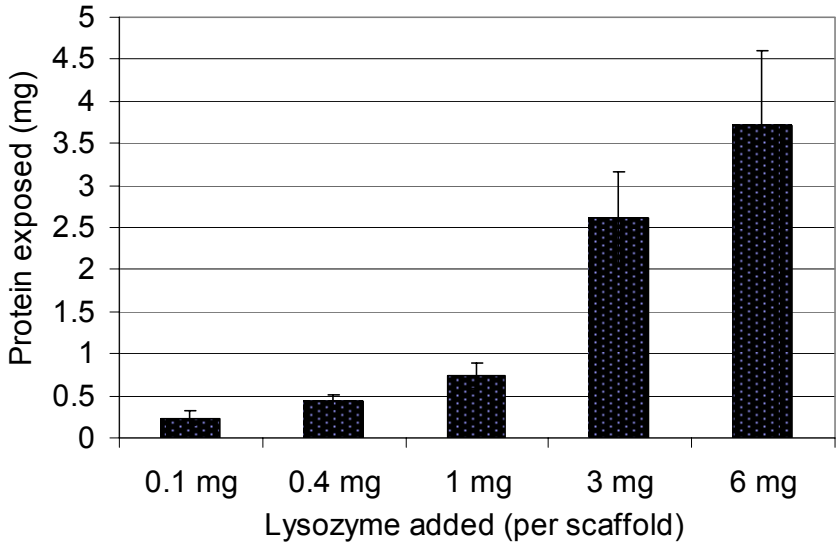

Figure 13. Amount of lysozyme released from the surface of scaffolds after 24 hours of digestion.

4.2.2 Comparison of protein releasing

Before the scaffolds were ground, they were covered with a glass-like surface. Thus the imprinted protein was not exposed well. The comparison of the amount of protein released before and after grinding of scaffolds is shown in Figure 14. Both sets of data show an increase in protein released according to the amount of protein loaded (after 3 hours of digestion). However, the amount of lysozyme exposed after grinding the

samples was over twenty times greater than that before grinding ($p>0.05$: 0.1 mg loading, $p<0.01$: 1 mg loading). Thus removing the glass-like surface helps expose potential protein recognition sites.

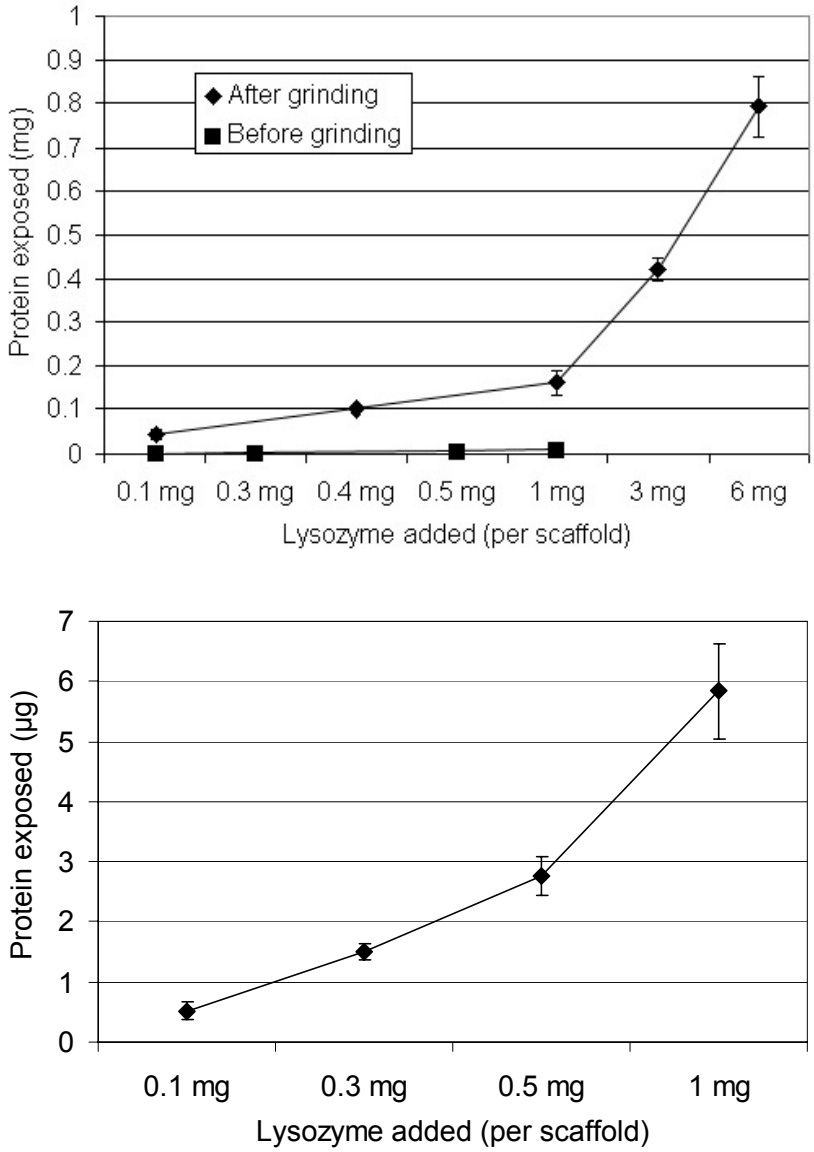

Figure 14. Comparison of the amount of protein exposed to the surface before and after removing the glass-like parts (top). The bottom graph magnifies the results before removing the glassy covering.

4.2.3 RNase A-imprinted scaffolds

RNase A was also imprinted into scaffolds. A mass of 0.05, 0.1 or 1 mg of RNase A was loaded. The protein exposed at the surface of the scaffolds was measured after 3

and 24 hours of digestion as well. Figure 15 shows that RNase A-imprinted scaffolds also released more protein when the amount of RNase A loaded increased. Approximately 3% of RNase A added to the scaffolds was available on the surface after 3 hours, and approximately 9% of loaded protein was accessible after 24 hours of digestion. Thus, the amount of RNase A released after 24 hours was approximately three times greater than the amount after 3 hours. If comparing the results of protein loading between lysozyme- and RNase A-imprinted scaffolds, the amount of lysozyme released was approximately six times more than that of RNase A after 3 hours. The amount was almost ten times more after 24 hours.

