Comparison of fibroblast and vascular cell adhesion to nano-structured poly(lactic-co-glycolic acid) films

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Abstract: The success of small diameter vascular grafts may be attributed to the ability to accurately mimic the nano-structured topography of extra-cellular matrix components of natural vascular tissue. Using this knowledge, the goal of the present study was to develop synthetic biomaterials that promote vascular cell adhesion and growth, while subsequently limiting fibrous tissue formation. For this purpose, poly(lactic-co-glycolic acid) (PLGA) with increased nanometer surface roughness was created by treating the surfaces of conventional PLGA with NaOH. Cell experiments on these surfaces indicated that nano-structured PLGA enhanced vascular smooth muscle cell adhesion and growth, while decreasing endothelial cell and fibroblast adhesion and growth, compared to their conventional counterparts. These favorable results were attributed to the selective adsorption of vitronectin. In combination, results of the present in vitro study provided evidence that nano-structured surface features have the potential to significantly improve the efficacy of small diameter vascular implants.

Key words: Nano-dimensional, vascular cells, biomaterial, cell growth, adhesion, PLGA.

INTRODUCTION

When implanted, a biomaterial initiates a complex reaction by the body, which is dependent on material factors such as surface chemistry, chemical composition, and topography (to name just a few). This reaction can lead to rejection, fibrous encapsulation, or tissue integration. Designers of implants must, therefore, factor in and attempt to control the body's reaction to a biomaterial by selecting material parameters (topography, chemistry, composition, etc.) that promote the desired response.

In the case of vascular grafts, this desired response is commonly referred to as graft healing. This process includes fibrin deposition/degradation, monocyte-macrophage recruitment, and cell layer generation, with the ultimate goal being complete endothelialization of the luminal space of the graft. Unfortunately, this goal has not been fully realized in all currently used vascular grafts. Specifically, while some materials have been used with adequate success in replacing portions of the aorta ("Antiplatelet" 1994), once vessel diameters decrease to less than 6 mm, synthetic vascular graft failures increases to greater than 40% (Sayers et al 1998). Frequently, these failures are attributed to thrombosis and poor healing characteristics (Bos et al 1998).

Many of these problems are non-existent in autograft procedures involving use of the patient's own vein. This is because the autograft material provides a good thrombosis resistant surface, improved healing characteristics (compared to synthetic materials), and mechanical properties similar to the original artery due to the graft's intact cellular makeup. It is the cells of the vasculature that are key in retaining these properties.

There are three major types of cells that are found in vascular tissue: endothelial cells, smooth muscle cells, and fibroblasts. The endothelial cell layer lines all blood vessels, and its function varies depending on anatomical location and size of the associated vessel. Smooth muscle cells contribute to the mechanical behavior of the vessel, while fibroblasts are active participants in creating connective tissue and are instrumental in the wound healing response. Specifically, in the normal wound healing response, fibroblasts synthesize proteins (such as collagen and proteoglycans) that are a part of the natural repair process (Ratner

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and Hoffman 1996). Although fibroblast production of matrix proteins is necessary for normal wound healing and tissue regeneration, it can also lead to an undesirable fibrous encapsulation of the implant. When such fibrous encapsulation occurs, the body walls off the offending material in an effort to isolate it from the rest of the nearby tissue; the resulting scar tissue is usually non-functional. A more favorable reaction is tissue regeneration. During this process, the granulation tissue is remodeled by parenchymal cells into native tissue that retains its function. In the case of vascular tissue, this would include a viable endothelial cell lining and smooth muscle cell layers. Applications involving current synthetic vascular grafts often elicit the former, undesirable response from fibroblasts (Salacinski et al 2001).

This may be due, in part, to the fact that commonly implanted vascular materials focus on graft development at the macro- and micro-scale while native vascular tissues are composed of nano-dimensional components (Goodman et al 1997). For example, Goodman et al showed that the luminal space inside a blood vessel exhibits a complex topography with measurable features down to 100 nm. Addionally, the extra-cellular makeup of vascular tissue is composed of a network of proteins that exhibit nano-dimensional characteristics (Ayad et al 1994; Wilson et al 1996). Therefore, the current study sought to investigate the adhesion and growth of vascular endothelial cells, vascular smooth muscle cells, and fibroblasts on nano-dimensional poly(lactic-co-glycolic acid) (PLGA).

