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SURFACE MODIFICATION OF PLGA BIOMATERIALS FOR SITE-DIRECTED IMMOBILIZATION OF GROWTH FACTORS

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ABSTRACT OF THESIS

SURFACE MODIFICATION OF PLGA BIOMATERIALS FOR SITE-DIRECTED IMMOBILIZATION OF GROWTH FACTORS

Biodegradable polymer materials, specifically poly(lactic-co-glycolide) (PLGA) can be used as bone replacements for bone regeneration. Scaffolds can be prepared to be porous to induce bone growth into a scaffold so that it is replaced with natural tissue as the polymer degrades. However, simply using PLGA will result in formation of scar tissue rather than regeneration of natural bone. Therefore focus has turned to attaching growth factors to the PLGA molecules to elicit a specific cellular response when the implant is placed in the body. Site-directed immobilization utilizes specific groups on both the biomaterial and biomolecule so that growth factors can be oriented in a specific manner for increased cellular response. In this research, exposed carboxyl groups on a non end-capped PLGA were modified with bishydrazide spacer molecules of varying length for the eventual attachment of a biomolecule via carbodiimide chemistry. The number of hydrazide groups attached to the surface could be controlled to investigate the effects of the spacer length on protein immobilization. Both vascular endothelial growth factor (VEGF) and parathyroid hormone (PTH) were used in these studies. These two molecules have different target cells and actions, although both can play a role in bone formation. Both molecules have carbohydrate residues that were oxidized with periodate to form aldehyde moieties that were able to react with the hydrazide spacers to form a stable bond between the spacer and protein. The use of a spacer enhanced the binding accessibility of the protein as compared to randomly adsorbed protein. The shortest and longest of the spacers resulted in the highest amount of protein, with corresponding results for antibody binding. The modification of PLGA functional groups with a spacer molecule indicates that this material could be used for site-directed immobilization for any application, simply by tailoring the reaction between the biomaterial and biomolecule.

KEYWORDS: PLGA, surface modification, growth factors, drug delivery, scaffold

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THESIS

Jessica Bennett Lynn Sharon

The Graduate School
University of Kentucky
2005

SURFACE MODIFICATION OF PLGA BIOMATERIALS FOR SITE-DIRECTED IMMOBILIZATION OF GROWTH FACTORS

THESIS

A thesis submitted in partial fulfillment of the requirements
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1. INTRODUCTION

Bone is a unique site for implants as loading directions and forces vary depending on where implants are located in the body [1]. Used as bone replacements, these substitutes must be able to withstand high loads and activity levels in a range of directions. For orthopedic repair, the age range of patients is large, as is the activity level, forcing any implant to be able to withstand many loading conditions [2]. Bone constantly undergoes different reactions in its modeling and remodeling due to the forces and stresses put on it [3]. This bone regeneration is not able to heal critical size defects, secondary to diseases, tumors and nonunion fractures. Critical size defects are large defects resulting in competition between bone and soft tissue to repair the defect [4]. Of the 6.5 million bone fractures suffered annually in the U.S., 10-15% of these are nonunions [5]. These nonunion fractures often require surgical intervention because the fracture is not able to heal properly.

Bone grafting procedures are very commonly used to treat bone defects; in fact more than 500,000 of these procedures are performed each year in the U.S [6]. There are a few types of natural bone grafting techniques, autografts, allografts and xenografts. An autograft uses the patient's own bone from another site, whereas in an allograft, a donor tissue from the same species is used and bovine tissue is used in xenografts. There are major limitations to all of these surgeries. Autografts require two painful surgical sites with a limit on the amount of bone that can be taken from the donor site, usually the iliac crest [7]. Allografts can be characterized by limited supply, possible rejection of donor tissue and possible transmission of disease. Xenografts increase the risk of disease transmission, as well as rejection of donor tissue from another species [3]. Therefore the focus of treating bone defects has turned to the development of synthetic substitutes [7].

Synthetic bone substitutes range from permanent to resorbable materials and include metals, glass, ceramics, polymers and composites of all of these materials. However, these materials were not originally designed for contact with the human body and fibrous

tissue can be formed when they come into contact with the body. The goal of a bone graft is to restore function to the site of implantation, and ideally the defect will be replaced with native bone not fibrous tissue, so a regenerative response to the implant is desired [8]. To improve response to any of these materials, the presence of specific proteins, e.g., growth factors, can be used to control the body's response to the implant immediately after implantation [9].

The presence of specific proteins, such as bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor (IGF), can recruit cells to the site of the defect and signal specific cellular responses, in order to promote the healing of bone defects [9, 10, 11]. These growth factors can be delivered using a systemic or local approach. Growth factors can be delivered more efficiently if they are placed directly on the surface of the implant, rather than a systemic delivery. Because of the short plasma half-life of these molecules, it is difficult to maintain an effective dose of growth factors at the defect site using systemic delivery [12, 13]. Therefore, it is more appropriate to use a local delivery approach, which can maintain a specific dose of the growth factors or drugs at the defect site.

One method to deliver growth factors to the site of the implant is to simply adsorb protein onto the surface of implant. This technique is not at all controllable, so it is more useful to have an immobilization method. One advantage to certain immobilization techniques is that they can allow for specific orientation of growth factors so binding sites on the growth factor are easily accessible to cells that encounter the implant [14]. There are two different methods for immobilization: physical and chemical pathways. The physical method utilizes electrostatic, hydrophobic/hydrophilic and van der Waals forces. This may result in protein leaching as well as multiple binding points between the protein and surface. The chemical method is carried out through the formation of a strong covalent bond between the protein and the surface. This can result in a change in the structure of the immobilized protein, although this can be done in a controlled manner, to create a site-directed immobilization [14]. Expanding this chemical method, a spacer molecule between the protein and the implant surface can be used. Using a spacer between the

material and the biomolecule can prevent crowding of the biomolecules and can help with accessibility of the protein for interaction with the cells. Using the spacer arms will allow for organization of the growth factors in such a way as to mimic the extracellular matrix of cells [15].

The objective of this research study was to develop methods for site-directed immobilization of bone growth factors on a biodegradable polymer. This was accomplished by using specific functional groups on the polymer and spacers molecules. Spacers were attached to the carboxyl groups on the polymer chains, and then growth factors were attached to these spacers in an oriented manner.

2. BACKGROUND AND SIGNIFICANCE

More than 500,000 bone grafting procedures are performed annually in the United States; these bone grafting procedures are most commonly autografts, allografts, xenografts and biomaterial implantation to repair a bone defect [6]. Autografts use a sample of bone from the patient receiving the graft. Of course there are many limitations to this technique, including the requirement of two surgical sites as well as donor site morbidity, although this is still the gold standard for bone defect repair [3, 16, 17]. Allografts use a donor tissue from another subject of the same species. Disadvantages of allografts include rejection of donor tissue, transmission of blood-borne diseases and infection as well as a limited supply of tissue [3, 16, 17]. Xenografts use an implant material from an animal of a different species. There are many drawbacks to using this treatment due to disease transmission, as well as possible rejection of donor tissue [3, 16, 17]. Many synthetic materials have been used for implantation into the body. However, since they were not designed for contact with the human body, a repair response rather than a regenerative response is the end result [8, 18]. If the response is a repair, the defect site is replaced with scar tissue, in contrast to regenerative results in which the defect is replaced with native tissue. Synthetic materials may also be characterized by long-term failure due to a mismatch in the mechanical properties between the implant and the body's natural tissue [19].

In order for a synthetic material to be appropriate for use as a biomaterial, there are several criteria for creating a biomaterial which can elicit a specific response from the body. These include: biocompatibility, specific architecture, osteoconduction, chemotaxis, angiogenesis and vascularization, controlled delivery of protein/drug, and administrative issues [20]. Biocompatibility is an important factor for any substance not natural to the body to be used for *in vivo* applications, whether it is permanent or temporary. It is defined in terms of the success of the implant to fulfill its intended function and is considered a two way process. For bone implants, the biomaterial must be able to withstand load bearing cycles without fatigue or wear, transfer appropriate loads

from the implant to bone. Both the entire implant and the implant-bone interface must be evaluated for the criteria of biocompatibility to be met [3]. Cyto-compatibility is important for *in vitro* testing of interactions between materials and cells. The implant must not cause harm to the body in the form of cell or tissue death from toxicity and must not elicit an immunological response [21]. These problems are usually tested using *in vitro* techniques. Specific architecture is an important aspect which changes based on where the implant is to be used. For example, biomaterials which will be used in load-bearing applications must have a specific shape to match the defect as well as high amount of strength. Non-load bearing applications also require a specific shape to match a defect but do not require as high of strength as load-bearing applications [22]. However, a bone replacement must not only have the same amount of strength as the natural material it is replacing, it must actually have a higher amount of strength. This is because implants lack the ability to repair localized damage due to fatigue [23]. In addition, the architecture of biomaterials must have porosity to allow for bone growth into the scaffold and transport of nutrients and waste products within the implant [21]. Osteoconduction is a three-dimensional process of promoting bone formation and is observed when an implant is placed into or adjacent to bone tissue [24]. In the process cells proliferate into the material, whether through porosity or simply on the surface, and begin either a repair or regenerative response [8]. Chemotaxis is the directional movement of cells in response to a chemical stimulus. Chemotaxis is required for cells to bind to receptor sites on the implant to perform a specific function, such as building bone [25]. Angiogenesis and vascularization are the formation of new blood vessels and their proliferation into an implant, which is an important aspect of wound healing. The presence of blood cells results in better healing, because when any implant is placed in bone an injury occurs [9]. If a specific response from the implant is either required or desired, it may be desired to use the biomaterial as a vehicle for protein or drug delivery. If this is the case, the biomaterial must be able to release the protein or drug at specific intervals and maintain the concentration over a desired period of time [26]. Administrative issues are important for the ease and quality control of preparation of any biomaterial. The biomaterial must be able to modified to be a specific size or shape, as well as control of any modification of the biomaterial.

2.1 Biomaterials

Currently there are permanent and biodegradable synthetic implants used in orthopedic surgeries to treat bone defects. Common uses of permanent implants include hip and knee replacements and some screws and plates for fracture fixation. Some biodegradable materials may be converted to water-soluble material through both physical and chemical processes. These biodegradable implants are often used in fracture nonunions as a filler material until the defect may be replaced with host tissue. They can also be used as drug delivery devices and coatings on permanent implants to improve bone attachment to an implant [26]. Even though these biodegradable implants are not permanent, they still must be biocompatible with natural tissues. They must be biocompatible from the initial time of implantation until they have completely degraded [24]. This requirement complicates the use of certain biodegradable implants as they may release acidic byproducts that can be harmful to tissues and cells as they degrade [21]. However, it is important to note that using a biodegradable implant eliminates the long-term disadvantages of using permanent synthetic material because degradable implants are usually replaced with host tissue [19]. When any material comes into contact with the body, a strong and immediate reaction by the body is necessary for the implant to be incorporated into the body and serve the function for which it was intended.

As previously mentioned, most of the current implant materials were not designed for implantation into the body. However, some of these materials may be more useful for implantation into the body than others, in that some materials may be able to elicit a specific and desired host response when they are implanted. Metals, such as titanium, are widely used for orthopedic implants into bone, but these metals have such different properties than bone that the material can actually weaken the bone surrounding the implant through stress shielding [27]. Stress shielding occurs when load is transferred to the implant rather than the surrounding bone. Bone requires load in order to maintain its strength through the constant remodeling it undergoes in response to force. With reduced force, there is less need for bone to maintain its strength, and the ultimate result of the bone remodeling process is net bone loss [3]. Ceramics, such as hydroxyapatite and tricalcium phosphate, can be processed under high temperatures and pressures to form a

dense ceramic and are characterized by brittleness with low resistance to impact [17, 28]. These dense scaffolds may also degrade over a period of months to years [29]. These same ceramics can also be processed to be porous, but may result in rapid dissolution, as short as six weeks [30]. However, polymers are now being investigated for use as orthopedic implants. Poly(glycolic acid) (PGA) is an FDA approved polymer for resorbable sutures. Poly(lactic acid) (PLA) is a similar polymer that is used for screws and plates in bone repair devices. However, combining both of these polymers to create a copolymer, poly(lactic-co-glycolide) (PLGA), creates a strong resorbable implant for bone replacements with controllable properties. The crystallinity, strength and degradation are controlled by the ratio of LA to GA [31, 32]. It is also possible to modify the end of each polymer chain in order to change the polymer properties through function groups present on polymers [33]. Many PLGA chains have an aliphatic end-cap that makes the polymer more hydrophobic and drastically increases the length of degradation as compared to a polymer chain without an end-cap. While all PLGA chains are hydrophobic, the presence of an end-cap makes the polymer considerably more hydrophobic than PLGA chains without the end-cap. These more hydrophobic polymers also have a lower acid number as reported by Schrier [34]. When the carboxyl group is exposed, the acid number, which is an indication of the amount of basic solution required to neutralize one gram of PLGA, may increase by a factor anywhere from 2 to 15 times [34]. These simple changes to the polymer chain, by addition or subtraction of an end-cap and the ratio of GA to LA, are important because they make the polymer a more versatile implant for many different applications, including orthopedic devices, dental implants, and even vascular implants. PLGA has been found to be osteoconductive and is therefore suitable for bone replacement in the form of microspheres, coatings and scaffolds.