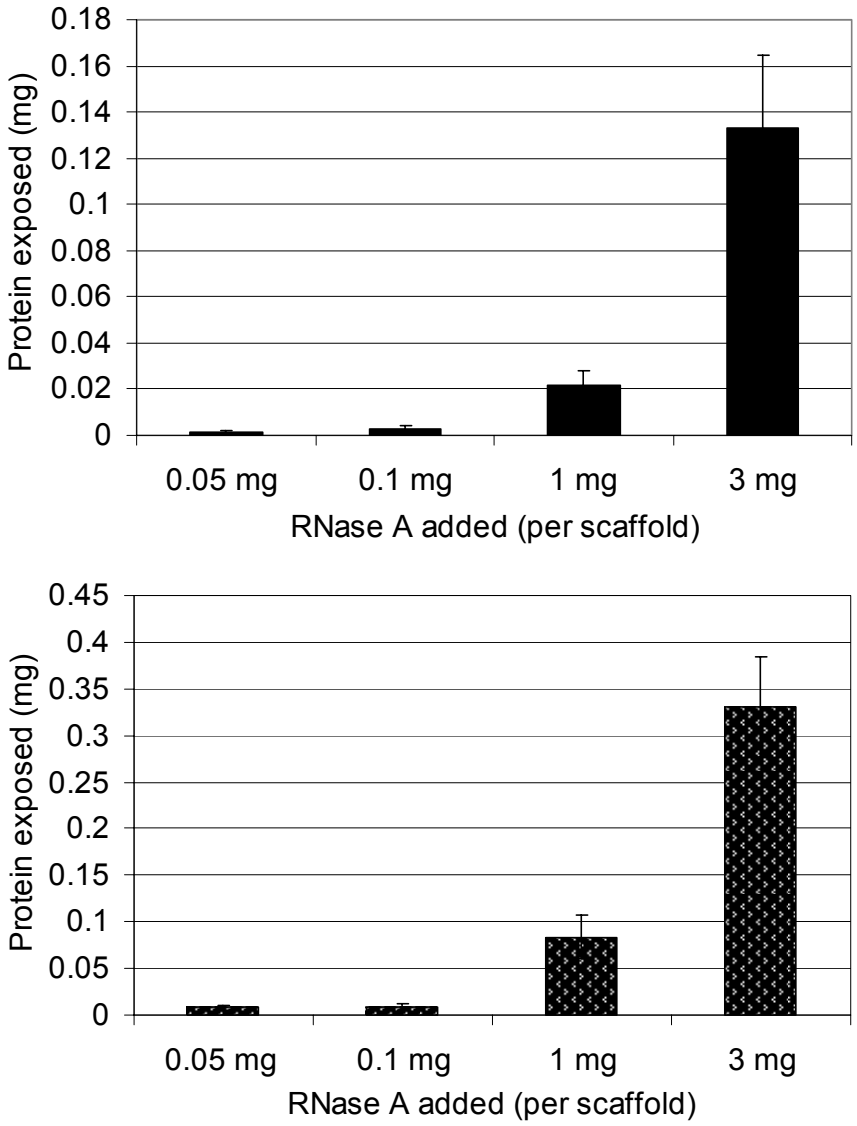

Figure 15. Amount of RNase A released from the surface after 3 hours (top) and after 24 hours of digestion (bottom).

4.3 Surface Area

The surface area of scaffolds was measured by nitrogen gas adsorption and calculated using the BET method. The results are shown in Figure 16. Seven types of samples (blank, 0.1, 1 and 6 mg lysozyme and RNase A loaded scaffolds) were tested. The average surface area of blank was 202.27 m^2/g, and surface areas of 0.1 mg of lysozyme and RNase A-imprinted scaffolds were 182.05 and 190.82 m^2/g. Average surface areas of 1 mg lysozyme- and RNase A-imprinted scaffolds were 129.3 and 174.07 m^2/g, and 6 mg of lysozyme- and RNase A-imprinted scaffolds were 186.06 and 239.76 m^2/g. However, there were no statistically significant differences ($p>0.05$). Table 1 shows the average amount of protein released from each type of scaffold that was measured its surface area. The amount of protein per unit area was calculated in Table 2. The amount of protein released from a unit area of 1 mg lysozyme-imprinted (0.055 mg/m^2) were almost six times greater than that of 0.1 mg lysozyme-imprinted (0.00885 mg/m^2). When compared the amount of protein release from a unit area between 1 mg and 0.1 mg RNase A-imprinted scaffolds, the former (0.003532 mg/m^2) was ten times greater than the latter (0.000319 mg/m^2).

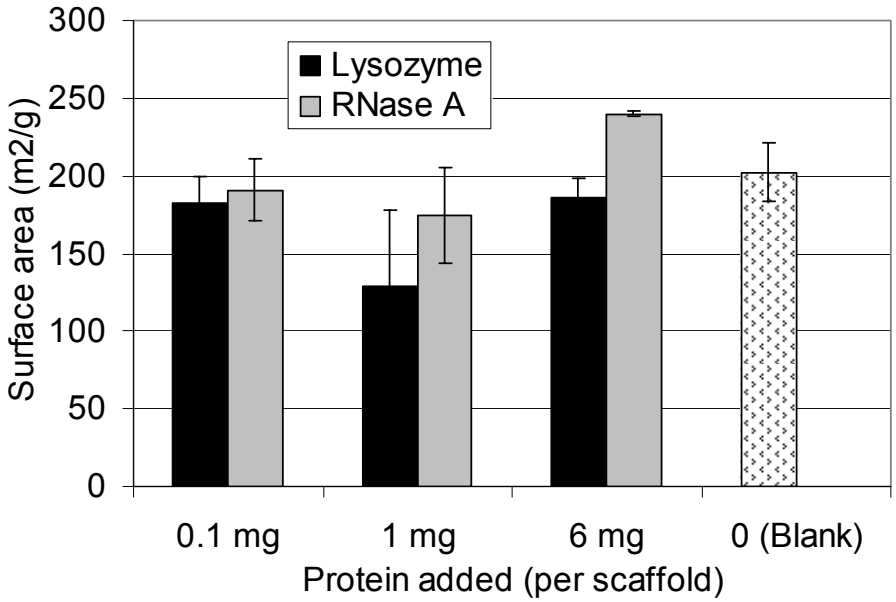

Figure 16. Surface area of blank and imprinted scaffolds.

Table 1. The amount of protein released from the surface of scaffolds.

Protein added	Imprinted scaffolds		Non-imprinted scaffolds
	Lysozyme (mg)	RNase A (mg)	Blank (mg)
0.1 mg	0.231536	0.008284	
1 mg	0.737036	0.083688	0
6 mg	3.715486	-	

Table 2. The amount of protein per unit surface area.

Protein added	Imprinted scaffolds		Non-imprinted scaffolds
	Lysozyme (mg/m^2)	RNase A (mg/m^2)	Blank (mg/m^2)
0.1 mg	0.00885	0.000319	
1 mg	0.055128	0.003532	0
6 mg	0.151969	-	

4.4 Porosity

As shown in Table 3, the porosity of each scaffold was approximately 40%, and the average pore size of blank was 6.19 μm, 0.05 mg RNase A-imrpinted scaffold was 5.56 μm, and 0.05 mg lysozyme-imprinted scaffold was 5.46 μm. There were no statistically significant differences of porosity, and differences of pore size between two samples ($p>0.05$).

Table 3. The porosity and pore size distribution of scaffolds.

Scaffolds	Blank (non-imprinted)	0.05 mg RNase A-imprinted	0.05 mg lysozyme-imprinted
Average pore size (μm)	6.19	5.56	5.46
Porosity (%)	43	42	41

4.5 Protein selectivity

In order to test the ability of the scaffolds to selectively bind their template molecules, scaffolds were imprinted with either lysozyme or RNase A and then exposed to solutions containing both proteins. These two protein molecules were similar in weight but different in chemical composition and shape.

4.5.1 Lysozyme-imprinted scaffolds

Protein selectivity was evaluated by measuring rebinding of both a particular protein and a competitor to the surface of imprinted scaffolds. According to the amount of protein digested from each sample (section 4.2), rebinding solutions were prepared. The scaffolds imprinted with 0.1, 1, or 3 mg lysozyme were exposed to solutions combining template (lysozyme) to competitor (RNase A) ratios of 1:0, 1:1, and 0:1. Figure 17 shows the result of protein selectivity test after 3 hours of digestion. When the rebinding solution includes only lysozyme, approximately 0.024 mg of lysozyme was rebound. Approximately 0.02 mg of RNase A was rebound when only competitor protein solution existed in the rebinding solution. The imprinted protein, lysozyme, was rebound 2.8 times greater than the competitor protein, RNase A when the template to competitor ratio was 1:1 in the rebinding solution ($p<0.01$).

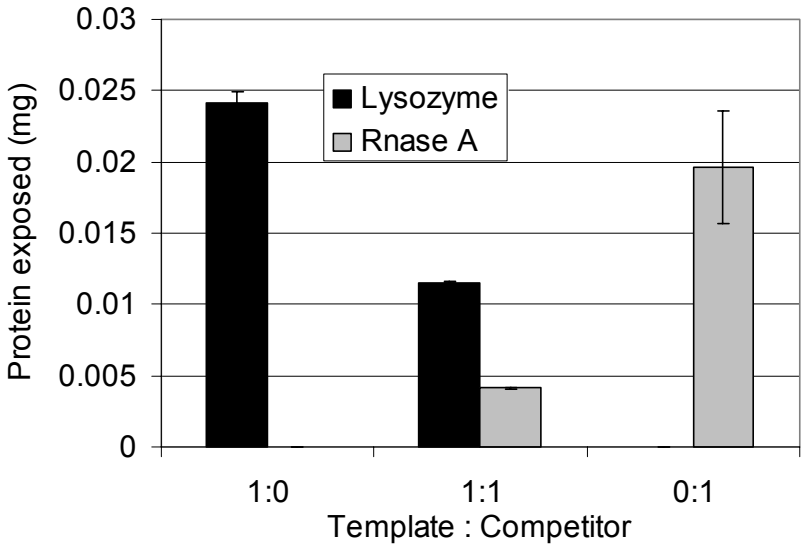

Figure 17. Competitive binding of protein to 0.1 mg lysozyme-imprinted scaffold after 3 hours of digestion.

