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Immobilization of glycoproteins, such as VEGF, on biodegradable substrates

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Abstract

Attachment of growth factors to biodegradable polymers, such as poly(lactide-co-glycolide) (PLGA), may enhance and/or accelerate integration of tissue engineering scaffolds. Although proteins are commonly bound via abundant amino groups, a more selective approach may increase bioactivity of immobilized molecules. In this research, exposed carboxyl groups on acid-terminated PLGA were modified with dihydrazide spacer molecules. The number of hydrazide groups available for subsequent attachment of protein was dependent on dihydrazide length, with shorter molecules present at significantly greater surface densities. The potent angiogenic glycoprotein vascular endothelial growth factor (VEGF) was oxidized with periodate and the aldehyde moieties allowed to react with the hydrazide-derivatized PLGA. Derivatization initially affected the amount of protein bound to the surfaces, but differences were substantially reduced following overnight incubation in saline. More importantly, use of shorter dihydrazide spacers significantly enhanced accessibility of immobilized VEGF for binding neutralizing antibody and soluble VEGF receptor. Furthermore, immobilized growth factor enhanced endothelial cell proliferation, with surfaces having the shortest and longest spacers stimulating greater effects. The present work has not only demonstrated an alternative approach to immobilizing growth factors on biodegradable materials, but the scheme can be used to alter the amount of protein bound as well as its availability for subsequent biointeractions.

Keywords

Protein immobilization; Glycoprotein; PLGA; VEGF

1. Introduction

Generally when the body encounters a biomaterial surface, the result is a repair response rather than a regenerative response. The repair response aims to restore function quickly, but the tissue formed does not mimic the normal structure and properties of the original [1]. Treating a wound site with growth factors can not only accelerate healing, but it may also promote regeneration of more native tissue. Oral delivery of growth factors is not ideal because of low bioavailability resulting from the peptide/protein's enzymatic degradation and poor absorption [2], and intravenous delivery of growth factors is not effective because of the short plasma half-life [3]. Therefore, local delivery of bioactive molecules is needed.

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The simplest way to deliver biomolecules to the tissue–implant interface is by dipping the material in a solution of protein before inserting it. Although some encouraging results have been reported [4–6], a major drawback with the adsorption method is that it provides little, if any, control over delivery, including retention and orientation, of molecules. Proteins are initially retained on the surface by weak physisorption forces, and then, depending on the implant microenvironment, which varies among anatomical sites and among patients, the molecules desorb from the surface in an uncontrolled manner to interact with cells.

An alternative approach is to chemically attach biomolecules to surfaces. Adhesive peptides, such as the Arg-Gly-Asp (RGD) sequence that binds to cell surface receptors of the integrin superfamily, have been the focus of much attention (e.g. [7–9]). Immobilization of proteins, such as growth factors, on the surface may provide more control over cell–biomaterial interactions. Whereas RGD peptides primarily mediate adhesion of cells to substrates, immobilized growth factors may be able to modulate subsequent cell functions, such as proliferation, differentiation, and activity, on biomaterial surfaces.

Most schemes used for immobilizing biomolecules involve formation of covalent bonds between surface-exposed functional groups of amino acids with suitable substrates [10]. Moieties commonly used for attachment include amino, carboxyl, and thiol groups. However, most proteins contain several of each type of these reactive groups. In contrast, proteins and peptides do not naturally contain aldehyde groups. Thus, introduction of aldehyde groups, such as by oxidation of carbohydrates, offers an alternative approach to tethering biomolecules on biomaterials.

Glycoproteins, such as vascular endothelial growth factor (VEGF), possess oligosaccharide chains that can be used for immobilization. VEGF is a key regulator of angiogenesis [11]. This 42 kDa glycoprotein is involved in all phases of neovascularization, including enzymatic degradation of the extracellular matrix, migration and proliferation of endothelial cells, and organization of the cells into tubules. Several groups have investigated use of VEGF for controlling tissue repair and tissue–biomaterial interactions in applications such as arterial graft endothelialization [12], bone grafting [13], in-stent intimal formation [14], and limb [15] and myocardial ischemia [16].

The objective of this study was to explore an alternative approach to immobilize growth factors on biodegradable surfaces. In particular, a method for binding glycoproteins to polymers was used. The amount, accessibility, and bioactivity of VEGF bound to poly(lactic-co-glycolic acid) (PLGA) were determined.