2.2 Growth Factors

Growth factors are designated as polypeptides that are able to transmit a signal in order to modulate cellular activities and promote cell proliferation [13, 35]. They play an important role in development and wound and fracture healing by recruiting appropriate cells to a specific site at necessary times. For example, immediately after a fracture has

occurred, natural wound healing begins. Initially, platelets aggregate to stop blood loss, while chemotaxis of neutrophils and monocytes into the blood clot occurs. Fibroblastic growth factor (FGF) stimulates fibroblasts to produce proteins to stabilize the fracture. Bone morphogenic protein (BMP) is found in mesenchymal cells in the hematoma and the periosteum adjacent to the facture. Transforming growth factor (TGF) and platelet derived growth factor (PDGF) are released by platelets and inflammatory cells into the fracture hematoma to stimulate proliferation of mesenchymal cells into the periosteum. PDGF is also found in macrophages near the periosteum two day after fracture but decreases after that [25]. Chondrocytes also organize the initial collagen matrix into a cartilage framework so that differentiated osteoblasts can remodel the framework into bone. These growth factors help to promote osteoblasts differentiation to build bone and repair the fracture. Concentrations of growth factors decrease as the bone matures. Healing continues over several months of bone remodeling [5].

Effects of growth factors are also concentration dependent and are able to influence both secretion and action of other growth factors. This is done through one growth factor stimulating another cell to produce and secrete a specific growth factor, often as part of a cascade of wound healing or other specific events involved in development. Growth factors are able to act on the cell that secreted them (autocrine), a nearby cell (paracrine), a distant cell of a different phenotype (endocrine) [9, 25].

As an effort to improve bone defect healing, growth factors have been added to implants prior to implantation. The adsorption of growth factors, including BMP, FGF, TGF, PDGF, (FGF), can improve osteointegration of the implant into bone [9, 25]. The release of these growth factors helps to regulate cellular activity. It has been shown that only BMP’s are able to differentiate mesenchymal cells into osteoblasts as well as stimulate cell growth and bone formation [9, 35, 16]. The concentration of the growth factors is also very important, as at time of implantation, a high concentration of BMP is required to encourage differentiation of recruited cells into osteoblasts, whereas the concentration of other growth factors is important further from the time of implantation for the osteoblasts to continue to build bone [25]. Biomaterials allow growth factors to be

maintained at the implant site for an extended period of time rather than in a systemic delivery when concentrations dissipate quickly.

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen and motogen and is often expressed in vascular processes. It is secreted by endothelial cells, osteoblasts and some macrophages and helps to stimulate the growth of any type of blood cells during angiogenesis and vasculogenesis processes [36, 37]. VEGF is a key regulator in angiogenesis. VEGF was initially identified through its actions on vascular endothelial cells, although, it is also expressed in the inflammatory responses, which is a natural part of wound healing [37]. It has also been shown to be chemotactic for monocytes and osteoblasts [36]. Some preliminary studies have shown that in the presence of BMPs, VEGF may play a role in bone regeneration through aiding in recruitment of mesenchymal stem cells, enhance cell survival and prevent cartilage formation in the place of bone formation [38]. In addition, VEGF has been found to be useful in bone regeneration through the process of angiogenesis. The presence of VEGF on PLGA scaffolds enhanced mineral tissue regeneration in a rat cranium critical defect. This enhancement of mineral tissue regeneration resulted in increased regeneration of the osteoid matrix [39].

Parathyroid Hormone (PTH) is normally secreted by the parathyroid gland in response to low plasma calcium level. PTH serves to regulate calcium homeostasis in three ways: stimulating bone resorption, increasing calcium absorption in the intestines and increasing calcium and phosphate resorption in the kidneys [40]. When there are high levels of PTH, bone resorption begins resulting in bone mass loss. However, an absence of PTH results in lack of bone growth. It has been determined that low, intermittent doses of PTH stimulate osteoblastic activity to replace bone loss [12]. PTH also works to increase bone mass by acting as an inhibitor of normal bone resorption [40]. The 34 amino acid sequence of PTH (1-34) is the section that is thought to have these effects [40]. The challenge to using PTH in cases of bone loss is to maintain a very low level dose of PTH or intermittingly high doses [12].

Systemic oral delivery of growth factors is not ideal due to low bioavailability based on the protein's enzymatic degradation and poor absorption. Intravenous delivery of growth factors has also been explored, but again is not very effective because of the short plasma half lives [25]. For example, PDGF can not be detected in the circulation after IV delivery, due to its half life of two minutes when delivered in this form [13]. Because concentration of the growth factor plays a role in its behavior, more frequent dosing by both of these systemic delivery methods would be required to reach an effective dose in the body [34]. Parathyroid hormone (PTH) has been found to have bone forming effects if a high dose is present for a short period of time or if a low dose is maintained for a short amount of time, otherwise bone resorption effects are seen [12]. Low doses can be maintained at the implant site if PTH is adsorbed onto the surface of the implant. Fracture nonunions have been shown to have poor bone stock, scar tissue and vascularity, and a one-time dose of BMP is not sufficient enough to stimulate bone formation. In contrast, maintained delivery of BMP directly to the site of implantation stimulates bone formation more than a systemic delivery [10, 41]. However, simply adsorbing growth factors onto the implant does not allow for controlled drug delivery, so the focus of drug delivery has turned to immobilizing growth factors onto or within the implant.

2.3 Immobilization Techniques

Currently there are several ways to attach growth factors to biomaterials. Some are very simple techniques, while some are more difficult to accomplish, but may or may not yield better results. The three ways of attaching a biomolecule are through the use of physical adsorption, physical "entrapment", and covalent attachment [3]. The most simple of these techniques is physical adsorption. This is usually carried out by simply soaking the implant in a solution of the molecule to be attached, and the biomolecules attach to functional groups on the surface, in addition to the possibility for liquid to be maintained within pores of some implants. This method allows for no control over the amount or orientation of biomolecules on the implant. Physical entrapment is not ideal because of the high shear forces and strong solvents that are often required for the development of biodegradable implants [34]. Conditions such as these can damage the protein, thereby

reducing its bioactivity and may also reduce the loading efficiency of the protein onto the biomolecule [34]. Finally, covalent attachment of proteins is carried out using hydrogels, solid surfaces and soluble polymer conjugates, all with functional groups which allow the biomolecule to interact and form a stable, covalent bond [3, 33]. This can lead to permanent or temporary presence of the biomolecule. For example, attaching a large biomolecule to a solid surface can lead to a long standing retention. Using a biodegradable system, such as PLGA, the biomolecule can be released as the polymer degrades and the biomolecule can advance the replacement of the implant with native tissue [42].

Random approach to protein immobilization results in some of cell-receptor binding sites being inaccessible to cells and possibly directed towards the biomaterial rather than cells. Random immobilization can be the result of very simple methods such as soaking a biomaterial in a protein solution. Covalent attachment, through surface exposed functional groups can lead to the immobilization of protein onto the surface of a biomaterial in which the biomolecules are randomly oriented or have the same orientation, as shown in Figure 1. This is a temporary or permanent localization of the biomolecule onto a biomaterial (or support). In the schematic with randomly adsorbed protein, the cell binding sites are all oriented in a different direction, which could make it difficult for cells to see and interact with the cell receptor binding sites, whereas in the oriented immobilization, all of the binding sites are directed in the same way, making it easy for cells to respond to these sites [14]. Immobilization is becoming widely used because the molecule (or cells) can be attached in such a controlled way so as to elicit a specific response from the body when attached.

One method of immobilization utilizes carbodiimide (CDI) chemistry in one or two steps to couple amines and carboxyl groups [33, 43, 44]. CDI is one of the more useful methods for modifying carboxyl groups. However, diazoalkane and carbonyldiimidazole processes can also be used. Diazoalkanes are amides and esters which are able to react spontaneously with carboxyl groups to form ester bonds. However, at acidic pH, cross-reaction with sulfhydrl groups can occur, and as pH increases the reaction becomes less

specific. Carbonyldiimidazole has two leaving groups of aculimidazole that react with carboxyl groups and produces carbon dioxide and imidazole. The carboxyl group can then react to form amide or ester bonds. CDI is used to mediate the formation of amide bonds between carboxyl groups and an amine. The reaction forms an intermediate, *o*-acylisourea that is highly reactive. The carbonyl group of this ester is attached by an amine nucleophile to form an amide bone [45]. In the single step version, both CDI and the biomolecule interact with the biomaterial simultaneously. However, in the two step process, first the CDI molecule interacts with the biomaterial to activate groups for protein attachment, and is removed. Following removal of CDI solution, the biomolecule is allowed to interact with the biomaterial. In both cases, protein immobilization is the result. This immobilization allows retention of the biomolecule on the surface when in contact with medium [43].

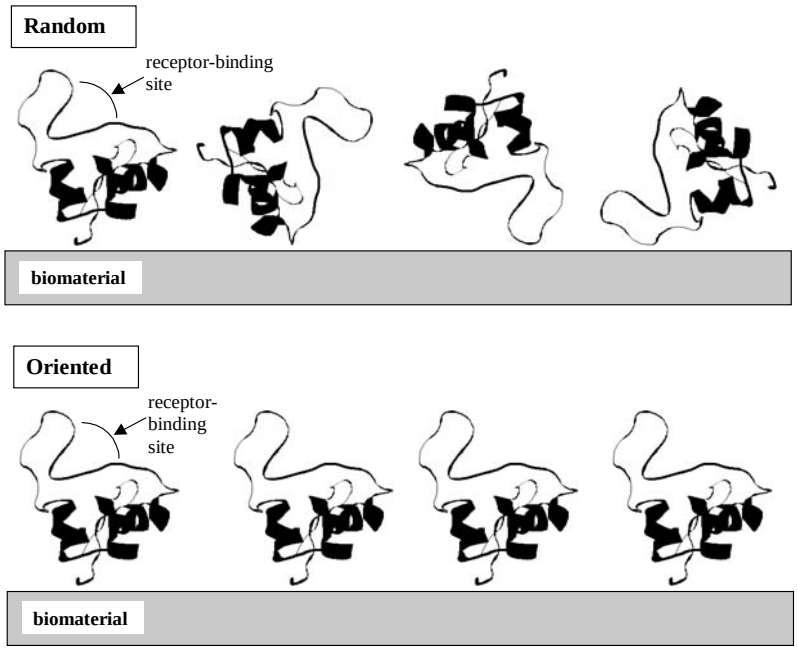


Figure 1: Schematic depicting protein adsorption, randomly (top image) with receptor binding sites oriented in different directions and oriented immobilization (bottom image) with all of the binding sites oriented in the same way

In a study by Puleo *et al*, BMP was immobilized onto the surface of a titanium alloy. The alloy was functionalized with different amine concentrations for immobilization of the BMP molecule. The immobilization was carried out using a two-step CDI process to activate the amine groups on the alloy surface. Then BMP was attached to the activated intermediate groups. BMP was also randomly adsorbed onto the surface of the titanium implant by soaking the implant in a solution of BMP for two hours. Initially the alkaline phosphatase activity was highest on surfaces with randomly adsorbed protein; however, after overnight incubation to remove loosely bound protein, the immobilized surfaces had a significantly higher activity [43].

Site-directed immobilization results in biomolecules being attached to surfaces of implant materials in a controlled and specific oriented manner. This allows for these growth factors/proteins to be oriented in such a manner that all of the cell receptor-binding sites on the biomolecule are accessible to the cells. Site directed immobilization is achieved by altering the surface of the biomaterial and/or the biomolecule. This can be done using specific functional group on the biomaterial to be reactive with specific functional groups on the biomolecule. For example, a glycoprotein has at least one oligosaccharide unit that is linked covalently to carbohydrate residues on amino acids. These carbohydrate residues can be specifically oxidized to aldehydes. A condensation reaction with hydrazide groups on the biomaterial results in hydrazone bonds between the protein and biomaterial [14].

Site-directed immobilization is already very controlled; though a spacer molecule can be used between a biomolecule and the biomaterial surface. The use of a spacer molecule is illustrated in Figure 2. The spacer is simply a molecule that is homo- and hetero-bifunctional, such that it may attach itself to the biomaterial and biomolecule. Use of spacers reduces steric hindrance for both large and small biomolecules, allowing for a greater amount of specific bioactivity. Attaching small molecules further away from the biomaterial surfaces allows cells to react with receptor-binding sites that have become more accessible. For large molecules, the use of a spacer can help to prevent interaction and crowding between the individual molecules. Using a spacer that is too short may not

prevent the crowding of the molecules as they would be so close to the biomaterial surface. Also, using too long of a spacer may prevent loss of the biomolecule and poor cell proliferation into a biomaterial. The use of a biodegradable spacer can also control the release of the biomolecule from the surface of the biomaterial. Thus, these spacers can be designed to operate for orthopedic coatings and implants, diagnostic tools and potential drug delivery systems by creating a material with both a traditional purpose as well as a biological function instead of a simple block of material implanted into the body.