The amount of lysozyme and RNase A rebound to the samples was also measured after 24 hours in Figure 18. The amount of lysozyme rebound to the surface of the scaffold was approximately 0.09 mg when the ratio of lysozyme and RNase A was 1:0 in the rebinding solution. When the rebinding solution included only RNase A, the amount of RNase A rebound was approximately 0.05 mg. Thus almost twice the amount of template protein was rebound to the surface ($p<0.05$). Lysozyme was rebound almost 2.5 times more than RNase A on the surface when the amount of template and competitor proteins was the same in the rebinding solution ($p<0.03$).

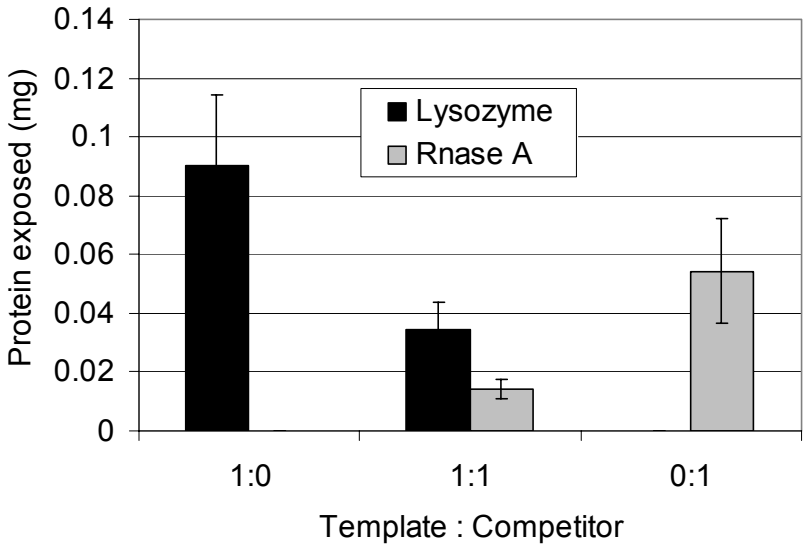

Figure 18. Competitive binding of protein to 0.1 mg lysozyme imprinted scaffolds after 24 hours of digestion.

Samples imprinted with 1 mg lysozyme were also tested for their selectivity for 3 hours of digestion (Figure 19). When only lysozyme existed in the rebinding solution, approximately 0.04 mg was rebound to the surface, and approximately 0.03 mg of protein was rebound when the rebinding solution included only RNase A ($p<0.001$). The amount of lysozyme rebound to surface was similar to the amount of RNase A rebound in the rebinding solution which included template (lysozyme) and competitor (RNase A) in the same ratio ($p>0.05$).

The result shown in Figure 20 after 24 hours demonstrates approximately 2.6 times more template molecules were rebound than competitor molecules when the ratio of rebinding solution was 1:1 at template to competitor ($p<0.03$). When there was only template protein or competitor protein in the rebinding solution, the amount of template protein rebound to the surface was three times greater than that of competitor protein rebound ($p<0.001$). Thus results show that the scaffolds bound more lysozyme (template) than RNase A (competitor) when both molecules were in the rebinding solution after 24 hours, and indicate the enhanced protein selectivity after 3 hours of digestion.

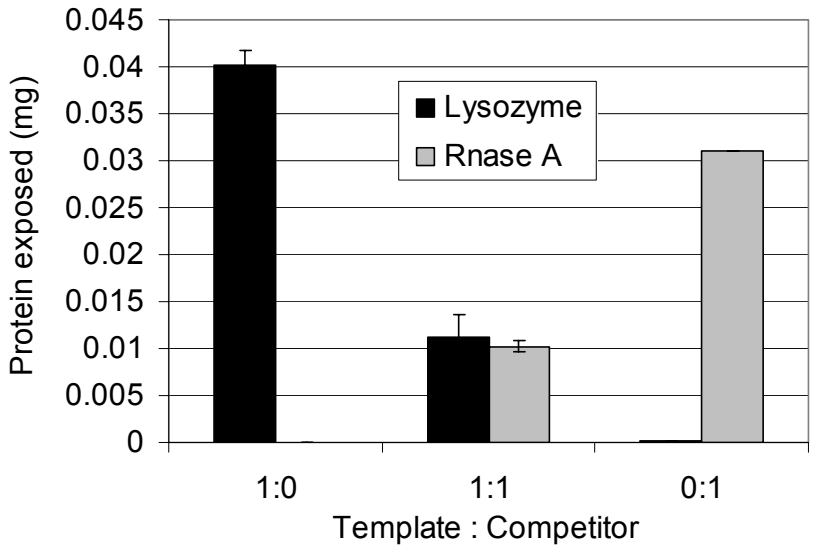

Figure 19. Competitive binding of protein to 1 mg lysozyme-imprinted scaffolds after 3 hours of digestion.

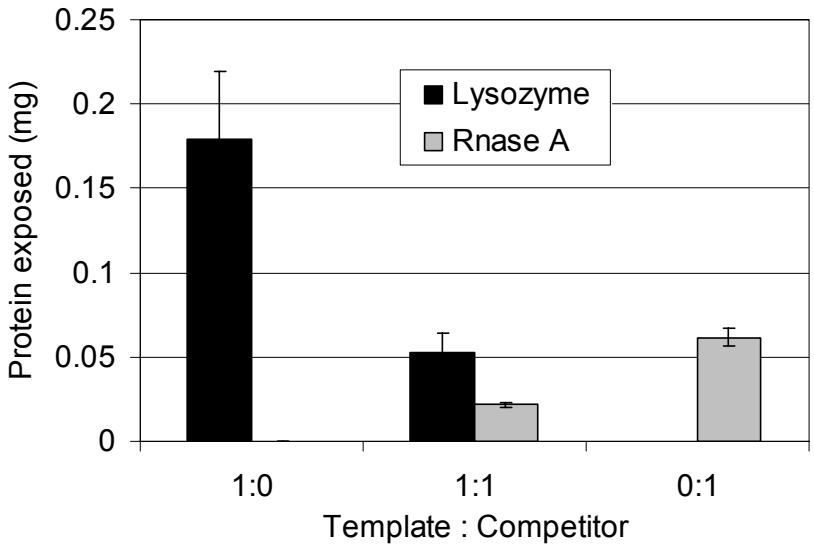

Figure 20. Competitive binding of protein to 1 mg lysozyme- imprinted scaffolds after 24 hours of digestion.

In order to test the selectivity of samples with high protein loading on the surface, samples imprinted with 3 mg of lysozyme were tested (Figure 21 and 22). In the 1:0 rebinding solution containing only template (lysozyme), the amount of template protein rebound was approximately 0.13 mg after 3 hours of digestion, but after 24 hours, the amount was approximately 0.7 mg, which was almost five times greater. When the ratio was 0:1 (RNase A only), the amount of competitor protein (0.21 mg) rebound to the surface after 24 hours was almost 2.5 times more than after 3 hours (0.085 mg) of digestion. At high protein loading the amount of protein bound to the surface was very different after 3 and 24 hours, and the difference was greater than that at low protein loading. The amount of lysozyme bound to the surface after 24 hours of digestion was almost three times more than RNase A when rebinding solution included both proteins with the same ratio 1:1 ($p<0.01$). High protein loading scaffolds show that the amount of competitor binding to the surface decreases. Thus non-specific binding relatively decreases slightly in high protein loading scaffolds.