2. Experimental procedures

2.1 Surface preparation

Non-end-capped (acid-terminated) PLGA (5050 DL 2A; Alkermes, Wilmington, OH) was used in these studies. Although porous scaffolds are also being examined, results for PLGA-coated coverslips are presented because their more uniform surfaces enabled more accurate comparison between treatments. Coverslips (12 mm diameter) were coated with approximately 30 μ l of 10% w/v PLGA solution in methylene chloride and allowed to dry overnight in vacuum; all samples had comparable surface area.

2.2 Surface modification

Four dihydrazides of varying length were investigated as spacer molecules: oxalic (Aldrich, Milwaukee, WI), succinic (Aldrich), adipic (Sigma, St Louis, MO), and sebacic dihydrazide (TCI America, Portland, OR). Each of these has the same basic backbone, with a carbon chain of different length at its center. The spacer arms were 2 (oxalic; C2), 4 (succinic; C4), 6 (adipic;

C6), and 10 (sebacic; C10) carbon atoms long (Fig. 1). Based on results from a pilot study, a dihydrazide concentration of 0.057 mM dissolved in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES; Sigma) at a pH of 6.0 was used. Samples were placed in wells of a 24-well plate along with 0.5 ml of one of the dihydrazide solutions. To activate the surfaces, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC; Sigma) and N-hydroxysuccinimide (NHS; Fluka, Buchs, Germany) were added. This solution was prepared to contain EDAC and NHS at a molar ratio of 5:2 in 0.1M MES, pH = 6.0 [17,18]. After adding 0.5 ml of the solution, plates were gently shaken at room temperature for 2 h. All liquid was then aspirated from the plate, and the samples were rinsed three times with deionized water. The derivatization reactions are shown in Fig. 2.

2.3 Quantification of hydrazide groups

To measure the number of hydrazide groups available on each PLGA sample, surfaces were reacted with 0.1% 2,4,6-trinitrobenzene sulfonic acid (Sigma) in 3% sodium borate solution at 70°C for 5 min. Hydrolysis with 1 M NaOH at 70°C for 10 min produced a yellow-orange color, measured at 450 nm, that was proportional to the number of trinitrophenyl groups and therefore to the number of dihydrazide groups [18]. Separate standard curves were generated for each dihydrazide, because their presence causes a shift in the color spectrum of the product.

2.4 Protein attachment

Glycosylated rhVEGF₁₆₅ (R&D Systems, Minneapolis, MN; expressed in Sf21 insect cells) was reconstituted in PBS at a concentration of 40 µg ml⁻¹. Oxidation was carried out by allowing 250 µl of 10 mM periodic acid (Aldrich) to react with 250 µl of the VEGF solution in the dark at room temperature for 45 min. The reaction was quenched by adding 500 µl glycerol (Sigma). The oxidized VEGF was purified using 30 kDa cutoff centrifugal filters (Millipore, Billerica, MA). After the final rinse, VEGF was resuspended first in 500 µl of PBS before increasing the volume to 10 ml. A volume of 250 µl of the purified, oxidized VEGF (approximately 0.1 µg) was added to each PLGA sample and allowed to react at room temperature for 45 min. Rather than examine the dose-dependent effects of immobilized VEGF, the present study bound a maximal amount on the surface to enable focus on consequences of the immobilization scheme on availability and activity of protein. VEGF was also randomly and directly bound to carbodiimide-activated PLGA surfaces by interaction of 250 µl of unoxidized VEGF solution with each sample; unoxidized protein was used to reduce the potential for crosslinking of VEGF molecules or binding to the antibody or receptor used for measuring Binding Accessibility. Except for the soluble receptor and bioactivity experiments, two sets of samples were prepared: one was immediately used for analysis, and the other was incubated in PBS at 37°C overnight before analysis. The approach for glycoprotein oxidation and attachment is shown in Fig. 3.

2.5 Protein detection

The amount of VEGF protein bound to PLGA surfaces was measured using a MicroBCA assay kit (Pierce, Rockford, IL) following the manufacturer's instructions. Immobilized VEGF was allowed to react with a working reagent solution that undergoes a color change, measured at 570 nm, in the presence of protein. Standard curves were generated using serial dilutions of bovine serum albumin (BSA).