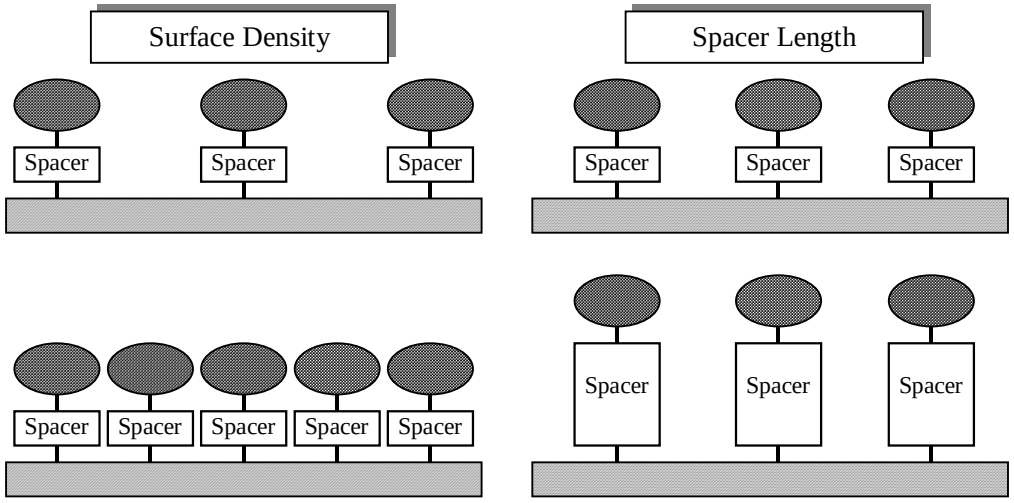


Figure 2: Schematic of protein attached to biomaterial using spacers with varying surface density (left) and varying spacer length (right)

While PLGA simply by itself has been shown to be osteoconductive, there is not a strong therapeutic response by the body to the polymer implant into bone [17]. PLGA offers very little reason for cells to be recruited to the surgical site, other than for repair response. However, the functional groups at the end of the polymer can be very useful in creating a controlled implant for repair of bone defects [33]. A polymer without an aliphatic end cap exposes a carboxyl group at the end and makes modification simple by altering this group. Therefore each polymer chain can be modified to attach a specific growth factor to its end and control the concentration and orientation of these immobilized growth factors. For example, in a study by Yoo *et al*, hyaluronic acid (HA)

was immobilized on the surface of PLGA for the purpose of cartilage regeneration. The PLGA scaffolds were prepared by blending PLGA with an amine-terminated PLGA-PEG di-block polymer. The HA molecules were chemically conjugated to the surface-exposed amine groups on the scaffolds, and allowed to interact with the PLGA surface, thereby immobilizing HA onto the surface in an oriented manner. When scaffolds were seeded with chondrocytes, enhanced cellular attachment was seen for the modified PLGA surfaces in comparison to unmodified. Collagen synthesis was also improved [46].

In a study by Hu *et al* chemically inert PLA porous films were activated with plasma NH_3 and then an RGD sequence or poly(L-lysine) was attached in an oriented manner. Following attachment of RGD or PLL the films underwent cell culture with osteogenic precursor cells (OPCs) and alkaline phosphatase (AP) activity and extracellular calcium were measured. Attachment of the RGD resulted in a higher amount of AP activity than did the PLL groups. Calcium levels were also higher after 28 days of incubation for scaffolds with attached RGD groups. These levels are proportional to the number of differentiated bone cells. The presence of the RGD groups resulted in a significantly higher number of bone cells present in comparison to films with immobilized PLL or control. These scaffolds were only tested *in vitro* so further testing is required to determine if the same results would be found *in vivo* [47].

PLGA releases protein from the scaffold through a bulk process of degradation when low levels of growth factors are used [48]. Using a spacer between the polymer and the growth factor allows for even more control by preventing overcrowding effects from too much of the growth factor and also makes it easier for cell receptors to respond to the presence of the growth factors.

2.4 Significance

The need for bone biomaterials is rapidly rising with an aging population. No one biomaterial has been proven ideal for bone implantation. The addition of an immobilized growth factor, drug or protein onto the surface of any biomaterial can help with any

application in the body. By using a functional group at the end of a polymer, or any resorbable material, the surface can be modified to allow for the presence of specific proteins or drugs that are delivered directly to a specific site. Using a spacer allows for a controlled response by the cells near the implant and can affect healing of the specific wound or diseased site. By altering the biomaterial, proteins, spacer length and density, this idea can be applied to any material implanted into the body.

3. MATERIALS AND METHODS

3.1 Polymer

The polymer investigated was poly(D,L-lactide-co-glycolide) (PLGA), obtained from Alkermes (Wilmington, OH). This particular polymer (DL 2A) is a 50:50 copolymer ratio of lactic and glycolic acid, and is non-endcapped, meaning that it has an exposed carboxylic acid group at the end of the chain, which will be used for further surface modification steps. The structure is shown in Figure 3.

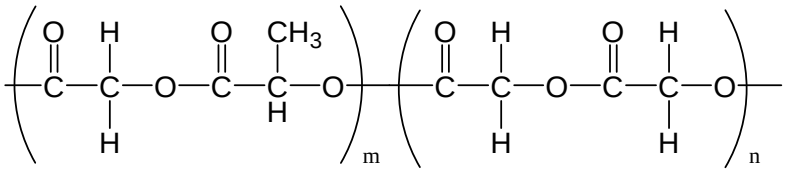


Figure 3: Structure of PLGA used in these studies

The molecular weight of the polymer was 11 KD. This polymer can easily be made into coatings, microspheres and scaffolds for various applications. The glass transition temperature, which was important for preparing scaffolds, was 41.3°C.

3.1.1 Coatings

Coatings were used for experimentation due to their ease of preparation and measurements. Glass coverslips with a diameter of 12mm were investigated following a simple coating technique. A 0.117% (w/v) polymer solution was made by dissolving 200mg into 1.7ml of methylene chloride (Sigma-Aldrich, St. Louis, MO). The solution was vortexed and sonicated at 25W for 30 seconds. Approximately 30 to 40μL of this solution was then dropped onto the top surface of each coverslip. Each coverslip was

allowed to air dry for about 30 minutes and then vacuum-dried overnight or until ready for use.

3.1.2 Microspheres

Microspheres were prepared using a water/oil/water (double) or water/oil (single) emulsion technique. The 0.117% (w/v) polymer solution was prepared in the same fashion as in the coatings method, by dissolving 200mg or PLGA into 1.7ml CH_2Cl_2. For double emulsion techniques, 200μl of water was added to the polymer solution. For both double and single emulsion techniques, the polymer solution was vortexed to dissolve the polymer and then sonicated at 25W for 30 seconds. The solution was then added dropwise to a 1% polyvinyl alcohol (PVA) (Sigma) solution. The PVA solution was spinning at 700rpm, which was a fast enough rate to allow delivery of the polymer solution directly to the bottom of the beaker. The rate was then slowed to approximately 600rpm and allowed to spin overnight until all of the CH_2Cl_2 had evaporated. Microspheres were vacuum filtered using #40 Whatmann filter paper, washed with deionized water and then vacuum-dried overnight and stored in the freezer until ready for use.

3.1.3 Scaffolds

Scaffolds were prepared using a simple sintering process adapted from Borden *et al* [7]. Prepared microspheres were gently disaggregated from one another using a mortar and pestle. Next, they were sieved to achieve a similar size range to one another, of about 50-150μm. Two different masses of microspheres were used for scaffold preparation, both 50mg and 25mg.

The height of the scaffold was dictated by the size of the mold, mass of microspheres, temperature and time of heating. The 50mg scaffolds were prepared by adding this mass of sieved microspheres to a mold with a diameter of approximately 6.2mm. Initially 25mg scaffolds were also prepared using this mold, but later a mold with a diameter of

4.2mm was used for the smaller scaffolds. As mentioned previously, the glass transition temperature of the polymer was 41.3°C, so microspheres were heated in the mold at 50°C for one, two or three days. This time and temperature was chosen to achieve a balance between porosity and compressive strength.

3.2 Material Characterization

3.2.1 Scanning Electron Microscopy (SEM)

Microspheres and scaffolds were both examined using SEM techniques. Microspheres were mounted onto a graphite "sticky pad" on an SEM stub. Scaffolds were fractured and then mounted onto the stub using colloidal graphite. Each sample stub underwent gold sputter coating in an argon atmosphere using the Emscope sc 400. Samples were then examined with the Hitachi S-3200 (Tokyo, Japan) using an accelerating voltage of 5kV.

3.2.2 Mechanical Testing

Mechanical testing was carried out using a system created in the Center for Biomedical Engineering. Linear force was applied through a stepper motor (model 801B-AM), digital linear actuator with 45 lbs maximum linear source (Eastern Air Devices Corp., Dover, NH). Displacement was measured with a DC linear variable differential transformer (model 0244-00000; Transtek, Tuscon, AZ) with a maximum working range of ±1 inch and had 0.5% error introduced by non-linearity over its working range. Load was measured by a subminiature load cell (Entran, Fairfield, NJ) that introduced 1% error for non-linearity over its working range and exhibits a 2.5% thermal sensitivity shift at 50°C. The system was computer-controlled using LabView (National Instruments, Austin, TX). Samples were loaded in compression until failure occurred. Compression data was recorded and then transferred to a Microsoft Excel spreadsheet as force and displacement. Within known height and diameter of the scaffold, a stress-strain plot was generated to calculate the ultimate compressive strength and compressive modulus. An example of the force-displacement plot generated from the apparatus is shown in Figure 4.

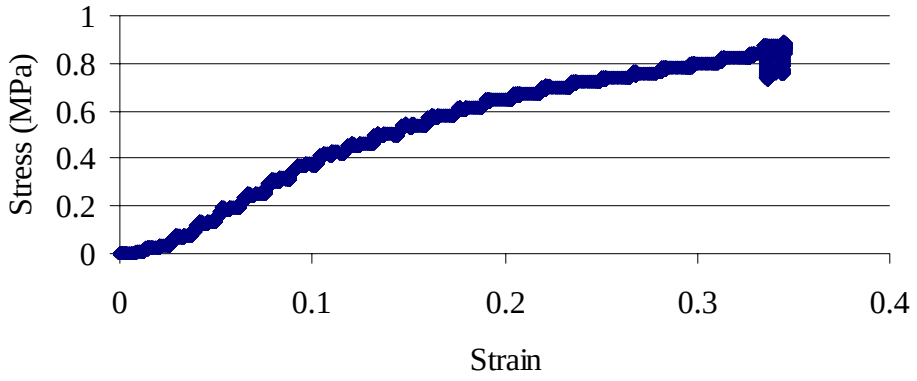


Figure 4: Stress-strain plot for compression testing of a 50 mg scaffold heated for three days at 50°C

3.2.3 Porosity

The porosity of scaffolds was measured using the Archimedes method in which the scaffolds were immersed in 2ml ethanol and shaken slightly in addition to repeated cycles of vacuum to force ethanol into the pores. Initially, 2ml of ethanol was placed in a small graduated cylinder; this volume of ethanol was recorded as V_1. A scaffold was then placed in the graduated cylinder; the total volume of ethanol and scaffold was recorded as V_2. The scaffold was then removed from the ethanol. The remaining amount of ethanol in the graduated cylinder was recorded as residual volume, V_3. The porosity of the scaffold was calculated using the following equation [49].

$$\varepsilon(\%) = \frac{V_1 - V_3}{V_2 - V_3} * 100$$

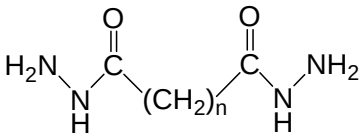
3.2.4 Surface Area

Surface area was measured through nitrogen gas adsorption. Scaffolds were placed under vacuum for a minimum of three days at room temperature and then outgassed overnight at 25°C to remove any air from the scaffold. In order to measure the surface area of the scaffolds, six scaffolds were used in each tube to ensure that a high enough surface area was available for measurment using the TriStar 3000 from Micromeritics (Norcross,

GA). The samples were outgassed at 25°C for 24 hours. Surface area was calculated using BET isotherms.

3.3 Surface Modification

Modification of the carboxyl groups of PLGA was carried out using both coverslips and scaffolds. Four different bishydrazides of varying length were investigated: oxalic (Aldrich, Milwaukee, WI), succinic (Aldrich), adipic (Sigma) and sebacic bishydrazide (TCI America, Portland, OR). Each of these has the same basic backbone, with varying carbon chain lengths at their center. The structures are shown in Figure 5.



Oxalic dihydrazide
(C2, n=0)

Succinic dihydrazide
(C4, n=2)

Adipic dihydrazide
(C6, n=4)

Sebacic dihydrazide
(C10, n=8)

Figure 5: Structure of bishydrazide spacer arms

The bishydrazide concentrations investigated were 0.002, 0.011, 0.018, 0.057, 0.115, 0.184mM dissolved in 0.1M 2-(N-morpholino)ethanesulfonic acid (MES) (Sigma) at a pH of 6.0. One scaffold or coverslip sample was placed in each well of a 24 well plate. Then 0.5ml of one of the bishydrazide solutions was added to the well. A solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC) (Sigma) and n-hydroxysuccinimide (Fluka, Buchs, Germany)was used to activate surfaces. This solution was prepared to be 16mg/ml in a 0.1M MES (pH = 6.0) buffer solution. The EDAC/NHS was prepared to be a 5:2 EDAC: NHS molar ratio and 0.5ml of this solution was allowed

to react with the PLGA surfaces [43, 45]. Plates were then shaken at room temperature for two hours. After activation the bishydrazide molecule attaches at the end of the PLGA via a nucleophile reaction mechanism by adding 0.5ml of hydrazide solution to each coverslip. A schematic of this entire reaction is shown in Figure 6. After complete reaction, all liquid was then aspirated from the plate and the samples were rinsed three times with deionized water.

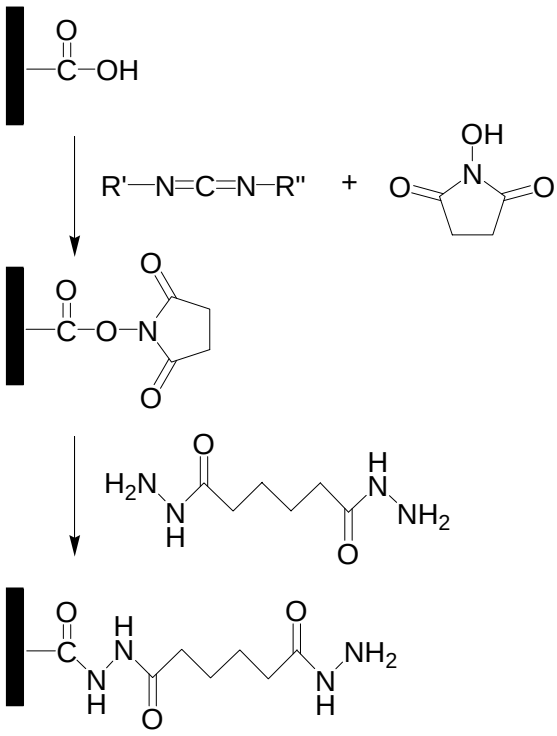


Figure 6: Schematic representation of bishydrazide attachment to PLGA surface using CDI chemistry

3.3.1 Quantification of Hydrazide Groups

To measure the number of hydrazide groups bound to each PLGA sample, modified surfaces were reacted with 2,4,6-trinitrobenzene sulfonic acid (TNBS) (Sigma). Samples were incubated at 70°C for 5 minutes with 0.1% TNBS in a 3% sodium borate solution (Sigma). This resulted in the formation of a trinitrophenyl group, which was then hydrolyzed by reaction with 1M NaOH at 70°C for 10 minutes. This hydrolysis produces

a yellow-orange color that is proportional to the number of trinitrophenyl groups and therefore to the number of bishydrazides groups [43]. Color development was measured at 450nm. A standard curve was generated for each of the bishydrazide, because their presence causes a shift in the color development of the assay.