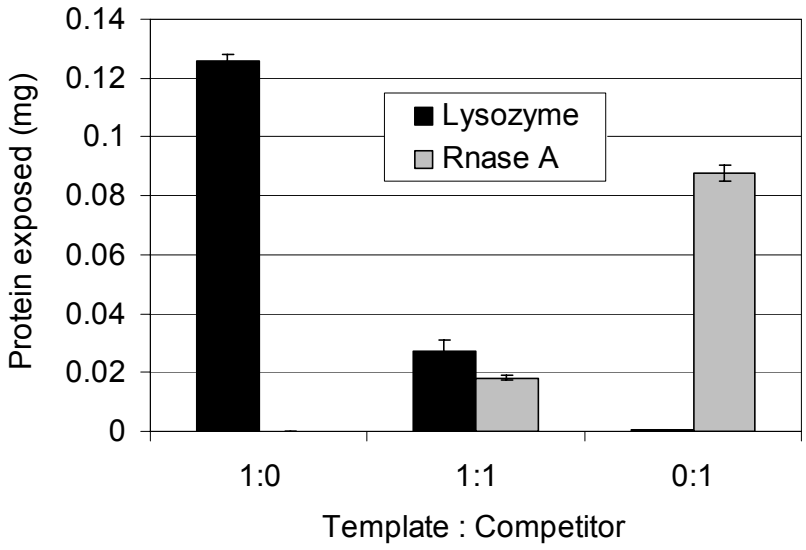

Figure 21. Competitive binding of protein to 3 mg lysozyme imprinted scaffolds after 3 hours of digestion.

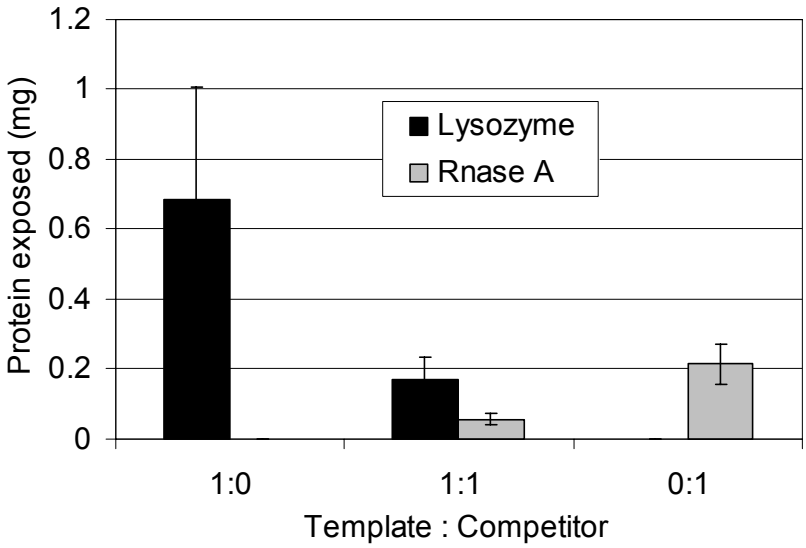

Figure 22. Competitive binding of protein to 3 mg lysozyme imprinted scaffolds after 24 hours of digestion.

4.5.2 RNase A-imprinted scaffolds

RNase A was used as a template protein, and lysozyme was used as a competitor protein. Scaffolds imprinted with 0.05 or 0.1 mg of RNase A were exposed to solutions containing template to competitor at the ratios of 1:0, 3:1, 1:1, 1:3 and 0:1. For low protein loading, scaffolds imprinted with 0.05mg RNase A were tested. There were no statistically significant differences after 3 hours of digestion (Figure 23). Thus protein selectivity of RNase A-imprinted scaffolds was analyzed with the results after 24 hours (Figure 24). At the ratio of 1:0, the amount of RNase A rebound was approximately 0.99 μg, and approximately 0.27 μg of lysozyme was rebound at the ratio of 0:1. Over three times more template molecules were bound than competitor molecules ($p<0.001$). Comparing the amount of protein digested in the rebinding solution combining template protein to competitor protein at the ratios of 3:1 and 1:3, twice more template protein than competitor protein was rebound ($p<0.001$). Almost no lysozyme was detected when the ratio was 3:1, and similarly no RNase A was shown at the ratio of 1:3 after 3 and 24 hours of digestion due to their little rebinding. As shown in section 4.2 at low loading, there was no significant difference between the amount of protein bound after 3 and 24 hours of digestion. The difference between the amount of template and competitor rebound was similar at the ratio of 1:1 ($p>0.05$).

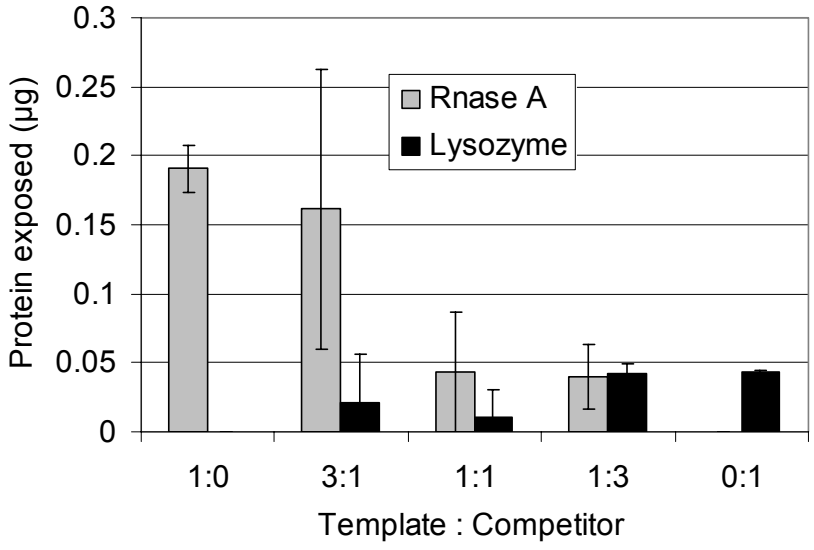

Figure 23. Competitive binding of protein to 0.05mg RNase A imprinted scaffolds after 3hours of digestion.

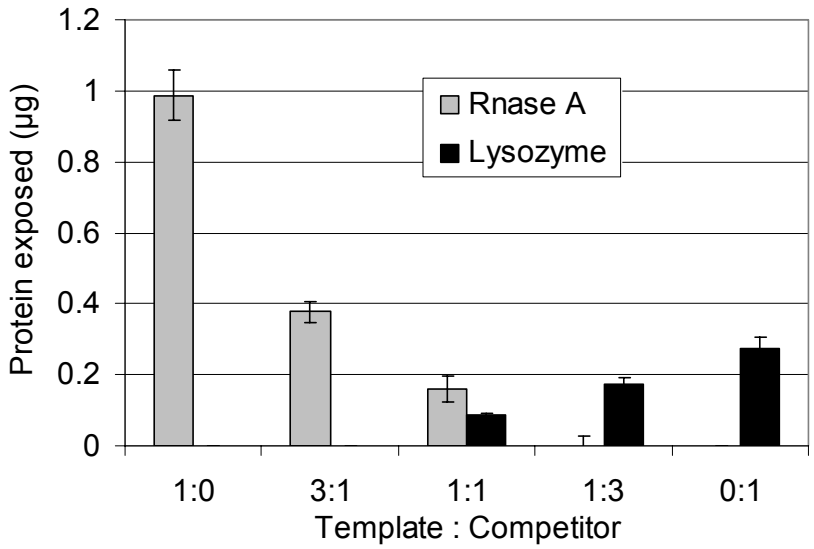

Figure 24. Competitive binding of protein to 0.05mg RNase A imprinted scaffolds after 24 hours of digestion.

Scaffolds imprinted with 0.1 mg of RNase A showed more significant protein selectivity results than that with 0.05 mg of RNase A after 3 hours (Figure 25). In Figure 26, the amount of RNase A rebound was approximately 1.8 μg at the ratio of 1:0, and approximately 0.9 μg of lysozyme was rebound at the ratio of 0:1 ($p<0.001$). At the ratio of 3:1, the amount of RNase A rebound was approximately 1.2 μg, and the lysozyme rebound to the surface was approximately 0.5 μg at the ratios of 1:3 ($p<0.001$). Then rebinding solution contained both proteins with the same ratio 1:1, the amount of RNase A rebound was almost 0.6 μg and lysozyme rebound was approximately 0.35 μg ($p<0.02$). Therefore, the amount of template protein (RNase A) rebound to the surface was almost twice greater than did competitor protein (lysozyme). Most of the protein selectivity results demonstrate that the protein-imprinted scaffolds had the ability to selectively bind their template molecules.