Moreover, this study aimed to elucidate the mechanism of cellular recognition to these nano-dimensional surfaces, since the rejection/acceptance of an implantable material is determined by such surface events. Specifically, when a material is exposed to proteins in solution (as is the case in blood or serum-containing medium), a monolayer of proteins immediately forms at the exposed surface. For example, work by Bale et al (1989) and Horbett (Horbett et al 1994) have shown that, of all plasma proteins, vitronectin preferentially adsorbs to biomaterial surfaces. Additionally, vitronectin has been identified at sites of vascular wound healing (Jang et al 2000). For this reason, the selective adsorption of vitronectin from serum proteins was investigated on nano-dimensional PLGA.

In the long term, results from the current study could be used in the future design of vascular graft biomaterials with nano-topographies; these materials have the unique potential for better integration with surrounding tissue in vivo and also improved retention of seeded vascular cells ex vivo.

MATERIALS AND METHODS

Substrates

Poly(lactic-co-glycolic acid) formulations

PLGA samples were prepared by dissolving (at 50–60 °C) 0.5 g of PLGA (50/50 wt.%; 12–16.5 × 10^3 MW; poly(lactic acid)/poly(glycolic acid), Polysciences, Inc.) in 8 mL of chloroform (Mallinckroft; Paris, KY) for 40 min. This solution was poured into glass petri dishes, allowed to sit overnight, and was then transferred to a vacuum (15 in. Hg) oven for 2 days at room temperature. Some of the resulting polymer films were left untreated (labeled conventional), while others were treated with 10 N NaOH for 1 h (labeled nano-structured). The resulting films were then cut using a scalpel into 0.5 cm × 1 cm × 0.5 cm strips and were sterilized by soaking in ethanol for 24 h followed by exposure to UV light for 1 h.

Reference substrates

Reference substrates used in all cell experiments were borosilicate glass coverslips (Fisher). Coverslips were etched in 1 N NaOH for 1 h according to standard procedures (Webster et al 2002).

Surface characterization

Surface topography of PLGA formulations was qualitatively and quantitatively evaluated using scanning electron microscopy and atomic force microscopy (AFM) (Miller et al 2003; Thapa et al 2003). Briefly, qualitative topography was evaluated using a JOEL JSM-840 scanning electron microscope using standard techniques after samples were sputter coated with gold at room temperature. Quantitative evaluation of surface roughness was accomplished using a NanoScope IIIa atomic force microscope (Digital Instruments Inc., Santa Barbara, CA). Height images were acquired in tapping mode at a frequency of approximately 300 kHz. Scans of size 1 μm × 1 μm were acquired at a scan rate of 1 Hz and 256 scanning lines. Changes in surface area (μm^2 per unit of scanned area (1 μm^2)) and root mean square surface roughness (nm) were recorded as a percent change from untreated conventional PLGA.

Cellular adhesion and growth

Cells

Rat aortic smooth muscle cells and rat aortic endothelial cells were purchased from VEC Technologies (Rensselaer, NY) and were used at population numbers 6–10 without further characterization. Human skin fibroblast cells were obtained from the American Type Culture Collection (CRL 1502) and were used at population numbers 1–10 without further characterization. Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone) and 1% penicillin/streptomycin (P/S, Hyclone) was used to culture smooth muscle cells and fibroblasts, while endothelial cells were cultured in MCDB-131 Complete Medium (VEC Technologies). All cells were incubated under standard cell culture conditions (that is, a sterile, humidified, 95% air, 5% CO_2, 37 °C environment).