3.4 Protein Attachment

Two different proteins were attached to the modified biomaterials of interest. Recombinant human vascular endothelial growth factor (VEGF) (R & D Systems, Minneapolis, MN) was selected because it plays an important role in the angiogenesis phase of wound healing [37]. It has also been found to play a role in bone regeneration when in conjunction with BMPs [38]. Parathyroid hormone 1-34 fragment (PTH) (Sigma) was also explored because of the presence of an N-terminal serine and its ability to stimulate bone formation. After attachment, the amount of protein and protein accessibility were measured with an immunoassay.

3.4.1 VEGF Attachment

VEGF is a glycoprotein and the carbohydrate residues near the middle of the chain can be oxidized to aldehyde moieties with periodate [45, 50]. This particular protein is expressed in Sf21 insect cells using a baculovirus system to allow for oxidation of asparagine, at approximately the 75^{th} amino acid residue [37, 51]. VEGF was reconstituted in PBS at a concentration of 40μg/ml. Oxidation was carried out by allowing 250μl of 10mM periodic acid (Aldrich) to react with the 250μl of the VEGF solution, in the dark at room temperature for 45 minutes. The reaction was quenched by adding 500μl glycerol. The solution was then filtered by centrifuging for a total of 100min at 10,000rpm, and washing with PBS. The VEGF was then resuspended in 500μl of PBS, the final volume was then increased to 10ml. A volume of 250μl of the resuspended oxidized VEGF was added to each PLGA sample, allowing the aldehyde groups to react with the hydrazide groups at neutral pH to form stable hydrazone bonds [52]. VEGF was also randomly adsorbed onto an unmodified PLGA surface by interaction of 250μl of VEGF solution with each sample. The protein was allowed to react with each surface for 45 minutes at

room temperature. One set of samples was used for immediate data collection. A second set of coverslips was prepared at the same time, and immediately following protein attachment, the plates were incubated overnight at 37°C. A schematic representation of glycoprotein oxidation and attachment to the modified surface is shown in Figure 7.

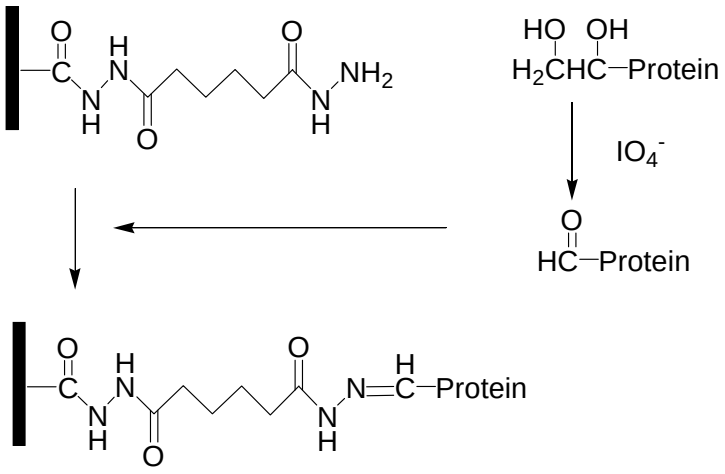


Figure 7: Schematic representation of VEGF attachment to bishydrazide modified PLGA surfaces

3.4.2 MicroBCA Analysis

The amount of VEGF protein bound to the PLGA surface was measured using a MicroBCA kit (Pierce, Rockford, IL) following the manufacturer's instructions. The VEGF on the surfaces was allowed to interact with a working reagent solution that undergoes a color change in the presence of protein. A standard curve was generated using dilutions of a bovine serum albumin (BSA) standard. Color development was measured at 570nm.

3.4.3 Binding Accessibility

The availability of VEGF for potential binding with cell receptors was assessed using an immunoassay technique. All samples were blocked with a protein solution, BSA/PBST, solution to prevent nonspecific antibody binding. BSA/PBST is a 0.5% BSA solution

prepared in a solution that is 0.05% Tween 20 in PBS. A primary antibody to VEGF, anti-VEGF purified mouse monoclonal IgG_{2B} (R & D Systems), was reconstituted in PBS and then diluted at a ratio of 1:500. A volume of 0.5ml of this primary antibody was allowed to react with the samples for 30 minutes at 37°C and then washed with PBST three times. The secondary antibody, alkaline phosphatase activated goat anti-mouse IgG (Sigma), was diluted with PBS at a ratio of 1:500 and 0.5ml of antibody solution was added to each sample. The secondary antibody was then allowed to react with the surface for 45 minutes at 37°C and washed with PBST three times. A solution of 0.1% Sigma 104 phosphatase (Sigma) in 10% diethanolamine (Chempure, Houston, TX) at pH 9.8 reacted with the samples for a minimum of 20 minutes but ranging up to 60 minutes to ensure maximum color development. Color development was measured at a wavelength of 410nm.

3.4.4 PTH Attachment

PTH is a peptide that has an N-terminal serine that can be oxidized to an aldehyde moiety with periodate [45, 50, 53]. PTH was reconstituted in PBS at a concentration of 40μg/ml. Oxidation was carried out by allowing 250μl of 10mM periodic acid (Aldrich) to react with the 250μl of the PTH solution, in the dark at room temperature for 45 minutes. The reaction was quenched by adding 500μl glycerol. The solution was then filtered by centrifuging for a total of 100min at 10,000rpm, and washing with PBS. The oxidized PTH was then resuspended in 500μl of PBS, the final volume was then increased to 10ml. A volume of 250μl of the resuspended oxidized PTH was added to each PLGA sample, allowing the aldehyde groups to react with the hydrazide groups at neutral pH to form stable hydrazone bonds [52]. PTH was also randomly adsorbed onto an unmodified PLGA surface by interaction of 250μl of PTH solution with each sample. The protein was allowed to react with each surface for 45 minutes at room temperature. A schematic representation of peptide oxidation and attachment to the modified surface is shown in Figure 8.

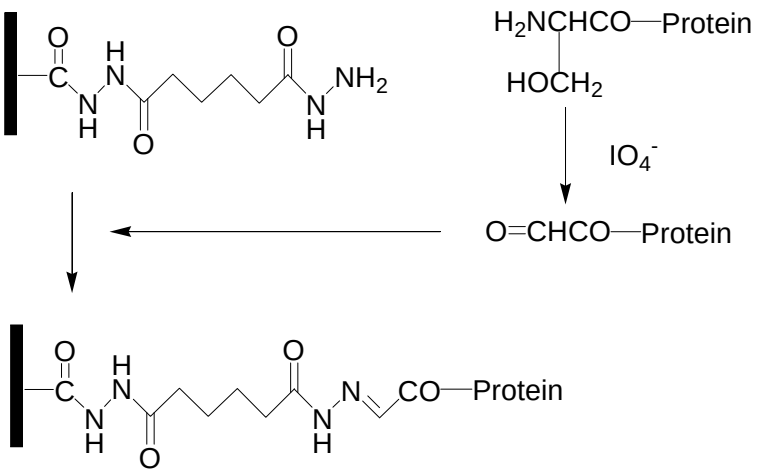


Figure 8: Schematic representation of PTH attachment to bishydrazide modified PLGA surfaces

3.4.5 MicroBCA Analysis

The amount of VEGF protein bound to the PLGA surface was measured using a MicroBCA kit following the manufacturer's instructions. The PTH on the surfaces was allowed to interact with a working reagent solution that undergoes a color change in the presence of protein. A standard curve was generated using dilutions of a BSA standard. Color development was measured at 570nm.

3.4.6 Binding Accessibility

The availability of PTH for binding with cell receptors was measured using an immunoassay technique, using the same procedures as in the VEGF experiments. In these experiments, however, two different polyclonal antibodies to PTH were used. One of the antibodies has a binding epitope at the N-terminus of the peptide (sc-9676) and the other had a binding site at the C-terminus (sc-9677) (Santa Cruz Biotechnology, Santa Cruz, CA). All samples were blocked with BSA/PBST to prevent nonspecific antibody binding. One of the two antibodies was diluted with PBS at a ratio of 1:500 and was allowed to react with the samples for 30 minutes at 37°C and then washed with PBST three times. The secondary antibody, alkaline phosphatase activated goat anti-mouse IgG (Sigma),

was diluted in PBS at a ratio of 1:500 was then allowed to react with the surface for 45 minutes at 37°C and washed with PBST. The remainder of the procedure was the same as in the VEGF binding assessment.

3.5 Statistical Analysis

Statistical analysis was performed using INSTAT3 software (Graphpad Software Inc.). One-way ANOVA was performed to determine if there were statistical significances in the differences between mean values of measured parameters for the various experimental groups. The Tukey Kramer multiple comparison test was used to compare the different experimental groups.

4. RESULTS

4.1 Material Characterization

Initially microspheres were prepared by double emulsion and simply adding polymer solution dropwise to a spinning solution of 1% PVA. This resulted in microspheres which were broken and fragmented, as shown in Figure 9. A few intact microspheres seemed to have high porosity, but most of the microspheres were too fragmented to be used in preparing scaffolds, as the porosity of the scaffolds would be changed by the presence of these fragments. Instead, other techniques were explored to find a protocol that would yield intact particles.

Figure 9: Fractured microspheres prepared from a double emulsion technique

Using a single emulsion technique, but delivering the polymer solution directly to the bottom of a 1% PVA solution by stirring the solution at a minimum of 700rpm, resulted in spherical particles, as shown in Figure 10. The microspheres had a very large range of

sizes from 10-250 μm. However, since all particles were spherical, this method of microsphere preparation was settled on, as the particle size could be controlled in the preparation of scaffolds. Using smaller microspheres with larger microspheres and even fragments could result in some of the smaller microspheres “clogging” the pores that would be formed by using microspheres of the same size range. In order to prevent this, microspheres were sieved using mesh sizes 45, 60 and 80 (US Standard sizes) to generate a range from 50-150 μm, which is demonstrated in Figure 11. These microspheres were used to prepare scaffolds.