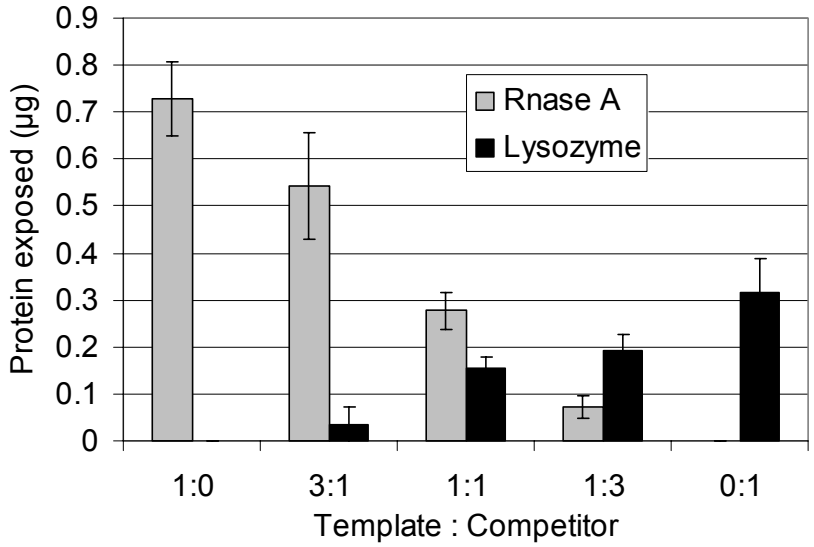

Figure 25. Competitive binding of protein to 0.1 mg RNase A-imprinted scaffolds after 3 hours of digestion.

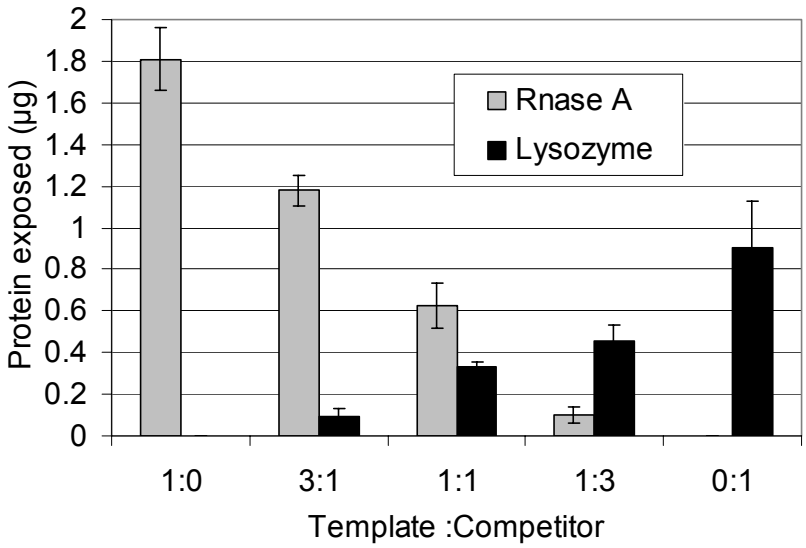

Figure 26. Competitive binding of protein to 0.1 mg RNase A-imprinted scaffolds after 24 hours of digestion.

4.6 Cytocompatibility

4.6.1 SaOS-2 cells

SaOS-2 osteoblastic cells were seeded onto blank and lysozyme-imprinted scaffolds. After the culture period of 1, 3 and 7 days, the cytocompatibility of the polysiloxane scaffolds was determined. The results in Figure 27 indicate that SaOS-2 cells proliferated on both blank and lysozyme-imprinted polysiloxane scaffolds ($p<0.001$). There were no statistically significant differences between the blank and imprinted scaffolds. Then, cell cultures were assayed for synthesis of alkaline phosphatase. Figure 28 presents the result of AP assay for blank and lysozyme-imprinted materials. There is possible decrease of AP activities on imprinted scaffolds at 7 days, but no statistically significant differences could be shown on the cells cultured on blank and imprinted materials ($p>0.05$).

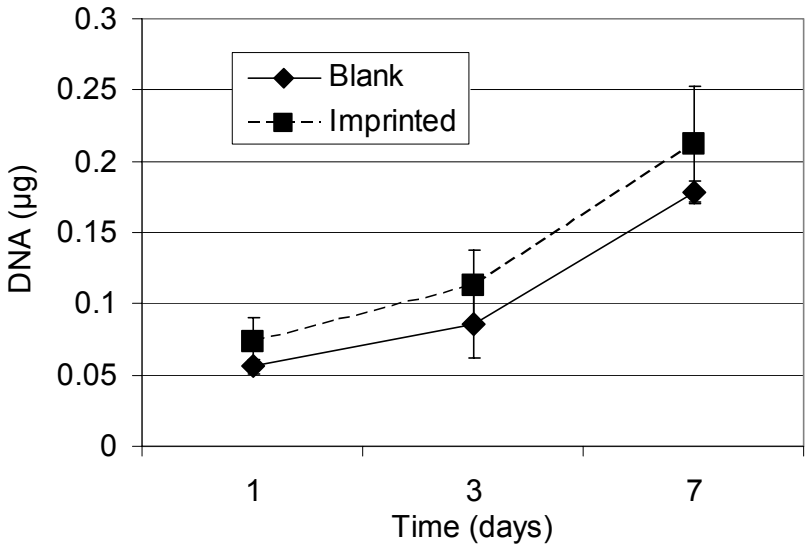

Figure 27. DNA contents for SaOS-2 cells cultured on scaffolds.

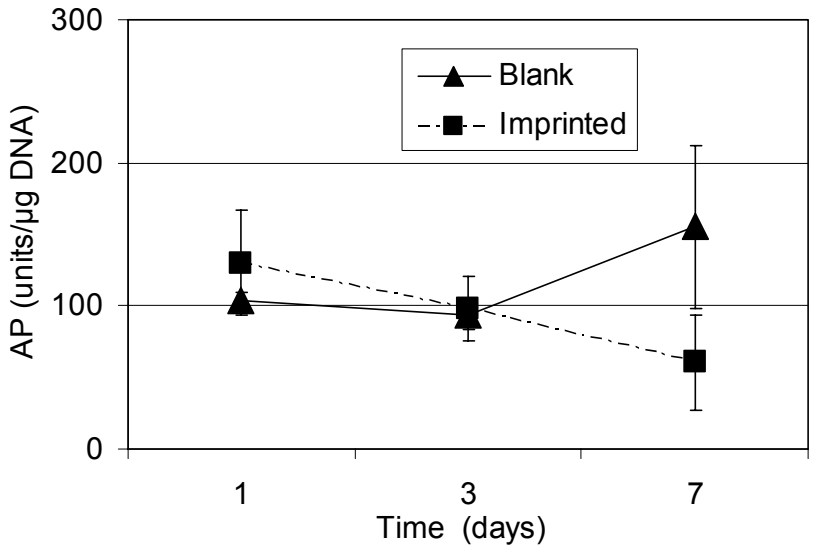

Figure 28. Alkaline phosphatase activity for SaOS-2 cells cultured on scaffolds.

4.6.2 Scanning Electron Microscopy

Morphology of a SaOS-2 cell cultured on the blank scaffold for 1 day is shown in Figure 29. The image of the cell surface is characterized by scanning electron microscopy. The cell was flattened and spread widely on the surface of a scaffold. The cell is well attached to the surface, and the edge of the cells is occupied with lamellipodia and filopodia.

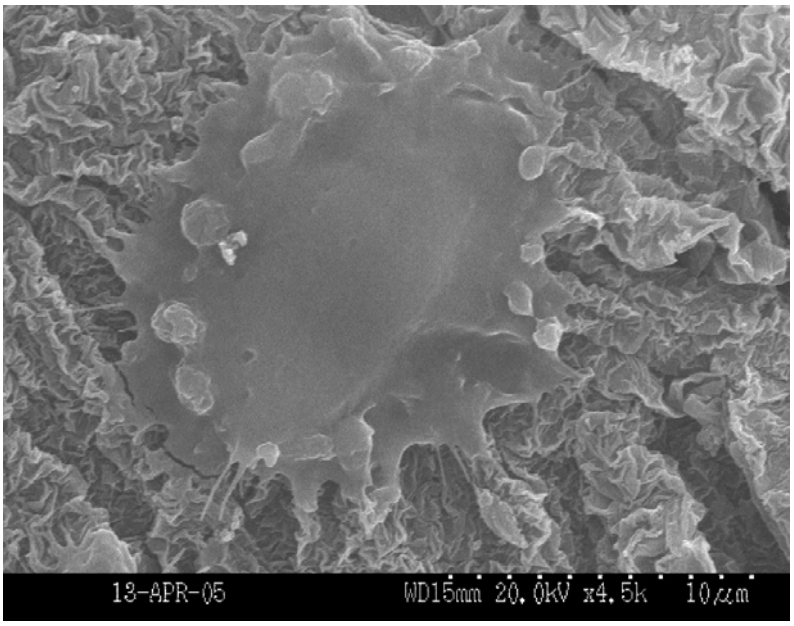

Figure 29. SEM image of a SaOS-2 cell on a blank scaffold after 1 day.