2.6 Binding accessibility

The availability of VEGF for potential binding to cells was assessed through binding of: (1) a neutralizing antibody and (2) soluble VEGF receptor. To prevent nonspecific binding to surfaces, all samples were first blocked with a solution of PBS containing 0.5% BSA and 0.05%

Tween 20. For sVEGF receptor, samples were examined only after overnight incubation to remove weakly bound protein.

Neutralizing antibody—A primary neutralizing 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 0.5 ml volume of this primary antibody was allowed to react with VEGF-treated and control samples at 37°C for 30 min and then washed three times with PBS containing 0.05% Tween 20 (PBST). A secondary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma), was diluted in PBS at a ratio of 1:500, and 0.5 ml of the antibody solution was added to each sample and allowed to bind at 37°C for 45 min. After washing three times with PBST, a solution of 0.1% Sigma 104 phosphatase (Sigma) in 10% diethanolamine (Chempure, Houston, TX) at pH 9.8 was reacted with the samples for a minimum of 20 min, but ranging up to 60 min, to ensure maximum color development, which was measured at a wavelength of 410 nm.

Soluble VEGF receptor—Soluble recombinant VEGF R2/Fc chimera (R&D Systems) was reconstituted in PBS and diluted to 40 ng ml⁻¹. A 0.5 ml volume of sVEGF R2 was incubated with VEGF-treated and control samples at 37°C for 30 min. After washing three times with PBS containing 0.05% Tween 20 (PBST), 0.5 ml of alkaline phosphatase-conjugated goat anti-human IgG (Fc specific) (Sigma; diluted 1:40,000) was added to each sample and allowed to bind at 37°C for 45 min. Color was developed as described previously for the neutralizing antibody.

2.7 Bioactivity

Biological activity of immobilized VEGF was determined by measuring mitogenesis of endothelial cells [19]. Bovine aortic endothelial cells were seeded onto VEGF-treated samples at a density of 5000 cells per well in DMEM supplemented with 1% FBS (GIBCO, Gaithersburg, MD). For comparison, cells seeded on unmodified PLGA were cultured in either low serum medium only or medium supplemented with 10 ng ml⁻¹ of VEGF. Soluble oxidized VEGF could not be tested directly because of its binding to proteins in the serum and on the cell surface. After 3 d at 37°C, cell growth was assessed via DNA content. Cells were rinsed twice with PBS and then lysed by sonication in a high salt solution (0.05M NaH₂PO₄, 2M NaCl, and 2mM EDTA). DNA standards were prepared by serial diluting calf thymus DNA in the high salt solution. Hoechst 33258 (final concentration, 0.5 µg ml⁻¹; Sigma) was added to DNA standards and samples and allowed to react in the dark for 10 min. DNA contents were determined by measuring fluorescence (λ_{ex} = 356 nm, λ_{em} = 458 nm) and then normalized to growth on derivatized PLGA, which yielded results comparable to untreated, control polymer.

2.8 Statistical analysis

Data are presented at mean $\pm$ standard deviation. A minimum of six replicates was used for each experiment. One-way analysis of variance (ANOVA) was conducted using InStat (Graphpad Software, San Diego, CA). Post-hoc comparisons were made using the Tukey–Kramer test when the p -value was significant ($p < 0.05$).

3. Results

As shown in Fig. 4, using the same molar concentration of each dihydrazide spacer molecule resulted in different numbers of hydrazide groups available for subsequent binding to protein. The overall trend was for decreasing availability of functional groups with increasing length of the spacer. With the shortest spacer molecule, oxalic dihydrazide (C2), more than five times larger quantities were bound compared to the other spacers ($p < 0.01$). The numbers of hydrazide groups for the other spacer molecules were not statistically significantly different.

The amount of protein bound to each surface is presented in Fig. 5. Initially, the greatest quantity of protein bound to samples derivatized with the shortest and longest dihydrazide spacer chains, C2 (oxalic) and C10 (sebacic), respectively. The C2 molecule resulted in binding of significantly more protein than did the C4 (succinic) or C6 (adipic) spacers ($p < 0.05$). The amount of VEGF on the hydrazide-modified surfaces was generally larger than on underivatized PLGA. The C2- and C10-modified samples had an amount of protein that was significantly different from that of protein randomly bound 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.