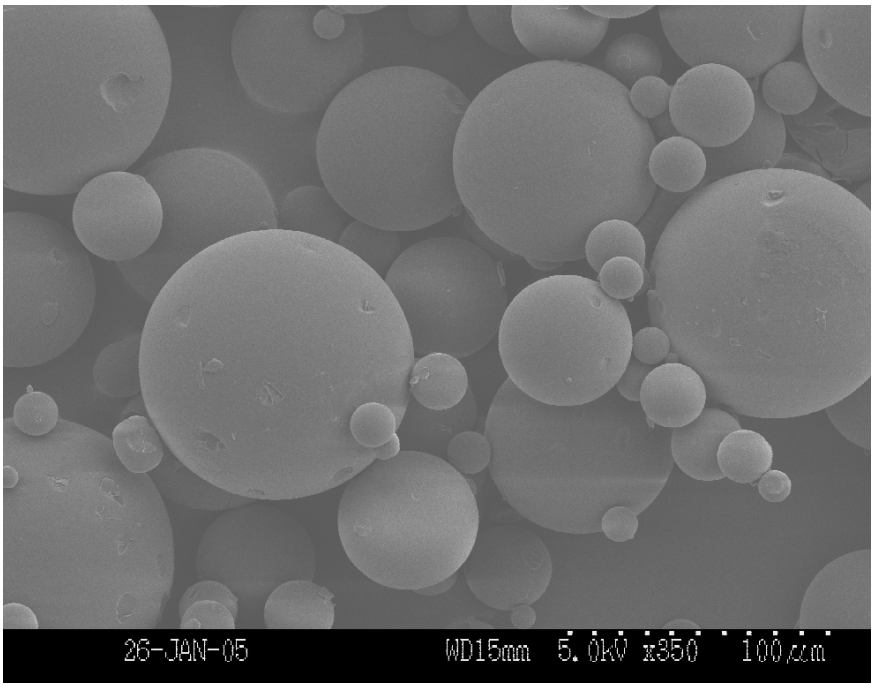


Figure 10: In tact microspheres prepared from a single emulsion technique

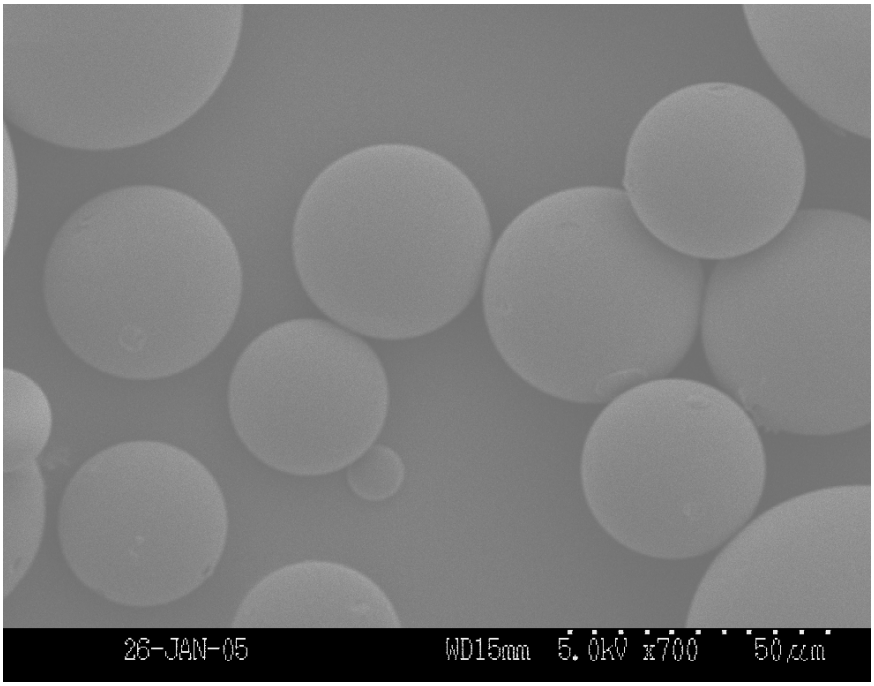


Figure 11: Sieved microspheres used to prepare scaffolds

The initial scaffolds were prepared by placing 50mg of the sieved microspheres into a mold and then heating at 50°C for three days. This temperature was chosen, because it was found to be high enough above the polymer's glass transition temperature of 41.3°C to yield a porous scaffold with reasonable strength, determined qualitatively. Using too high of a temperature, even for a short period of time, yielded a very glass-like scaffold, with very low porosity, measured using a displacement method. As mentioned previously, Archimedes method was used to quantify porosity. However, instead of water as the solvent for immersion, ethanol was used due to the hydrophobicity of the polymer. Using ethanol instead of water resulted in more of the liquid entering the pores of the scaffolds to allow for better measurement [49]. The 50mg scaffolds prepared by heating at 50°C for three days, were of diameter 5.8 ± 0.16 mm with a height of 1.8 ± 0.11 mm. These had an approximate porosity of 70%. The approximate ultimate compressive strength of these scaffolds was 0.2893 ± 0.377 MPa and a compressive modulus of 4.4742 ± 3.69 MPa. Porosity was confirmed by SEM, as shown in Figure 12. These scaffolds showed high amount of porosity, through the fusion of adjacent microspheres. This fusion allowed for the creation of many pores and several pores of 100μm and larger

in size was found. This pore size is required for osteons to build bone in these pores of the scaffold [54].

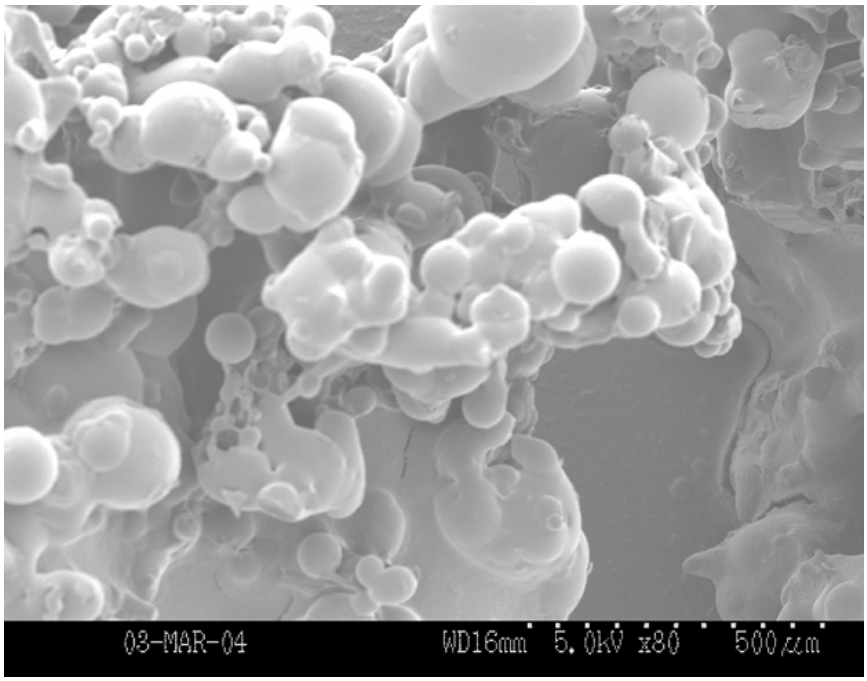


Figure 12: SEM of 50mg scaffold prepared by heating for three days at 50°C

The 50mg scaffolds did seem desirable, until they were put into a cell culture medium prior to starting cell culture experiments. The 50mg scaffolds simply released too much acidic products as they degraded, which could not be controlled with buffers that were safe for cell culture. Thus, smaller scaffolds were necessary. A mass of 25mg of sieved microspheres were placed in the existing mold and heated at 50°C for three days. The result was a very thin scaffold of approximate height of 0.5-0.75mm, with some scaffolds having a ramp-like structure. These samples seemed to have very little strength, so a different mold had to be developed, and it was found that a diameter of 4 mm resulted in a strong scaffold that also had porosity. Scaffolds were prepared by heating at 50°C for one, two or three days. Other temperatures were explored qualitatively for strength to determine if the temperature had been too low or too high. When too high of a temperature was used (greater than 53°C), scaffolds were extremely glassy and seemed to have very little porosity. Temperature too low (less than 45°C) resulted in seemingly high

porosity but crumbled upon any force, including removing them from the mold. Scaffolds were examined with SEM and compression testing to determine the time of heating. Figures 13, 14 and 15 show SEM images from the different heating times. As shown in the SEM images, all scaffold preparations were found to produce porosity, with interconnected pores larger than the minimum 100μm. As shown in Figure 13, the scaffold that was heated for one day, the microspheres were only fused at a few contact points. More fusion was shown after heating for two days, as shown in Figure 14, and further fusion of adjacent microspheres after heating for three days, as shown in Figure 15. Increased fusion between microspheres should translate to higher strength in compression for scaffolds.

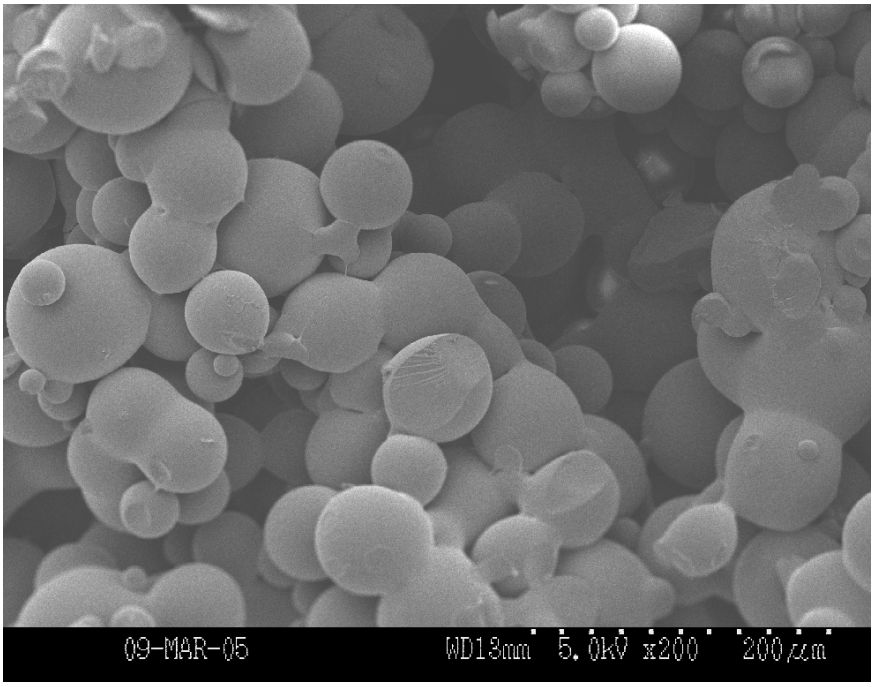


Figure 13: Scaffold prepared with 25mg of microspheres and heated for one day at 50°C

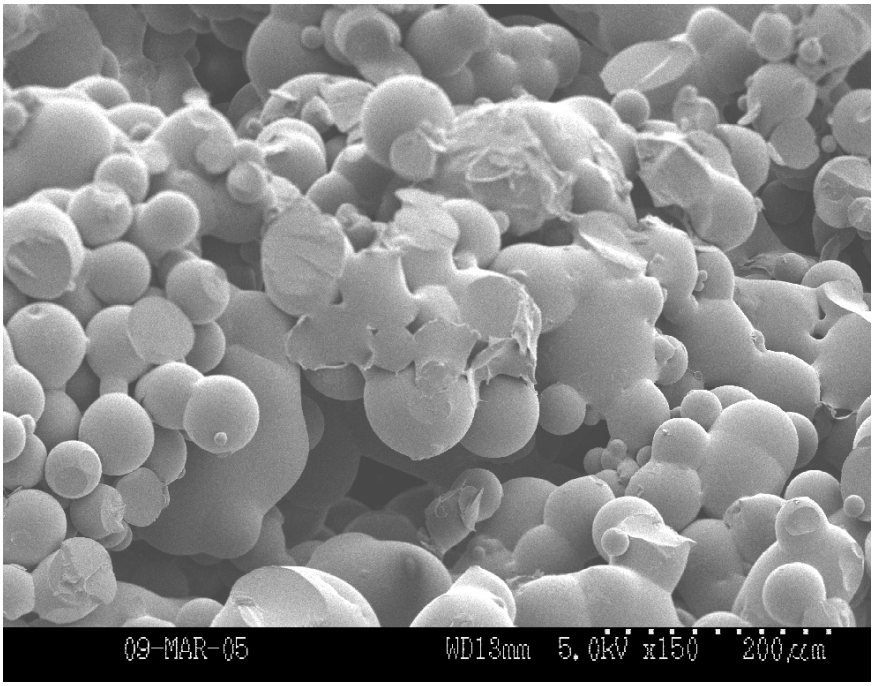


Figure 14: Scaffold prepared with 25mg of microspheres and heated for two days at 50°C

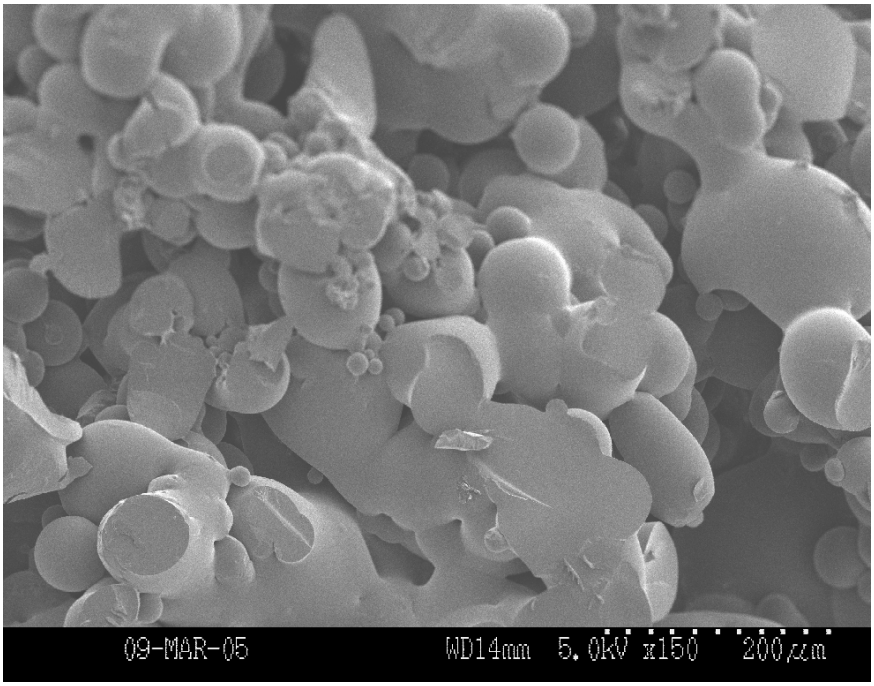


Figure 15: Scaffold prepared with 25mg of microspheres and heated for three days at 50°C

The 25mg scaffolds were tested in compression. Table 1 and Figure 16 summarize the results of this experiment. As the length of time that scaffolds were heated increased, the compressive strength and modulus both increased as adjacent microspheres fuse together. The scaffold heated for three days had a significantly higher compressive strength than the other preparations ($p<0.001$) and the compressive modulus increased with increasing length of heating.

Table 1: Compressive strength and modulus of 25mg PLGA scaffolds heated for one, two or three days at 50°C

Time of Heating	Compressive Strength (MPa)	Compressive Modulus (MPa)
1 day	0.085 ± 0.082	4.702 ± 4.677
2 days	0.292 ± 0.207	5.014 ± 3.061
3 days	0.661 ± 0.119	6.718 ± 1.468

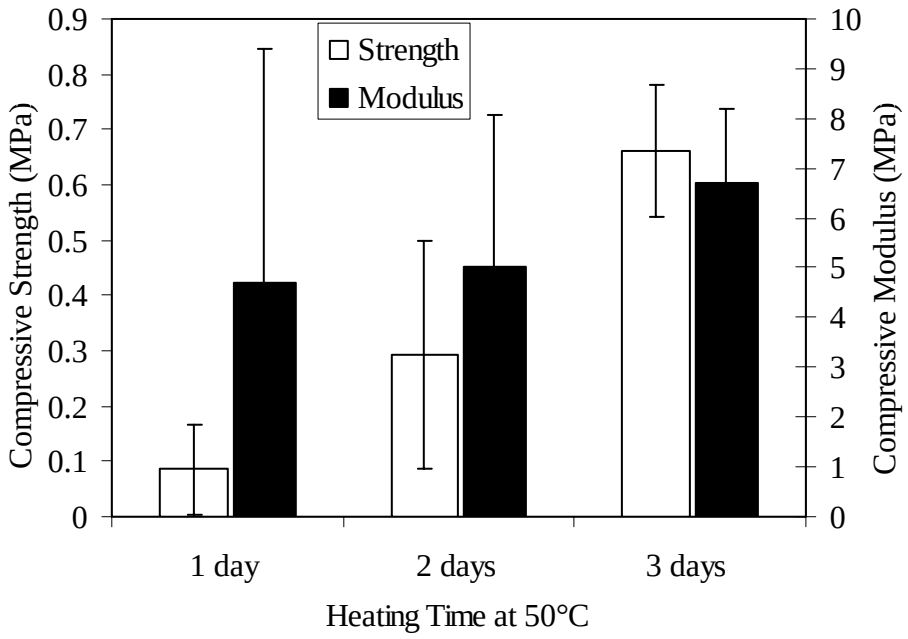


Figure 16: Compression testing results for 25mg scaffolds (*denotes significant difference)

Porosity of the 25mg scaffolds was measured in the same way as the 50mg scaffolds were. As the length of heating increased, the porosity decreased, but no scaffolds had porosity below 70%. There were no significant differences in the porosities. The porosity data for the three different heating times are presented in Table 2.