Figure 30 shows a cell that was seeded on a scaffold and cultured for 7 days. If cells migrate, the leading edge has moved forward and spread. Then the lamellipodia at the edge form a thin flat sheet and separated into two lamellae [50]. The shape of the cell in the image indicates the growth and migration of the cell. Thus the cell looks retracting at the right lamella and extending at the left edge of lamellipodia. It has been growing and moving to the left direction in the SEM image.

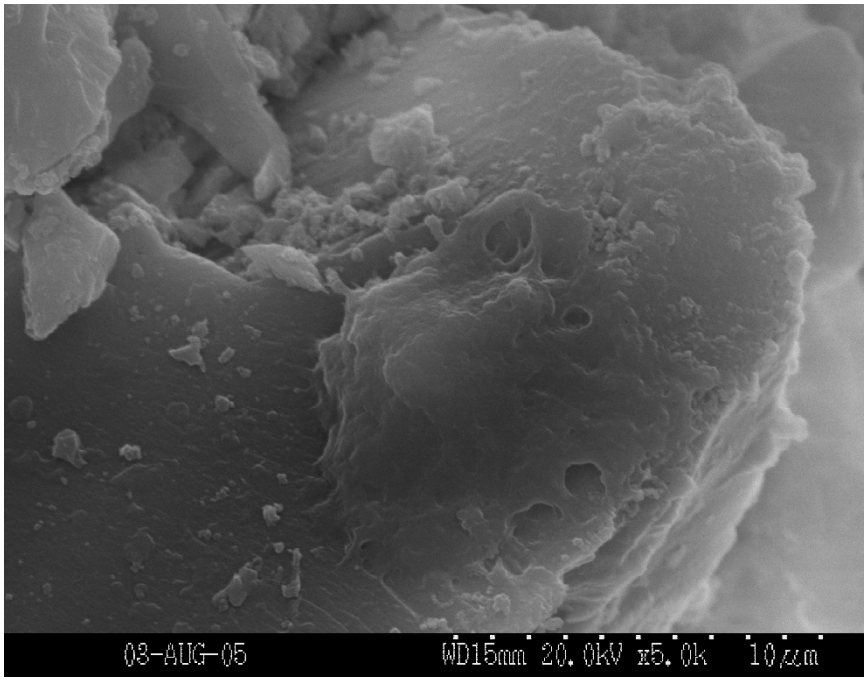

Figure 30. SEM image of SaOS-2 cells on the scaffolds after 7 days of culture.

4.6.3 C3H cells

Scaffolds, imprinted with 0.1 mg of RNase A, that showed gradual proliferation with SaOS-2 cells were subsequently tested using C3H cells. The cells were grown on the well-plate and RNase A-imprinted scaffolds up to 7 days. Because we did not see the statistically significant differences between blank and imprinted scaffolds (Figure 27 and 28), only one type of samples was chosen for the C3H experiment. The results of DNA assay show the growth of C3H cells (Figure 31). Statistical analysis of DNA content of C3H cells showed significant differences between control well plates and cells seeded on the scaffold each period (1-day: $p<0.05$, 3 and 7-day: $p<0.001$). C3H cell contents in the control wells increased significantly from 1 day through 7 days ($p<0.001$). Though cell

contents with the scaffolds revealed no statistically significant between 1 and 3 days, the cell contents significantly increased from 3 days to 7 days ($p<0.01$). The growth rate of C3H cells was generally slower than SaOS-2 cells during routine culture. Figure 32 indicates the result of AP assay for C3H cell activity. Even though the result of DNA assay shows more cells were grown on the well-plate than cells cultured on the scaffolds, the AP activity of the cells was not higher when cells were cultured on the plates ($p>0.5$).

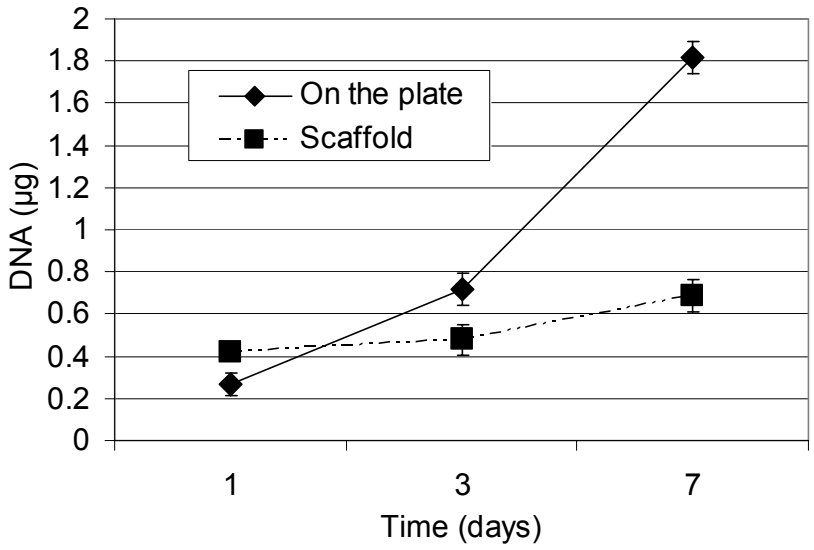

Figure 31. DNA contents for C3H cells cultured on the well plate and scaffolds.

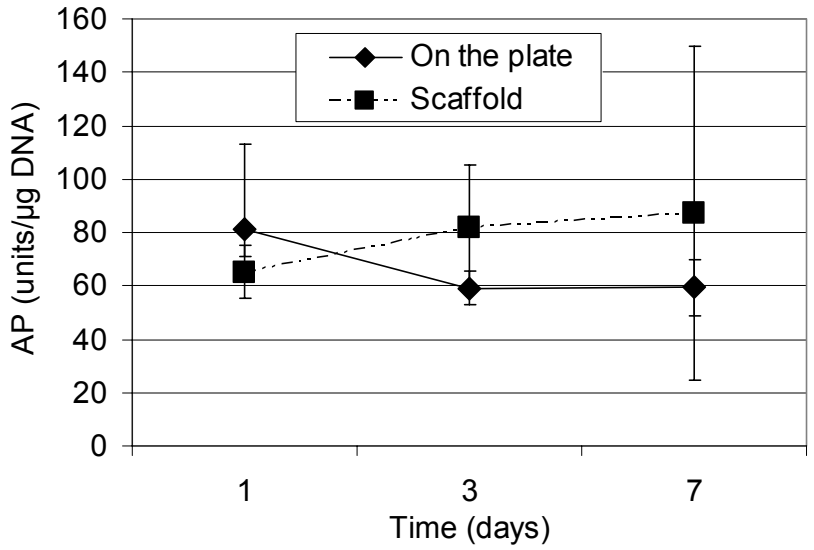

Figure 32. Alkaline phosphatase activity for C3H cells cultured on the well plate and scaffolds.

CHAPTER 5. DISCUSSION

Three dimensional polysiloxane scaffolds possessing micro and macropores have been designed to demonstrate their ability of protein-selectivity, and have been developed to be cytocompatible.