Adhesion and growth

For adhesion and growth experiments, vascular endothelial cells, vascular smooth muscle cells, and fibroblasts were seeded separately (3,500 cells/cm^2) in their respective

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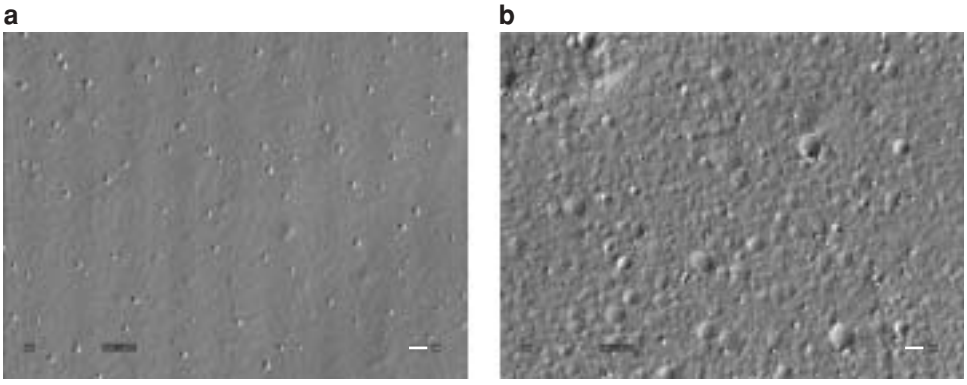

Figure 1 SEM images of conventional and nano-structured PLGA. SEM images confirmed that compared to (a) untreated conventional PLGA, (b) treated nano-structured PLGA had a higher degree surface roughness. Scale bar (lower right) is 10 μm.

media types onto the substrates of interest and were allowed to adhere for 4 h or grow for 5 days under standard cell culture conditions.

After cell adhesion and growth experiments, all substrates were rinsed with phosphate buffered saline (PBS) to remove non-adherent cells, fixed in 10% formalin (Fisher) or formaldehyde (Fisher), and stained with 0.1% Coomassie Brilliant Blue (BioRad). Light microscopy was used to count three (vascular smooth muscle and endothelial cells) or five (fibroblasts) random fields per substrate. Cell counts on each substrate were averaged and recorded as cells/cm^2. Experiments were run in triplicate and repeated at least three separate times.

Protein adsorption

PLGA substrates were separately exposed to either serum-containing media (DMEM) or serum-free media in the absence of cells overnight (Nikolovski and Mooney 2000). The substrates were then rinsed with PBS and blocked with 2% bovine serum albumin (Sigma) for 1 h. Each substrate was then incubated with anti-bovine vitronectin (1:100; Accurate Chemical) for 1 h. Following, the substrates were rinsed with Tris buffered saline–0.1% Triton X-100 (Sigma) and incubated with horse radish peroxidase conjugated anti-rabbit secondary antibody (1:100; Biorad). An ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) soluble substrate kit (Vector Labs, Burlingame, CA) was used to detect secondary antibodies spectrophotometrically, per manufacturer's instructions.

Statistics

All results were analyzed using student's *t*-test. Statistical significance was considered at $p < 0.05$.

RESULTS

Surface characterization

Figure 1 (Thapa et al 2003) shows scanning electron micrographs of the untreated and 10 N NaOH-treated PLGA substrates. SEM images show that with NaOH treatment, PLGA surface roughness increased dramatically. Additionally, quantitative results from AFM [Miller] provided statistically significant ($p < 0.05$) evidence of a 476% increase in surface roughness from untreated conventional to treated nano-structured PLGA. Surface area measurements were also increased (by 1.2%) from nano-structured to conventional PLGA; however, this increase was not as drastic as the change in surface roughness.

Cellular adhesion to PLGA formulations

Compared to untreated conventional PLGA, results indicated that fibroblasts, vascular endothelial cells, and vascular smooth muscle cells responded differently to treated nano-structured PLGA after 4 h (Figure 2). Specifically, results indicated that fibroblast and endothelial cell density was significantly decreased, while smooth muscle cell density was increased, on nano-structured compared to untreated conventional PLGA. For example, fibroblast and endothelial cell density decreased by 16 and 52%, respectively, while smooth muscle cell density increased by 28% after 4 h. Values are mean $\pm$ SEM; $n = 3$; $*p < 0.05$.

In addition, results provided evidence of significantly less fibroblast and endothelial cell numbers on treated nano-structured (compared to untreated conventional) PLGA after 5 days of culture (Figure 3). Specifically, there was a 35 and 53% decrease in fibroblast and endothelial cell number, respectively, on nano-structured PLGA after 5 days. In contrast, vascular smooth muscle cells had significantly higher cell density (by 25%) on nano-structured PLGA after the same time period.