Table 2: Porosity of 25mg scaffolds heated for one, two or three days at 50°C

Time of Heating	Porosity (%)
1 day	75 ± 10
2 days	74 ± 6
3 days	71 ± 7.5

The surface area was measured with nitrogen gas adsorption. Results of this are shown below in Table 3. The surface area decreased as heating time was increased.

Table 3: Surface area of 25mg scaffolds heated for one, two or three days at 50°C

	Surface Area	
Time of Heating	m^2/g	cm^2/scaffold
1 day	0.035 ± 0.0059	8.76 ± 1.48
2 days	0.012 ± 0.0053	3.13 ± 1.33
3 days	0.0094 ± 0.02	2.35 ± 5.00

Using results of SEM images, compression testing, porosity and surface area scaffolds should be prepared by heating 25mg of PLGA microspheres for three days at 50°C. In addition, acid released by these scaffolds is more easily controlled.

4.2 Surface Modification

The carboxyl groups at the end of the polymer chains were modified with bishydrazides using EDAC/NHS chemistry for both coverslips and scaffolds. Using coverslips coated

with a polymer solution allowed for simple measurements of surface area and ease of preparation.

Coverslips had a surface area of 113mm^2. Equimolar concentrations for each of the spacers were first used to ensure that each of the spacers had the same number of hydrazide groups to interact with PLGA. This allowed examination of the effect of length on the density of spacers could be studied. Using a concentration of 0.057mM, each bishydrazide was successfully attached to the PLGA surface. As shown in Figure 17, the same concentration for each of the spacers resulted in different number of groups. The shorter spacer molecule, oxalic bishydrazide, was bound in much larger quantities, at least five times more hydrazide groups than other treatments, despite an equal number of groups for each bishydrazide. In fact, the oxalic bishydrazide was significantly different ($p < 0.01$) than the other treatments. The other treatments were not significantly different from the others. Based on the differences in binding for each of the different spacer lengths, more concentrations were investigated.

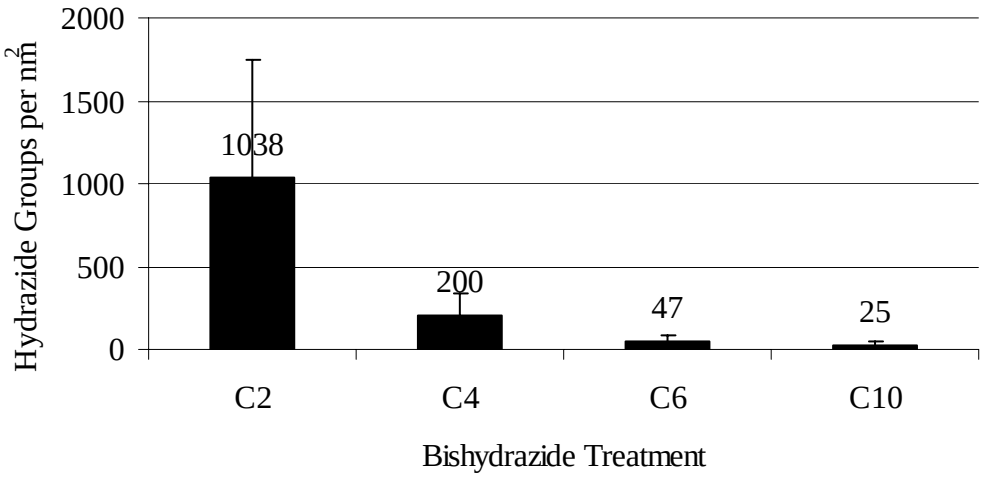


Figure 17: Surface modification using the same concentration for all bishydrazide spacers

Six equimolar concentrations for each of the bishydrazides were investigated so treatments that yield the same number of groups for each of the bishydrazides could be identified. However, due to differences in the lengths of the spacers, equimolar

concentrations over a large range did not yield the same number of groups per surface area. This is demonstrated in Figures 18 and 19 and summarized in Table 4.

A great deal of variability was found in the shortest and longest spacers due to solubility issues with these solutions. For concentrations of 0.018, 0.057 and 0.115mM, the C10 spacer, sebacic bishydrazide produced a significantly greater number of hydrazide groups bound to PLGA ($p<0.05$ compared to 0.018mM, and $p<0.001$ for 0.057mM and 0.115mM). In order to work with this spacer, though, solutions had to be prepared well in advance of use and with rapid spinning to ensure that all of the bishydrazide stayed in solution, and plates were shaken rapidly in order to keep most of the spacer in solution. C2, oxalic bishydrazide, also had slight problems with solubility, but was easier to keep in solution with extended spinning time and rapid plate shaking during the modification.

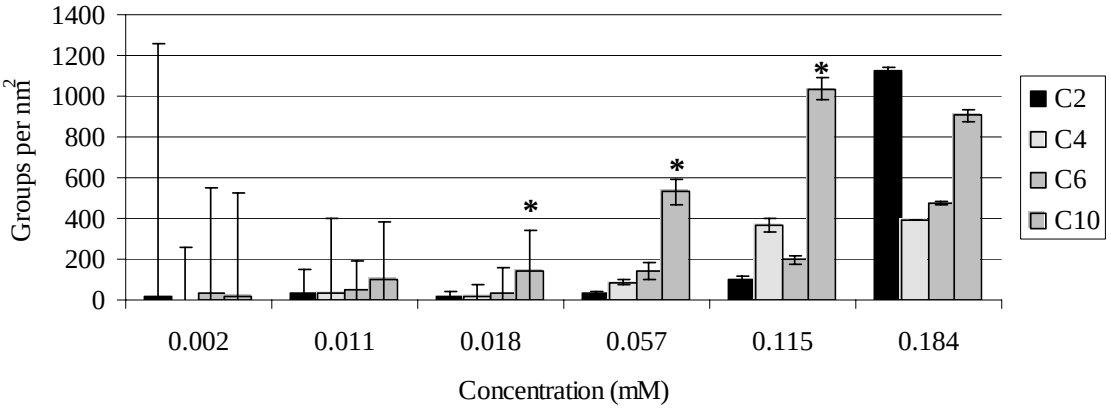


Figure 18: Surface modification of PLGA coverslips with varying concentrations of bishydrazides (*denotes significant difference from other spacers at same concentration)

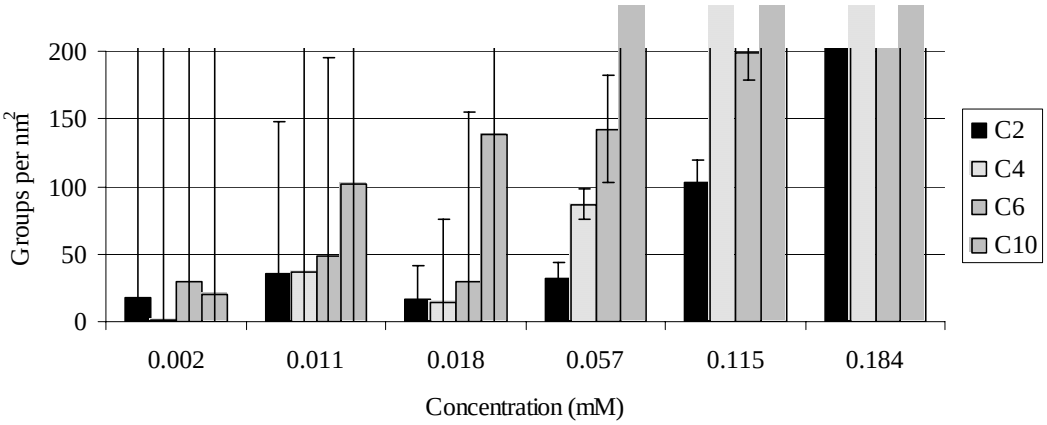


Figure 19: Expanded figure for surface modification of PLGA coverslips with varying concentrations of bishydrazides

Table 4: Summary of number of groups bound to PLGA surface for each of the bishydrazide spacers and each concentration

	Groups per nm^2			
Concentration (mM)	C2	C4	C6	C10
0.002	18	1	30	20
0.011	36	37	48	102
0.018	17	14	30	138
0.057	31	87	143	531
0.115	103	369	199	1036
0.184	1125	390	476	904

Based on the results from using many different concentrations for each of the bishydrazide spacers, concentrations could be chosen that would result in a similar number of hydrazide groups for each spacer length. With an equal number of groups for each of the spacers, the effect of the length on protein attachment could be studied. Using results from previous experiments and experience with the methods, bishydrazide concentrations shown in Table 5 were selected. Using these various concentrations

resulted in comparable number of groups for each of the spacers, as presented in Figure 20. Using different concentrations for each of the different spacers resulted in approximately 50 groups per nm^2 for each of the different treatments of the coverslips. The result was a control of the number of hydrazide groups per unit surface area despite varying lengths between the spacers. None of these groups were significantly different from each other.

Table 5: Concentrations for each of the spacers used to generate an equal number of hydrazide groups for each

Spacer	Concentration (mM)
C2	0.018
C4	0.057
C6	0.018
C10	0.011

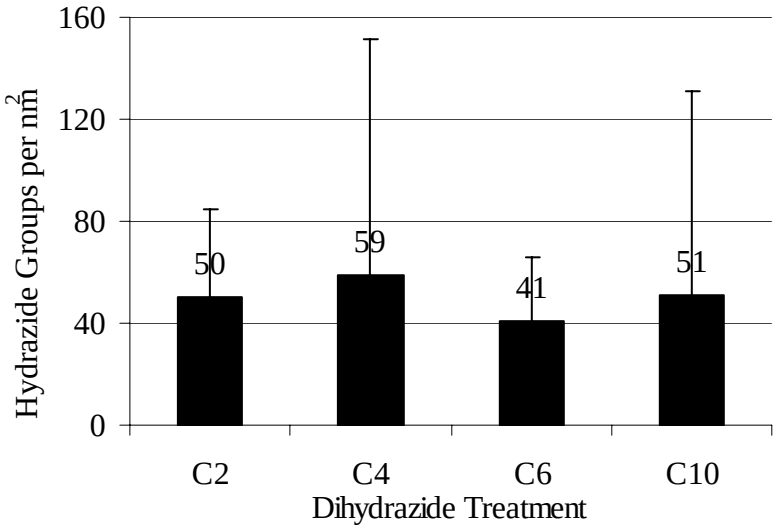


Figure 20: Equal number of groups per surface area for each spacer

4.3 Protein Attachment

4.3.1 VEGF Attachment

The success of binding VEGF to the surface of modified and unmodified PLGA coverslips was assessed using both the quantitative amount of protein bound as well as a technique to measure the accessibility of VEGF for binding to antibody. These results were measured immediately after attachment and after incubation for 12 hours in PBS at 37°C. VEGF was successfully bound to all modified and unmodified surfaces. In this experiment the number of hydrazide groups decreased as spacer length increased.

The amount of protein bound to each surface is presented in Figure 21. The amount of protein bound to coverslips was the highest for the shortest and longest spacer chains, C2 (oxalic) and C10 (sebacic), respectively. The C2 group resulted in a significantly higher amount of protein than the C4 (succinic) or C6 (adipic) ($p<0.05$). The amount of VEGF on the hydrazide-modified surfaces was generally larger than on untreated PLGA. The C2 and C10 modified coverslips had an amount of protein which was significantly different than that of randomly adsorbed protein on PLGA ($p<0.05$). After overnight incubation, the amount of protein bound to the modified surfaces decreased. Also, the variability within each group decreased as this loosely bound protein was reduced. Interestingly, the amount of protein bound to the surface of unmodified PLGA actually increased. The variability for adsorbed protein also increased.

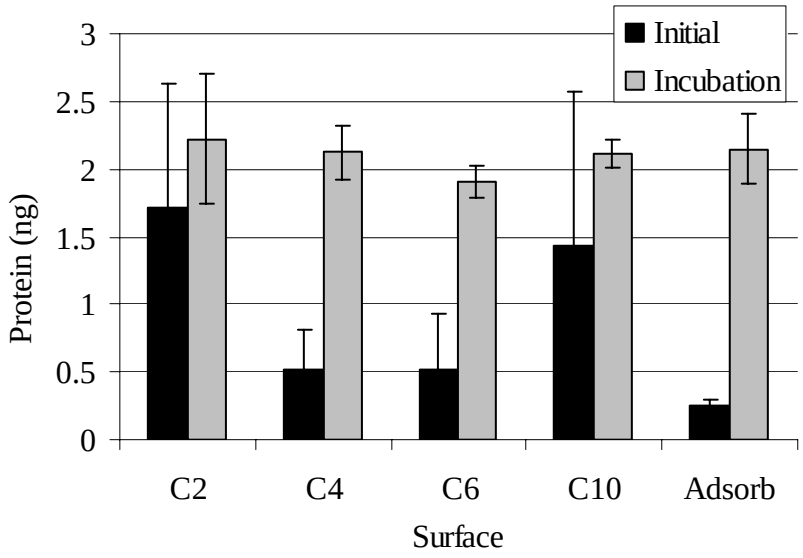


Figure 21: Amount of VEGF bound to modified and unmodified PLGA surfaces initially and following overnight incubation in PBS

The accessibility of VEGF for antibody binding is presented in Figure 22. Antibody binding was the greatest on the C2 modified surface and decreased as the spacer length increased. However, the amount of binding was not significantly different for any of the different treatment groups. A high amount of variability in antibody binding was observed for protein simply adsorbed on untreated PLGA surfaces. The accessibility of VEGF for antibody binding was also measured following overnight incubation. Both the initial and incubation binding results are presented in Figure 22. The accessibility for antibody binding was similar for all surfaces and variability within samples was also decreased following incubation.