5.1 Fabrication of scaffolds

5.1.1 Polysiloxane scaffolds

Molecularly imprinted polymers have been developed in several ways. Acrylic polymers were produced often using methacrylic acid and polyacrylamide as functional monomers [26, 51, 52], and thin-film polymers were designed using phosphorylcholine by a micro-contact approach [53]. Acrylic polymers have potential advantages of capacity, stability and cost effectiveness, but they lack sufficient thermostability and mechanical strength [51]. Polysiloxane (silica; SiO_2) materials have been developed in this study using a sol-gel process. The silica materials have been used to encapsulate biomolecules such as cytochrome c, myoglobin and hemoglobin in transparent porous silica glass matrices [54-56]. The entrapped biomolecules in a matrix would detect and react with reactant molecules by diffusion. Thus the materials can be useful for biosensors, chromatography, and immunoadsorbent [54, 57]. In order to deposit template protein into the polysiloxane materials for the first step of molecular imprinting, similar method to encapsulation technique was used. On the other hand, the template would be removed to imprint biomolecules to the polysiloxane materials and leave the recognition sites that only template molecule can bind by sol-gel processing. This process was used because the materials could be prepared easily by adding solutions to a silane base in a liquid phase. TEOS can be hydrolyzed and polycondensed into silica gel easily, and has been applied for this study [58]. APS was used as functional monomer with TEOS to imprint large biomolecules [59].

The one-step sol-gel process was performed in the beginning of this study with a TEOS to APS volume ratio of 3:1. Though Venton et al. fabricated polysiloxane polymer with the same ratio of TEOS to APS [59], the bulk type scaffolds used for present study

could not retain their structural integrity in a simulated biological environment, being a cell culture medium at 37 ºC. Moreover pH of the materials was too alkaline (ca. pH 11). Thus, the two-step sol-gel process was applied next, and the TEOS to APS scaffold volume ratio for the two-step process was determined to be 4 to 1. Approximately 80% of three dimensional scaffolds could retain their structure in a simulated biological environment during the whole experiments for a month, and the scaffolds need to be tested more than a month in the future work. The pH was still alkaline (ca. pH 9.5), but it could be controlled by changing external condition (described in section 3.6.1).

The properties of the scaffolds widely varied with gelling conditions. Huang et al. tested the effect of HCl on the gelation time in a two-step sol-gel process. A larger amount of HCl and higher base concentration lead to faster gelation [60]. In the first step, HCl has an effect on the property of the scaffolds in this study as well. The scaffolds become transparent glass-like materials if the pH of the solution is very low (ca. pH < 2). In the second step, when solution II was prepared in the mold vial, the time at which the reaction sat at room temperature determined the properties of the scaffolds as well. APS, used as a functional monomer, is very sensitive and it makes the scaffold color change (becomes white) when the APS in a liquid phase stays in a vial over 15 minutes. It seemed that the H_2O included in 0.1M SDS caused condensation of APS by hydrolysis. Thus the time during which the APS is exposed to the SDS plays an important role in changing the properties, especially the turbidity of the mixtures of solutions I and II. When the acidity of solution in the first step is high and the time at which solution II sits at room temperature is short, the scaffolds also become glass-like materials. If the scaffolds are immersed into a liquid, they fracture apart. The glass-like scaffolds are very hard materials, but liquid seeps through the microcracks in the glass-like scaffolds, making them break down and disintegrate into very small particles. Therefore, the polysiloxane scaffolds that were able to be imprinted with biomolecules were fabricated with 9.1% v/v of 0.1M HCl in the first step. The time when APS exposed to the SDS was fixed at 30 minutes before mixture of solution I and II.

The fabrication of scaffolds using sol-gel processing went through aging and drying steps after gelation. When the scaffolds were aged and dried from the evaporation

of the solvent phase, the vapor from evaporation created the glass-like surface around the scaffolds. The glass-like surface which covers the pores was then removed via grinding.

5.1.2 Imprinted Polysiloxane Scaffolds

Molecularly imprinted polysiloxane materials were prepared. As a long-term goal, scaffolds imprinted with a growth factor receptor may be expected to lead to biospecific and desired cellular responses. Molecular imprinting technique has been investigated for chromatography, which is a method to separate or analyze complex mixtures [30]. The technique has not been used to assess the ability of imprinted surfaces to control cell behavior. Some research applied molecular imprinting on antigen-antibody interaction with making synthetic antibodies. Due to the complication of antigen-antibodies interactions, no research could achieve similar capabilities to natural antibodies. There is, however, a high potential of molecular imprinting technique on artificial antibodies [61].

Some approaches used BSA, urease, cytochrome c, 2-aminopyridine and lysozyme as template molecules [25, 30, 59, 62]. In this study, lysozyme was applied as a model template biomolecule before actually using a growth factor due to its size and reasonable price. In addition, the molecular weight (14.3 kD), shape and structure (PDB code: 1AZF) of lysozyme have been also widely known [63]. RNase A (PDB code: 1AFU) was chosen as another model template biomolecule due to its comparable molecular weight (13.7 kD) with lysozyme. The binding sites would have identical geometry with a template molecule. Thus particular molecule which has similar shape to the binding sites would be recognized. In this case, the sizes of template and competitor should be similar. Then the different molecules could be compared by not their sizes but their geometries. When protein solution was added to solution II to fabricate molecularly imprinted polysiloxane scaffolds, the amount of protein could be varied from 0 to 10 mg per sample. However, the amount of protein solution affected the properties of scaffolds. The interaction between protein solution and silane mixture would affect condensation of polysiloxane. Therefore, the volume of protein solution added in the second step should be less than 10% of total volume per scaffold. When the amount of protein solution was higher, gelation time of the mixture got longer and the mixture did not gel properly. Thus

some gel included mixing of white and transparent parts, and some broken gel was observed. When such kinds of gels were observed, the gel did not dry completely after 48 hours in an oven. The strength of scaffolds was influenced by the amount of protein solutions, so the structure was smashed easily by gently pressing.

The properties of polysiloxane materials are affected by drying conditions. The pores could be collapsed and the connected network between functional monomer and crosslinker might be broken in high drying temperature [56]. Pooart et al. investigated the thermal stability of lytic activity of rhea and ostrich lysozyme. The enzymes had the thermal stability up to 40 ºC, and both lysozyme had the optimum temperature at 30~40 ºC. The group showed 20% of remaining activity after treatment at high temperature (80 ºC) [64]. Therefore, the drying temperature has been fixed at 40 ºC.

5.2 Protein loading

The amount of protein was easily altered by adding different concentration of protein solution. Addition of protein with higher loading on the scaffolds resulted in multilayers or piles of embedded protein. Lysozyme-imprinted scaffolds showed submicron-sized pores which were imprinted nanocavities on the surface after digestion of surface-exposed protein to the enzyme solution (Figure 6). In order to expose maximum number of imprinted binding sites, the scaffolds were digested with the protease solution rather than simply washed with water. If the template protein could be washed with water, it indicates that the binding strength between protein and the material is weak, and shows low protein retention on the surface [62]. Other researches also used strong washing solutions, but different methods to remove template using acetone, acetic acid [22], acetonitrile [29] and ammonia [30]. The nanocavities may indicate the imprinted binding sites on the surface in which the template protein can rebind. Thus the quantity of protein available on the surface of scaffolds is important. Protein loading experiments were executed to determine the amount of protein at the surface. However, the amount of lysozyme released from scaffolds was approximately 80% of the amount loaded after 24 hours of digestion. The amount of protein exposed to the surface and digestion increased with protein loading. RNase A-imprinted scaffolds were also

designed to measure the amount of protein at the surface. The results with RNase A used as a model template molecule were compared to the result with lysozyme as a template molecule. The amount of RNase A exposed to the surface was approximately 10% of the amount loaded after 24 hours of digestion. Thus, compared with lysozyme imprinted scaffolds, more protein was entrapped in the polysiloxane scaffolds. The released amount of protein from RNase A-imprinted scaffolds was increased with the amount of loading. We can expect that with greater protein loading, the more potential binding sites will be present on the surface of scaffolds. This would be discussed more in protein selectivity section.

The result in section 4.2 shows the difference between scaffolds before and after grinding. The amount of protein exposed to the enzyme solution was greater on the scaffolds from which the glass-like surface was removed. The glass-like layer that covered the surface of samples inhibited the protein release from the scaffolds. Thus the property of the surface can be one of the significant factors to determine the amount of binding sites on the scaffolds.