Results indicated that, compared to untreated conventional PLGA, vascular smooth muscle cell growth was enhanced, while endothelial cell and fibroblast density was significantly decreased, on NaOH-treated nano-structured PLGA after 5 days. Values are mean $\pm$ SEM; $n = 3$; $*p < 0.05$.

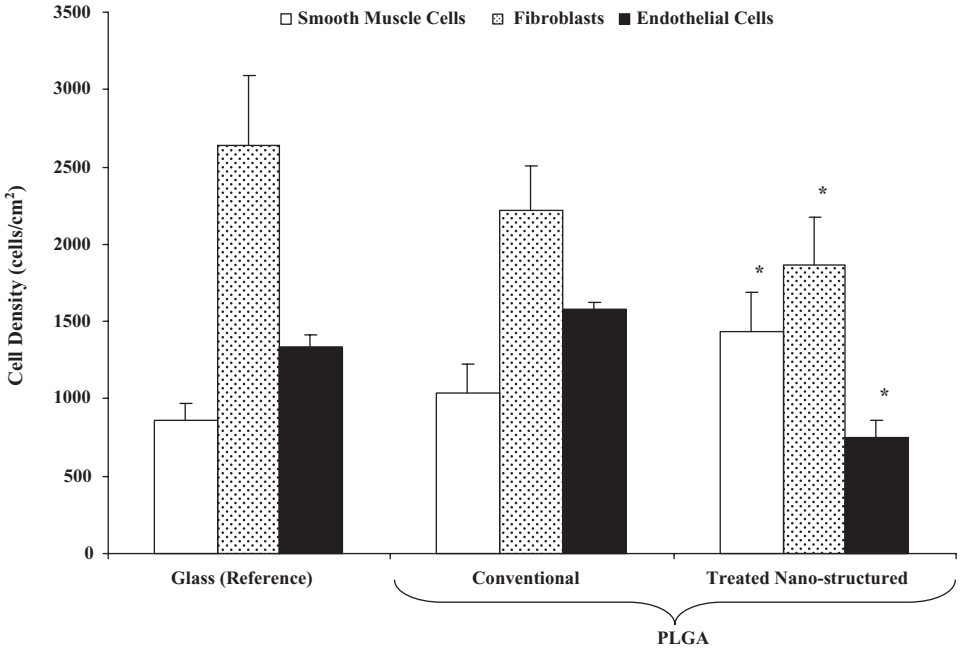

Figure 2 Endothelial cells, vascular smooth muscle cells, and fibroblasts show results that indicate, compared to untreated conventional PLGA, vascular smooth muscle cell adhesion was enhanced while endothelial cell and fibroblast density was significantly decreased on NaOH-treated nano-structured PLGA after 4 h.

Protein adsorption

PLGA films were exposed to serum-containing media overnight and specific antibodies to vitronectin were used to detect the relative amount of vitronectin on each surface. Results indicated that treated nano-structured PLGA adsorbed significantly (43%) more vitronectin when compared to untreated conventional PLGA (Figure 4). As a reference, both untreated conventional and treated nano-structured PLGA exposed to serum-free media were negative for vitronectin adsorption (data not shown).

Results indicated that, compared to untreated conventional PLGA, NaOH-treated nano-structured PLGA