The availability of immobilized VEGF for antibody binding is shown in Fig. 6a. Initial antibody binding was greatest on the C2-modified surfaces and decreased as the spacer length increased ($p < 0.05$). However, the extent of binding was not significantly different for any of the other treatment groups, although a slight increase was observed for C10-derivatized PLGA. Even though the initial mean level of antibody binding to randomly attached VEGF was intermediate between that for the C2 and C6/C10 surfaces, variability in binding was quite large. Following overnight incubation in buffer, antibody binding decreased for the C2 and C4 surfaces but was slightly increased for the C6 and C10 surfaces, however similar overall trends in antibody accessibility were retained. The increase was statistically significant for C10 ($p < 0.01$). On PLGA with randomly bound VEGF, antibody accessibility was dramatically reduced to a level significantly below that for all of the hydrazide-modified surfaces after incubation ($p < 0.01$).

Patterns for binding of sVEGF R2 to VEGF immobilized on PLGA were similar to those observed for interaction with a neutralizing antibody (Fig. 6b). Again, binding was greatest on surfaces with the C2 molecule as spacer ($p < 0.01$) and least following direct, random attachment to the surface ($p < 0.05$). Binding to VEGF on the C4, C6, and C10 surfaces, although showing a trend of increases for the shorter and longer chains, were statistically insignificantly different.

Endothelial cell proliferation on VEGF-modified surfaces largely followed results from binding of the neutralizing antibody and soluble receptor (Fig. 7). Treatment with soluble growth factor stimulated the greatest response (at least $p < 0.05$). Comparing the surface treatments, VEGF bound via the C2 spacer resulted in significantly greater DNA contents than did the C4 ($p < 0.01$), C6 ($p < 0.5$), and random immobilization ($p < 0.001$) surfaces. The effect of the C4 surfaces was comparable to that of the C6 and PLGA surfaces. VEGF randomly and directly bound to PLGA yielded a significantly lower response than did C10-derivatized surfaces ($p < 0.01$).

4. Discussion

VEGF was bound to both unmodified and hydrazide-modified PLGA surfaces. Immobilization was assessed by quantifying the amount of protein attached as well as measuring the accessibility of VEGF for binding a neutralizing antibody and soluble receptor. The ultimate test was assessment of bioactivity. Although use of spacer molecules had some benefit for the amount of protein attached, the primary effect was to enhance accessibility of the immobilized growth factor for stimulating proliferation of endothelial cells.

Although a comprehensive discussion of protein and peptide immobilization is beyond the scope of the present paper, excellent reviews are available (e.g. [20–22]). Biomolecules are commonly bound to surfaces via amino, carboxyl, or thiol groups on the protein and/or the substrate. Carbodiimide chemistry, which activates carboxylic acids that then react with nucleophiles, such as amines, results in an amide bond but without a spacer between the two reactive groups. Spacer molecules can be introduced by derivatizing the surface, such as with

aminoalkylsilanes or self-assembled monolayers, or by using a variety of crosslinking chemicals. Peptides containing terminal cysteine residues have been attached to surfaces using heterobifunctional crosslinkers possessing thiol- and amine-reactive moieties. Strategies to achieve “site-directed” immobilization have even employed recombinant DNA methodology to introduce selective binding sites in peptides and proteins.

The present approach to immobilizing growth factors on biodegradable substrates was adapted from methodology for making immunoaffinity columns [23,24]. This involves cleaving diols in carbohydrate chains by reaction with periodate to form reactive aldehyde groups, which will readily bind to free amino or hydrazide groups. In the present work, hydrazides were selected because they allow binding at neutral pH to form hydrazone bonds [23]. If desired, the hydrazone bond can be subsequently reduced, such as by treatment with sodium cyanoborohydride, to stabilize them in acidic environments, such as may be found during wound healing. Because the method does not involve carboxylate or amino groups on the protein, the molecule’s net charge and consequent effects on conformation are not altered. The approach described is amenable for use with any glycoprotein. VEGF was selected for demonstrating the immobilization scheme.

The most abundant form of VEGF is a 165 residue protein with an N-glycosylation site at Asn74 [25]. Studies using site-directed mutagenesis have shown that the carbohydrates do not affect function of the growth factor, but they are needed for secretion of the protein [26]. The glycosylation site lies between two receptor-binding regions, one for VEGFR-1 (Flt-1) toward the N-terminus (amino acids 63–67) and another for VEGFR-2 (KDR/Flk-1) toward the C-terminus (amino acids 82–93) [11,25]. Because the VEGF used in the present work was expressed in insect cells rather than bacteria, it possesses carbohydrate moieties near the middle of the chain that were oxidized to aldehyde groups.