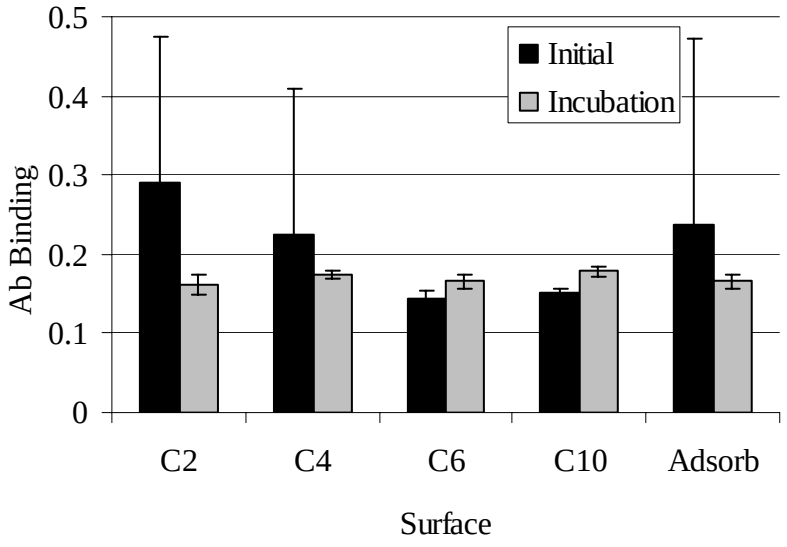


Figure 22: Antibody binding of VEGF bound to modified and unmodified PLGA surfaces initially and following overnight incubation in PBS

A comparison between the initial amounts of VEGF bound to the each surface and the accessibility of VEGF for antibody binding is presented in Figure 23. The shortest spacer, C2 had both the highest amount of bound protein as well as the highest amount of accessibility. Significant variability was seen for these two groups.

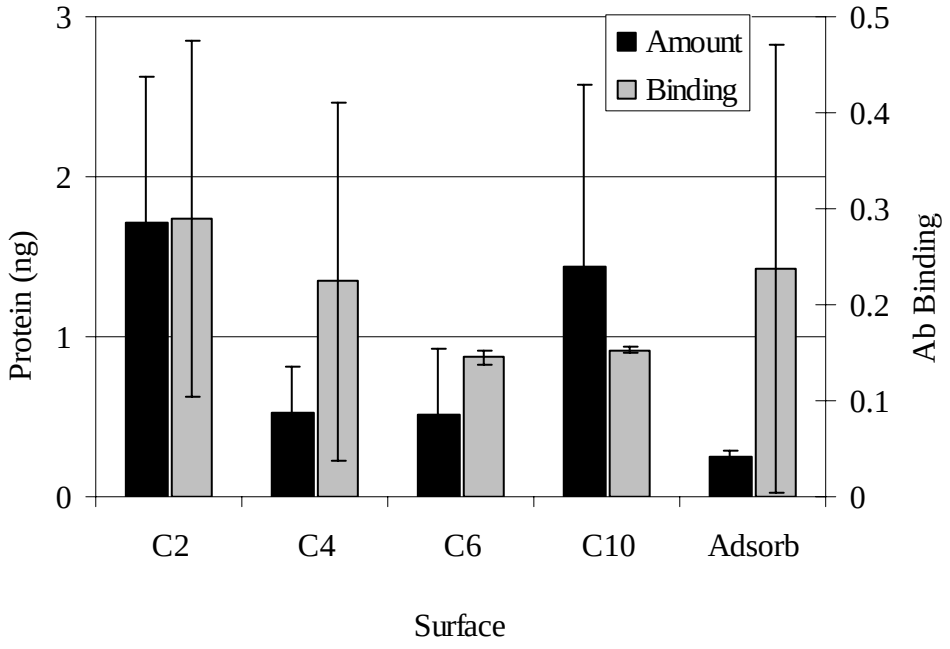


Figure 23: Amount of VEGF and accessibility of bound VEGF when bound to PLGA by bishydrazide spacers immediately following VEGF attachment

Figure 24 summarizes both the amount of bound VEGF as well as the accessibility for binding following 12 hour incubation in PBS at 37°C. All groups showed a similar amount of bound VEGF despite different numbers of hydrazide spacer groups. The accessibility for binding was also similar for all groups. Variability was also decreased with the overnight incubation.

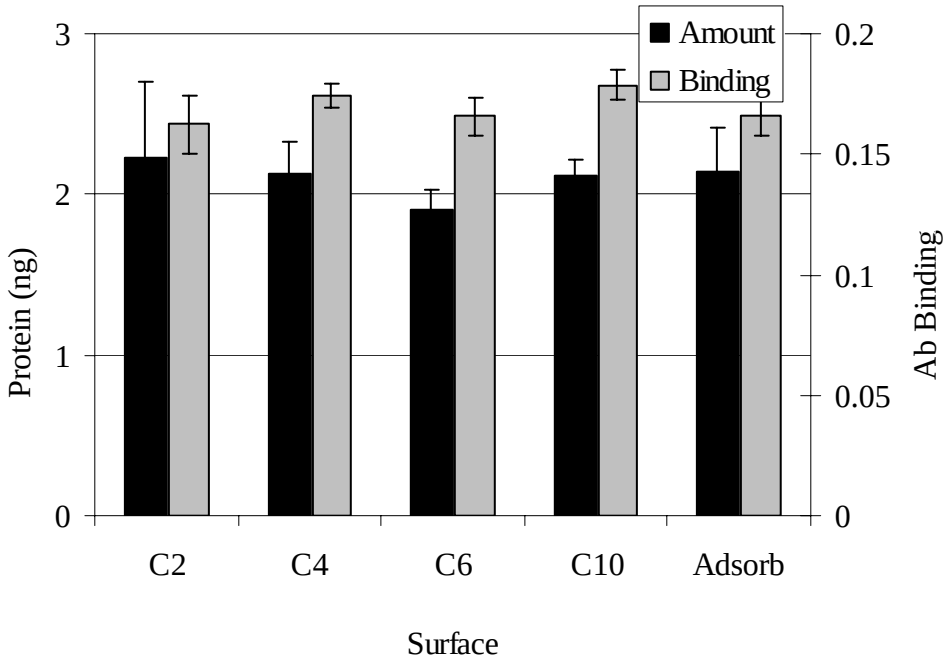


Figure 24: Amount of VEGF and accessibility of bound VEGF when bound to PLGA by bishydrazide spacers after incubation in PBS for 12 hours at 37°C

4.3.2 PTH Attachment

PTH was successfully bound to all modified and unmodified surfaces. The success of binding PTH to the surface of modified and unmodified PLGA coverslips was measured using both a quantitative amount of protein bound as well as a technique to measure the accessibility of PTH for binding to antibody. The modified surfaces were controlled such that all of the surfaces, regardless of the spacer length, had a similar number of groups per surface area, equal to approximately 50 hydrazide groups/nm^2. This allowed the study to determine the effects of the length of spacers on protein attachment.

The amount of PTH bound is presented in Figure 25. The amount of PTH attached to the four hydrazide-derivatized surfaces was statistically the same at about 0.5 µg. The spacer length did not play a role in the amount of protein that was able to bind to the modified

coverslips. PLGA with randomly adsorbed peptide had a similar amount. The use of a spacer in general did not improve protein attachment to PLGA coated coverslips.

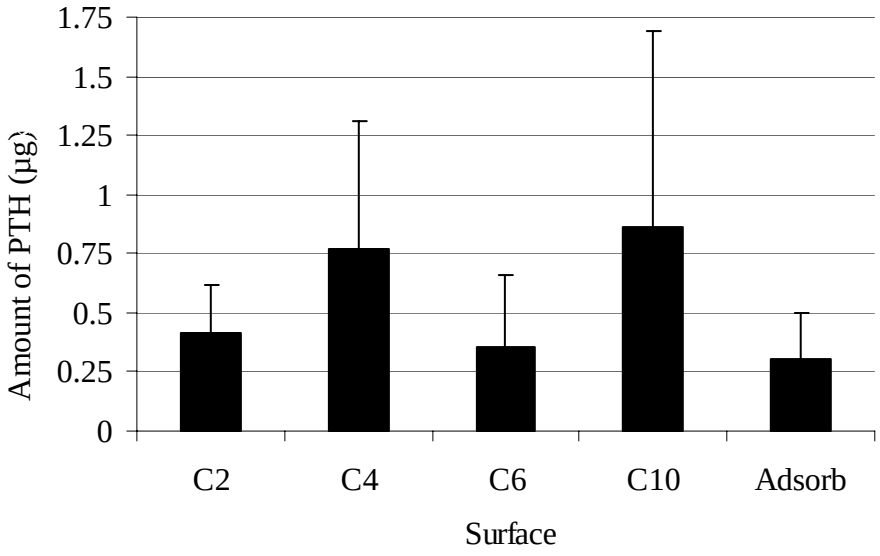


Figure 25: Amount of PTH bound to modified and unmodified surfaces using equal number of hydrazide groups for modification

The PTH antibody binding results are presented in Figure 26. In contrast to the amount of bound protein, the use of a spacer improved accessibility of the N-terminus, which was used for immobilization. Antibody binding to an N-terminal epitope was significantly greater on all four hydrazide-derivatized surfaces compared to the random surface. In contrast, availability of the free C-terminus of PTH was statistically similar on all five surfaces, but with a trend of lower accessibility on the random surface.

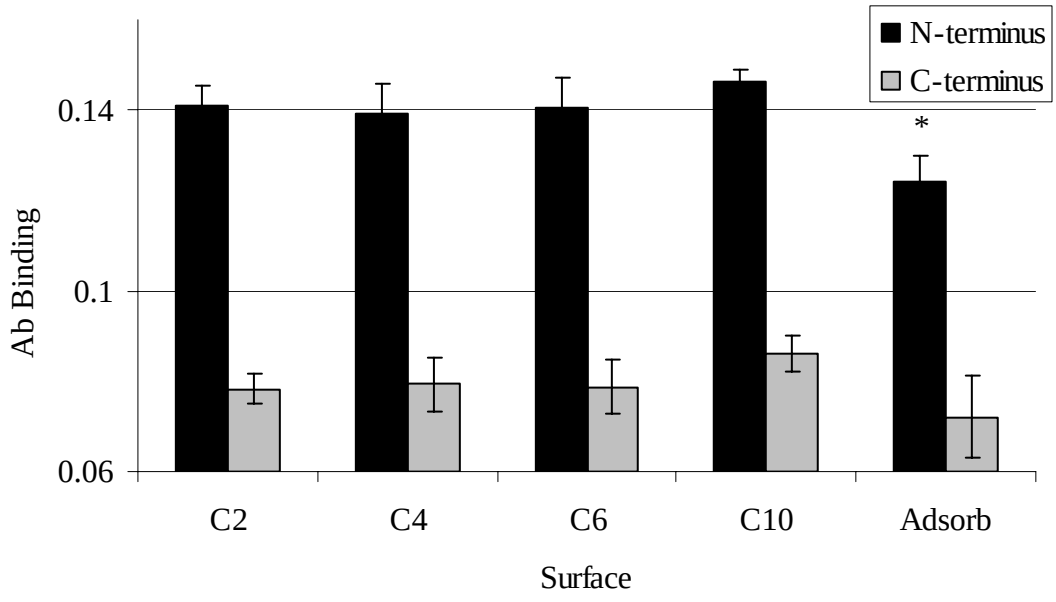


Figure 26: Antibody binding of PTH bound to modified and unmodified surfaces using equal number of hydrazide groups for modification (* denotes significant difference)

5. DISCUSSION

5.1 Materials

Microspheres showed very similar properties to those prepared using a non-loaded double emulsion technique, as reported by Schrier and DeLuca [20, 34]. Microspheres had a general nonporous surface with a spherical shape and varying size ranges. Microspheres were found to require specific conditions of preparation. Simply adding the polymer solution to PVA dropwise at the top of the beaker did not result in spherical particles, because when the polymer is added to solution, the force of the droplet hitting the surface must be high enough to break that drop into microspheres in the solution. Only adding to the top surface of the PVA solution resulted in fragmented and misshapen microspheres. However, if the polymer solution was added directly to the bottom of the PVA solution, the force was high enough to create spherical microspheres with a nonporous surface. As Ma *et al* found, the rate of spinning of the microspheres had an effect on the size range of particles formed [12]. Using a stir rate of 600rpm overnight resulted in microspheres within the desired size range for preparation of scaffolds to create a minimum pore size of 100μm required for bone formation into the scaffold [54].