5.3 Surface Area and porosity

Each scaffold may have different pores and pore area when it is fabricated. In order to obtain the average surface area of samples, nitrogen adsorption was applied to seven different kinds of samples. The results were compared with surface areas of blank and imprinted scaffolds, scaffolds with the three different protein amount, and lysozyme and RNase A imprinted scaffolds. All comparisons did not show any significant differences among the seven types of samples. Generally, macro pores (μm) existing on scaffolds are for cell and tissue ingrowth, and small pores (nm) are for cell adhesion and protein binding [65]. Thus, pore area, pore diameter and porosity of scaffolds were examined by porosimetry. Three different types of scaffolds, blank, 0.05 mg RNase A- and lysozyme-imprinted samples, were tested. There was no statistically significant difference in all results. The nanocavities revealed by imprinting with 0.05 mg of lysozyme and RNase A on surfaces may not affect total amount of both surface area and pore area resulting from nano-, micro-, macro-pores. Therefore, several different types of

samples should be tested in the future to check whether the amount of protein imprinted and types of protein give effects on porosity.

5.4 Protein Selectivity

Two proteins, lysozyme and RNase A, were labeled and imprinted into the scaffolds for selectivity tests. These proteins were used to prepare rebinding solutions which included either template protein or competitor protein, or both template and competitor proteins. Those proteins were labeled with three different dye compounds in order to distinguish between loaded and reloaded proteins.

Lysozyme or RNase A-imprinted scaffolds were tested in the rebinding solutions which included several ratios of template protein to competitor protein as shown in table 4. The template protein (lysozyme) rebound to the 0.1mg lysozyme-imprinted surface was two times greater than competitor protein (RNase A) after 24 hours. When RNase A was used as template protein, the template protein rebound to 0.05 and 0.1mg RNase A imprinted surface was also two times more than competitor protein (lysozyme). The template protein (lysozyme) rebound to the 1 and 3mg lysozyme-imprinted surface was three times greater than competitor protein (RNase A) after 24 hours. Overall the results showed that the template protein was preferentially bound to the surface of scaffolds when compared with the amount of rebound in the presence of a similarly sized competitor protein. Even though nonspecific protein adsorption, which was lysozyme bound to RNase A-imprinted materials and vice versa, was observed, still almost twice as much of the template was rebinding to the surface. Therefore, the polysiloxane materials with sol-gel processing show the protein selectivity.

When protein was highly loaded on the surface, the embedded protein was not equally distributed and showed multilayers or piles of the protein as mentioned earlier. The selectivity results, however, showed more binding of template protein than competitor. It may indicate there are more binding sites for specific template protein on the surface. In addition, more template protein was bound to the surface in high loading (three times greater) than low loading (two times greater). It shows a little more apparent protein selectivity on the high loading surfaces than low loading surfaces.

Hunnius et al. showed that imprinted sites in silica matrix change selectivity based on preparation procedures such as chemical composition. The group also described that the selective adsorption of organic molecules was not related to imprint effects and was predicted to be dependent on surface polarity of the porous materials [23]. Gore et al. developed molecular imprinting polymer using methacrylic acid as monomer, ethylene glycol dimethacryate as crosslinker and cholesterol as a template. The group showed non-specific binding onto the materials could be reduced by changing the hydrophobic crosslinker to hydrophilic glyceroldimethacrylate. Thus, the polymers showed very good selectivity for cholesterol as compared to other steroids [66]. Cummins et al. compared the selectivity between acrylic and sol-gel polymers in which both imprinted with 2-aminopyridine. The group used three different solutions, chloroform, acetonitrile and methanol, for rebinding study. Though there was high affinity of non-specific binding, sol-gel based polymers rebound more templates than did acrylic polymers, and also showed the potential ability of higher selectivity of sol-gel materials [22].

Shi et al. investigated the selectivity in protein recognition with imprinting lysozyme and RNase A that were the same proteins as our study. The group defined the protein ratio that decreased imprinted protein adsorption to 50% of its non-competing value as R50, and estimated of the relative affinity of the pair of protein containing lysozyme and RNase A. A larger R50 means a higher affinity of the imprinted protein for the surface. The lysozyme imprinted exhibited a 26-fold enhanced selectivity for lysozyme over the RNase A imprinted, and RNase A imprint was increased by 20-fold of R50 when adsorbing RNase A versus lysozyme [7, 62].

Table 4. The ratios of rebound templates to competitor for each imprinted scaffold.

Lysozyme-imprinted scaffolds	Template / Competitor (lysozyme) / (RNase A)		RNase A-imprinted scaffolds	Template / Competitor (RNase A) / (lysozyme)		
	1:0 / 0:1	1:1		1:0 / 0:1	3:1 / 1:3	1:1
0.1 mg	1.66	2.4	0.05 mg	3.6	2.15	1.87
1 mg	2.9	2.44	0.1 mg	2	2.57	1.86
3 mg	3.18	3.01				

5.5 Cytocompatibility

Molecularly imprinted surfaces of biomaterials may offer potential for controlling events at the tissue-implant interface. Cells are influenced by not only physical surface such as structures of scaffolds but also chemical characteristics of the scaffolds. Therefore if cell surface molecules are used as template molecules for the surface of scaffolds, the biomaterials could bind a specific cell type. This binding of cell surface molecules may lead to specific intracellular signaling events as well. In order to support all above hypotheses, cell culture experiments were used to demonstrate cytocompatibility of basic polysiloxane scaffolds by checking cell proliferation, growth, and morphology of the cells.

Bildirici et al. observed high levels of detachment of beads from cells occur when silica beads were used [67]. However, Dieudonné et al. had experiments with osteogenic precursor cells from rat bone marrow on pure titanium discs, silica gel bioactive glass discs and titania coated titanium by sol-gel technique. Initial growth up to day 3 was higher in pure titanium compared to sol-gel derived titania and silica gel. Cell growth rates were higher on the silica gel although initial cell adhesion was lower than the titanium substrates [68].

DNA and alkaline phosphatase activity were measured in order to estimate the growth and activity of cells. First, SaOS-2 osteoblastic cells were used to assess the cytocompatibility of both blank and imprinted scaffolds. SaOS-2 is an isolated and characterized human osteosarcoma cell line. Hence, the cell line could be good for initial cytocompatibility research because the growth rate is faster and expected viability is higher than other cells. The cells grew similarly on both materials for 7 days, and morphology of the cells using SEM showed the fine attachment of cells to the surface of scaffolds. AP activities of the cells did not show significant differences either.

Next, C3H cells were used for the cytocompatibility test for eventual use with bone morphogenetic protein (BMP) receptor. The cells were seeded on the well plate and 0.1 mg RNase A-imprinted scaffolds. Though more than twice of the cells existed on the well plate, cells were growing gradually on the scaffolds for 7 days, and AP activity of C3H cells did not show the significant differences between seeded cells on the plate and on the scaffolds.

CHAPTER 6. CONCLUSIONS

Molecularly imprinted polysiloxane scaffolds possessing micro and macropores were fabricated by sol-gel processing. The ratio of TEOS:APS was determined at 4:1 by observations of the strength of the scaffolds in biological environment. Thus the ratio was used for further investigations of the study. In order to enable cell ingrowth and tissue regeneration, the scaffolds needed to be macroporous as tissue engineering biomaterials. The foaming agent was applied to develop macroporous scaffolds. When protein-imprinted scaffolds were fabricated, the interaction between biomolecules and the polysiloxane chains produced an effect on the morphology of scaffolds surface.

After loading various amounts of protein into the scaffolds, the measured amount of protein accessible on the surface was approximately 80% of lysozyme loading and 10% of RNase A loading. The amount of protein exposed to the surface and susceptible to proteolytic digestion increased with each protein loading. Lysozyme and RNase A as either templates or competitor molecules were mixed in rebinding solutions due to their comparable size but differing chemistry. Even though nonspecific binding was observed at high ratios of competitor, the template molecules were preferentially bound to the surface of scaffolds in the rebinding solutions.

Initial cytocompatibility studies were conducted with SaOS-2 and C3H cells. The study demonstrated that the scaffolds support the adhesion, growth, and activity of cells.

The potential for molecularly imprinted polysiloxane scaffolds as tissue engineering biomaterials has been shown by these selectivity and cytocompatibility studies. Therefore, the developed polysiloxane scaffolds are novel biomaterials that may elicit controlled protein and cellular interactions at the interface between bone tissue and implant. As further investigations, development of imprinted scaffolds with cell surface molecules should be performed. Then, osteoblastic cell responses to the scaffolds need to be tested.

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