Based on how biological effects can occur via nondiffusible signaling, the term “artificial juxtacrine stimulation” has been used to describe the way immobilized biomolecules elicit events [22]. Although both soluble and immobilized molecules bind to receptors on cells, their effects differ. After binding soluble molecules, aggregated receptors are internalized and subsequently recycled or degraded. Immobilization prevents internalization and degradation and can inhibit down-regulation. Related to these differences, two important factors that affect the amount and activity of immobilized proteins are the surface density and length of spacer linking the biomolecule and substrate. Appropriate spacers will achieve high local concentrations, achieve multivalent interactions with cell surface receptors, and permit lateral diffusion and aggregation of receptors in the plane of the membrane [22]. Improper spacing of the proteins or insufficient distance from the surface can lead to steric hindrance and decreased bioactivity. Length of spacer was the focus of this work. Additional research is investigating effects of surface density of spacer molecules on growth factor binding and accessibility. Dihydrazides were selected for the present studies because of the ease of reaction with aldehydes at neutral pH as well as the availability of molecules differing in only the length of the hydrocarbon chain spanning the two hydrazide functional groups.

When comparing the effects of different spacer lengths, the shortest and longest spacers initially resulted in attachment of the most protein. Even though a significantly greater surface density of hydrazide groups was measured on C2 surfaces, it did not result in orders of magnitude more immobilized VEGF, most likely because of steric effects limiting interaction of protein molecules with the abundant functional groups. This is supported by the high area density of C2 molecules (20 nm^{-2}) exceeding a monolayer, likely because of swelling of the surface and derivatization of subsurface layers. Based on an estimated density of 23 carboxylic acid groups per nm^2 (data not shown), 1.4% (for C10) to 90% (for C2) of the hydrazides were subsequently used in the coupling reaction. For the longer sebacic dihydrazide (C10) chains, flexibility can

be a problem for surface modification, with the chain cyclizing to have both terminal hydrazide groups bound to activated carboxyl groups on the acid-terminated PLGA substrate, which then exposes the hydrophobic middle of the spacer molecule. This can lead to nonspecific hydrophobic interactions between protein and the C10-modified surface, more so than on the unmodified, carboxylic PLGA substrates. Thus, during overnight incubation in simulated physiological conditions, weakly and nonspecifically bound protein desorbed, leaving a significant amount of stably bonded VEGF. In contrast, the short chain oxalic dihydrazide (C2) molecules do not have the flexibility to bend over and cover the hydrophobic PLGA surface. They do, however, provide a high surface density of carbonyl groups that can form a large number of hydrogen bonds with VEGF molecules. The weakly and nonspecifically bound protein was lost during incubation in saline, again leaving the covalently bonded protein. To avoid adverse effects on the bound protein, more stringent washing conditions were not used immediately following immobilization. The smaller amount of unoxidized VEGF linked directly to carboxyl-terminated PLGA is similar to reduced binding of VEGF to acidic gelatin compared with other growth factors [27]. Because hydrophobic interactions also play a role in protein adsorption to PLGA, only a portion of the VEGF was lost during incubation in saline.

More important than the amount of a growth factor tethered to a surface is its availability for stimulating appropriate cellular responses. As a first step and to simplify determination of whether VEGF is accessible once immobilized on PLGA, binding of a neutralizing antibody and a larger soluble VEGF receptor was measured. To neutralize bioactivity of VEGF, the antibody must interfere with binding to VEGF receptors, the binding sites for which are on both sides of the N-glycosylation site at Asn74. The soluble receptor chimera used contains the extracellular domain of VEGF R2 (KDR/Flk-1), which is involved in mitogenesis of endothelial cells [25].

Initially, antibody binding was highest for VEGF immobilized via the shortest spacer, oxalic dihydrazide, and decreased with increasing length. As with findings for the amount of protein attached, these availability results likely relate to the length and flexibility of the spacer, which greatly influence the steric accessibility of the epitope for antibody binding. Whereas longer spacers would be expected to hold protein molecules further from the surface and therefore to increase accessibility of the antibody binding site, the greater flexibility reduced availability of VEGF. Bending of the central portion of the longer dihydrazide may have obscured the binding epitope. Desorption of weakly bound protein during overnight incubation in saline decreased the number of VEGF molecules on the surface, and therefore the amount of antibody bound on C2 surfaces was diminished. Although the total levels of antibody were reduced, trends in spacer-related differences were retained. Following immersion, molecular rearrangements on the C6 and C10 surfaces resulted in slight increases in accessibility of VEGF molecules for the antibodies. Similar trends were observed for binding of soluble VEGF R2 to the modified surfaces, with C2 spacer yielding the highest level of accessibility.