As previously mentioned, the initial scaffolds investigated were 50mg. When these scaffolds were placed into cell culture medium, they were found to release too much acid too quickly for the buffer to maintain a constant pH, which is required for control of cellular experiments. As polymer degrades it breaks into different monomers and oligomers of lactic and glycolic acids with terminal carboxyl groups exposed following a chemical hydrolysis of ester bonds, in which both the rate of release and amount of acid decreases the pH [42]. The pH must be controlled using a concentration of buffer that is not detrimental to the cells [42, 55]. The amount of acid released from PLGA implants is controlled in the body, but too much acid that is not controlled may cause an inflammatory response and negate the purpose of the specific implant [42]. If the amount of acid present is controlled *in vitro* in cellular experiments, it can be theorized that the amount of acid could then be controlled when the implant is placed *in vivo.* Because of

this problem for *in vitro* experiments, however, the mass of polymer was decreased from 50mg to 25mg. Initially 25mg scaffolds were placed into the same mold that was used to prepare the larger scaffolds. The use of this mold with the same preparation conditions resulted in a very thin scaffold. These thin scaffolds did have seemingly high porosity but were so thin that the strength would have been extremely low, in addition to difficulty in controlling the shape. A majority of these thin scaffolds had a ramp like top surface. Based on this, a new mold that would generate an acceptable size scaffold was prepared.

Scaffolds can be prepared a number of different ways, from salt leaching to gas foaming to simple heating [7, 56]. All of these methods result in stable scaffolds, but since heating is a very simple technique it was used for the preparation in this work. When temperature is increased, thermal fusion of adjacent microspheres at their contact points occurs [7]. The nature of this preparation technique allows for 100% pore interconnectivity due to the behavior microsphere fusion [7]. Even a slight increase in temperature above the glass transition temperature of the polymer can result in a scaffold with high porosity but very little strength. High temperatures result in a very glassy scaffold as expected, with very little porosity but high strength. Time was also found to play a role in the strength and porosity of the scaffolds. The challenge was to find a balance between the mechanical strength of the scaffold and a high porosity. Cancellous bone has been found to have a compressive modulus between 2 and 2000 MPa and approximately a 70% pore volume, depending on where the sample is taken from [7, 27, 54]. The mechanical strength was tested in compression. The 50mg scaffold had a compressive strength of 0.2893 MPa and a modulus of 4.4742 MPa. Borden *et al* reported sintered scaffolds, prepared by heating for 24 hours 5°C above T_g, with a compressive modulus of approximately 349MPa. However, these scaffolds were prepared using a PLGA with a ratio of LA:GA of 58:42 [7]. In another study, Borden *et al* presented the compressive modulus for PLGA with a ratio of LA:GA of 85/15 as approximately 272MPa. These scaffolds were prepared by heating microspheres for four hours at approximately 100°C above T_g [7]. These values are within the range of compressive modulus for cancellous bone, so these scaffolds could be used for a bone replacement. The porosity was 70% which matches the

approximate porosity of natural bone. This is important because bone needs to grow into the scaffold and a high amount of porosity is required.

The same conditions of heating were also explored for the smaller scaffolds. They were heated at 50°C for one, two or three days, and the final scaffold was tested for mechanical strength, porosity and examined using SEM. As expected, as mechanical strength increased the porosity decreased, which was in response to the heating time. All scaffolds appeared to maintain minimum pore sizes of 100μm as seen through SEM images. This is the minimum size pore to allow for bone growth throughout the scaffold which is the only way for the scaffold to be replaced with natural bone. The porosity of the scaffolds decreased with increasing time of heating. As the length of time increased, more adjacent microspheres were able to fuse together to form a structure with less porosity. However, in contrast, the longer heating times resulted in a stronger scaffold from the fusion of microspheres. Based on the high porosity and greater compressive strength, the scaffold that was heated for three days was selected for this study.

5.2 Surface Modification

Many PLGA chains are synthesized with an end-cap, so that there are no exposed functional groups. However, the polymer used in this study was non-endcapped, leaving an exposed carboxyl group at the end of the chain. This made modification of the PLGA chains simple with many and accessibility of functional groups. Surface modification was carried via CDI to couple varying length spacers of bishydrazide groups to the carboxyl group on PLGA. The four different lengths of the spacers varied in their ease of use. Variability is explained by solubility differences between the different spacers, for example, as the length increased the solubility decreased making it difficult to keep the spacer in solution. The increasing length of carbon chains and the use of an acidic solution only make the solubility issues more difficult to control. However, this was remedied by preparing solutions about an hour before needed and maintaining a high rate of stirring or shaking while the solution was in use.

Using the same concentration for each of the spacers resulted in a decreasing number of hydrazide groups with increasing spacer length. Because the concentrations were on a molar basis there were equal numbers of molecules available to react with PLGA for each different bishydrazide. With the decreasing number of groups it can be deduced that more competition between the longer spacers, as well as steric hinderance, resulted in fewer bishydrazide molecules attached. The bishydrazide molecule has a hydrazide group at each end, and with increasing length and less competition in dilute solutions it is possible for both ends of the spacer to attach to an activated carboxyl group at the end of the PLGA chain. These molecules are then not able to react with TNBS in a detection assay and therefore would not be detected. The shorter chains did not seem to have these problems.

Using several concentrations of each spacer allowed for the development for a large matrix of concentration and number of bishydrazide groups. This matrix was developed as a method for controlling the number of groups on any modified surfaces. The ultimate goal was to fix either the density or length independent of one another.

Control of the number of groups was demonstrated through the surface modification of coverslips for the eventual attachment of PTH. Different concentrations were used for each of the spacers to result in an average surface density of hydrazide groups of 50 per nm^2 of nominal surface area. The relative similarity in surface density allowed comparison of the effect of spacer length on peptide immobilization and antibody accessibility. The similarity in surface density of bishydrazide molecules also demonstrated that the length and density of any spacer could be controlled independently of one another. Effectively, the density could be fixed to study the dependence of protein binding on the spacer length, and vice versa.

5.3 Protein Attachment

5.3.1 VEGF Attachment

VEGF was successfully bound to both unmodified and hydrazide-modified PLGA coverslips. When comparing different densities and lengths of the spacers in response to protein attachment, the shortest and longest spacers resulted in the highest amount of protein. The high amount of protein bound to the C2 spacer likely relates to the number of hydrazide groups available for binding since surface density decreased as spacer arm increased. The C10 spacer also had a high amount of protein attached to it, due to the flexibility of the spacer for protein binding. However, it is also important to note that the amount of protein bound to each hydrazide-modified coverslip was higher than the amount of VEGF bound to untreated PLGA. It was then determined that the use of a spacer improved protein attachment regardless of the length or density of hydrazide groups. The amount of protein had been measured at two different time points, both immediately after protein attachment and after incubation overnight in PBS. The amount of protein attached to each coverslip did not change overall, but the variability in the groups did decrease, as was expected. If the protein was covalently bound to the surface of PLGA by means of a spacer molecule, a great deal of protein should not have been lost; however, the loosely bound VEGF molecules were desorbed from the surface. Therefore the use of hydrazide spacers for protein attachment onto PLGA was validated.

The antibody binding was also measured at both time points. The accessibility of protein for binding was the highest on the shortest spacer, oxalic bishydrazide, and decreased with increasing length. These results likely relate not only to the length and flexibility of the spacer but also the number of hydrazide molecules available for binding, since the surface density decreased as the spacer arm increased. Significant variability was found for the protein simply adsorbed onto the untreated PLGA surfaces. After incubation, the antibody binding was similar for all surfaces, and again the variability decreased. The decrease in variability was the result of loss of weakly bound protein from the samples.

Carbohydrate residues on VEGF were successfully oxidized to form aldehyde moieties for interaction with hydrazide groups attached to PLGA. However, in $VEGF_{165}$, the site of carbohydrate oxidation is close to the binding sites on VEGF. The oxidation site is at approximately the 75^{th} amino acid residue and the cell-receptor binding sites are located on both sides of oxidation site at approximately the 50^{th} and 100^{th} amino acids [51]. The oxidation of the carbohydrate residues could then result in some changes in the binding sites of the chain since they are so close. In addition, with the attachment site of the protein to the spacer so close to the binding sites, steric effects could cause some of the cell-receptor binding sites to be unavailable.

Use of a spacer arm between immobilized VEGF and PLGA can result in better availability of the growth factor for antibody binding, which may translate to better interaction with VEGF receptors on cells that encounter the modified surface.

5.3.2 PTH Attachment

For the experiments on PTH attachment, the spacer length was the primary variable under investigation. Based on previous experimentation, different concentrations of the bishydrazides were chosen to result in a similar number of groups on each derivatized surface. This was in order to investigate the effects of the length of different bishydrazides to determine its effect on peptide immobilization and antibody accessibility. Selected concentrations resulted in an average surface density of hydrazide groups of approximately 50 per nm^2 of nominal surface area for each spacer length. When using the similar density of groups, it was found that the amount of protein bound to the surface of each coverslips was approximately equal, as was the amount randomly adsorbed on PLGA.

Antibody binding was measured for two different PTH antibodies, one which measures the accessibility of the C-terminus of PTH and one which measured the N-terminus of PLGA. The C-terminus showed statistically similar binding on all surfaces but a trend of

lower accessibility on the random surface. In contrast, the accessibility of the N-terminus, which was used for immobilization, was significantly greater on all four of the hydrazide-derivatized surfaces in comparison to the random surfaces. This increased antibody binding may translate to better interaction with PTH receptors on cells that encounter the modified surface. Again, binding could have been further investigated with overnight incubation PTH. Most likely, the binding should not change for any of the modified surfaces and decrease for the random PLGA surface, as the protein attached to the spacers should be bound more tightly than the amount simply adsorbed on PLGA.

5.4 Context

The primary objective of this study was to determine the effects of site-directed immobilization of growth factors onto PLGA materials. However, in order to do this, PLGA materials had to be developed according to current literature. PLGA coatings, microspheres and scaffolds were fabricated using a single-emulsion technique and scaffolds were prepared using a sintering method similar to that reported by Borden, Schrier, and Ma [7, 12, 20, 34, 57]. Scaffolds were prepared to have compressive strength and porosity for use as a bone replacement similar to those of trabecular bone, though on the lower end of the range of compressive strength [7, 27, 54]. In order to achieve the goals of the study, coatings and scaffolds were modified to act as delivery vehicles for growth factors using site-directed immobilization.

Non end-capped PLGA provides available carboxyl group for modification of the polymer chain. Site-directed immobilization requires the tailoring of both the biomaterial and biomolecule. The surface modification of PLGA was performed using CDI, which is a widely used method of coupling an amine to a carboxyl group [33, 43, 46]. Bishydrazide spacer molecules were used to covalently attach a protein to the surface of PLGA, rather than randomly adsorb protein onto the polymer surface. Hydrazide modification of surfaces allows for attachment of periodate-activated carbohydrates, as used in this study. The use of the bishydrazide spacer results in more sensitivity to cell

binding sites and correlates to an increase in binding activity in comparison to unmodified surfaces, in a report by Veilleux [58]. Veilleux and Duran performed a study in which polystyrene plates were activated with hydrazide. The plates were then reacted with a periodate-activated alkaline phosphatase that normally would not be reactive towards polystyrene plates. The amount of protein immobilized on the activated surfaces was considerably higher than what would bind to an unmodified surface. In the present study, similar results were also observed in studying the binding accessibility of VEGF and PTH bound to the surface of PLGA using a hydrazide spacer. Currently there have not been many studies in the immobilization of VEGF or PTH onto PLGA scaffolds. Instead, these proteins have primarily been encapsulated into PLGA microspheres for a controlled release and gene therapy. Sofia *et al* used a silk based scaffold for immobilization of PTH hormone, using CDI chemistry. The scaffolds modified with PTH showed increased osteoblasts adhesion as compared to plastic. Thus, immobilization of PTH onto scaffolds was able to elicit a response towards bone regeneration [59]. The present research took the immobilization one step further to actually use site-directed immobilization of VEGF and PTH onto a scaffold through surface modification of PLGA using bishydrazide chemistry.

Using this site-directed immobilization with or without spacer molecules resulted in more protein bound to the modified surface rather than unmodified. Immobilization of growth factors or proteins, whether site-directed or random, results in a higher amount of bound protein as reported by Puleo and Yoo. Puleo *et al* coupled amine groups to the surface of titanium alloy, and then randomly immobilized BMP to the surfaces. This resulted in a higher amount of activity than protein that was simply adsorbed onto the alloy surface [43]. Yoo *et al* reported increase in collagen synthesis from immobilized HA onto the surface of modified PLGA implants. Cellular attachment to the implants was improved [46].

In these present studies, both VEGF and PTH were more accessible for antibody binding, indicating that they would be available for interaction with the receptors on cells. In addition, the covalent attachment of VEGF onto the PLGA surfaces allowed for protein

to remain bound following incubation in PBS. As Schrier reported, growth factor concentrations must be maintained at the site of implant in order to induce a specific cellular response [13, 20, 25]. The use of site-directed immobilization allows for control over the concentration of growth factor on the surface of the implant, and the material properties control the length of time that the growth factor is maintained at the site, as PLGA releases growth factors as it degrades [9, 34, 42, 48].

6. CONCLUSIONS

PLGA materials can be fabricated into coatings, microspheres and scaffolds for implantation into the body depending on the site of implantation. For bone biomaterials, PLGA scaffolds can be very useful because each scaffold can be fabricated to match a specific defect in bone, for example, filling in fracture nonunions. In addition, functional groups at the end of the polymer chain can be utilized for surface modification and growth factor attachment to the material prior to implantation.

Bishydrazide chemistry was used to modify the carboxyl group at the end of the polymer chain. Four different bishydrazides were successfully attached to the PLGA materials. These four spacers were of varying length, and the length and density of the surface groups were studied to determine each variable's effect on protein binding. The use of a spacer improved protein binding rather than protein simply adsorbed onto a PLGA surface. Short and long spacer arms had the highest amount of protein binding, with similar results for accessibility of the protein for antibody binding.

Both VEGF and PTH were used for protein binding measurements. Both of these proteins are very different in their target cells as well as purpose, but this furthers the applications of surface modified PLGA materials for site-directed immobilization of growth factors. Using different growth factors can allow the polymer biomaterials to be applied to type of implant, from vascular to bone implants.

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