Substantial antibody binding was observed for VEGF randomly immobilized on PLGA via any available lysine residue. Even though some molecules were oriented so as to mask the binding epitope, some protein molecules nevertheless presented the binding site without much obstruction. After overnight incubation, however, the weakly and nonspecifically bound VEGF molecules, which previously displayed the antibody binding site, were lost. In contrast, VEGF immobilized via the spacer molecules was retained on the surface with a superior mode of presentation. This is supported by the significantly lower binding of sVEGF R2 to randomly immobilized VEGF.

Although enhanced binding of soluble molecules to VEGF-modified PLGA was observed, the most important determination was whether proliferation of endothelial cells would occur. Even with the increased variability associated with bioassays, the present results demonstrated that

VEGF immobilized by binding its carbohydrate residues to dihydrazide spacers on PLGA-stimulated mitogenesis. In this short-term assay, the effect was up to five times greater than when VEGF was randomly immobilized to the polymer (50% increase for C2 surfaces compared to the 10% increase for direct attachment). Preliminary studies had shown that neither endothelial cells nor osteoblastic cells grew differently on derivatized PLGA (but without VEGF) compared to control polymer (data not shown). As expected, however, exposure of cells to soluble growth factor was a more effective stimulator of mitogenesis. As described previously, the carbohydrate chains of VEGF are attached to an asparagine residue lying between the two receptor binding sites. In combination with the much larger size of cells compared to the antibody and soluble receptor used to initially evaluate accessibility, steric hindrance undoubtedly decreased activity of the immobilized protein. Nonetheless, immobilization on biodegradable substrates enables retention of the growth factor in a desired site while also maintaining biological activity. Furthermore, the location of carbohydrates at different sites (e.g., more distant from the receptor-binding domain) on other growth factors may allow even greater bioactivity.

5. Conclusion

Delivery of growth factors directly to the tissue-implant interface has the potential to improve healing and lead to regeneration of tissue with a more natural composition and structure. One approach to delivering the biomolecules is by immobilization on biodegradable substrates. The present work has demonstrated an alternative approach to binding growth factors on biomaterials that avoids use of ubiquitous amino and carboxylate groups. Generation of aldehydes by oxidation of polysaccharide residues on the glycoprotein VEGF enabled stable binding to hydrazide-derivatized PLGA. Use of different dihydrazide spacer molecules altered the amount of protein bound as well as availability of the growth factor for antibody and soluble receptor binding. This finding of improved accessibility compared to randomly immobilized VEGF was directly related to better interaction with VEGF receptors on endothelial cells and therefore to enhanced proliferation of cells seeded on the modified surfaces.

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Oxalic dihydrazide
(C2, n=0)

Succinic dihydrazide
(C4, n=2)

Adipic dihydrazide
(C6, n=4)

Sebacic dihydrazide
(C10, n=8)

Figure 1.
Structure of dihydrazide spacer molecules having increasing length.

Figure 2.
Idealized scheme for dihydrazide attachment to PLGA.

Figure 3.
Idealized scheme for VEGF attachment to dihydrazide-modified PLGA.

Figure 4. Surface density of hydrazide groups available on PLGA following derivatization with dihydrazide spacers of increasing length.

Figure 5. Amount of VEGF bound to hydrazide-derivatized PLGA and directly on carbodiimide-activated PLGA surfaces initially and following overnight incubation in PBS.

Figure 6. Accessibility of VEGF immobilized on hydrazide-derivatized PLGA and directly on carbodiimide-activated PLGA surfaces. (a) Binding of neutralizing antibody before and after overnight incubation in PBS. (b) Binding of sVEGF R2/Fc chimera following overnight incubation in PBS.

Figure 7. Proliferation of endothelial cells seeded on VEGF bound to hydrazide-derivatized PLGA and directly on carbodiimide-activated PLGA surfaces following overnight incubation in PBS. For comparison, cells were also incubated with 10 ng ml^{-1} soluble VEGF.