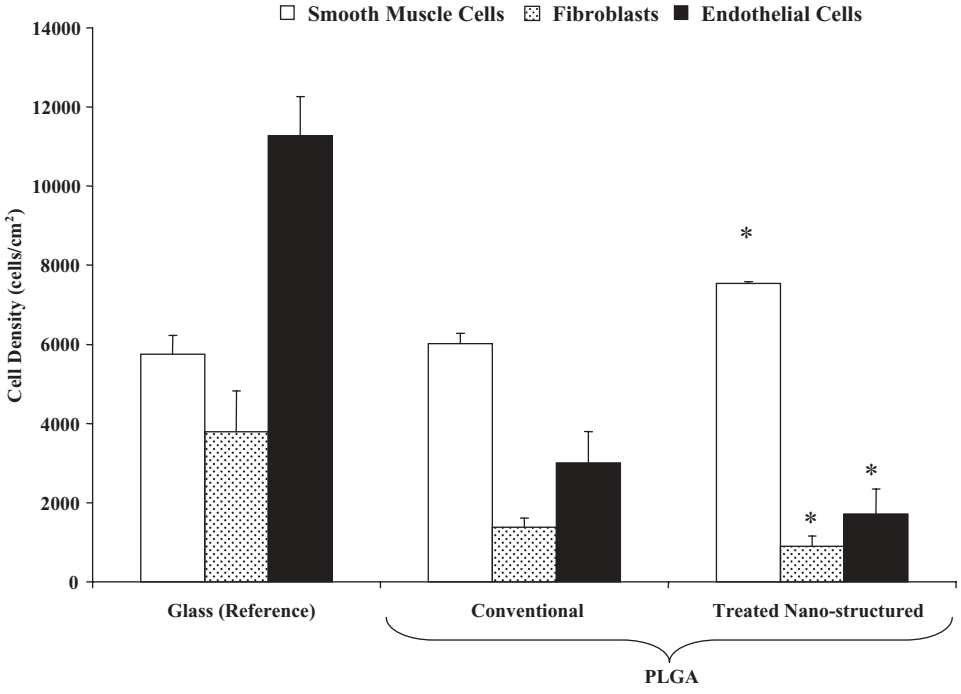

Figure 3 Endothelial cells, vascular smooth muscle cells, and fibroblasts proliferated differently on nano-structured PLGA.

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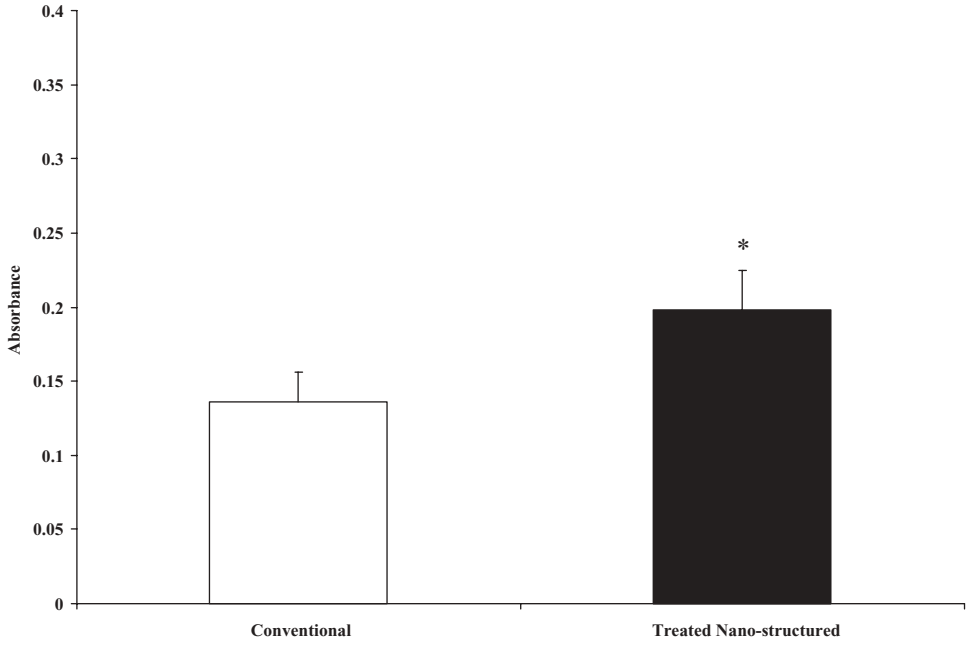

Figure 4 ELISA results indicated that nano-structured PLGA adsorbed more vitronectin from serum-containing media compared to conventional PLGA.

adsorbed significantly more vitronectin from serum-containing media. Values are mean ± SEM; $n = 3$; $*p < 0.05$.

DISCUSSION

Currently, vascular graft materials are manufactured with topographies that fail to match the micro- and nano-structural characteristics of vascular tissue (Goodman et al 1997). For example, native vascular tissue is composed of extra-cellular matrix proteins that exhibit nanometer characteristics (Ayad et al 1994; Wilson et al 1996). It is our hypothesis that mimicking these nano-dimensional characteristics in synthetic biomaterials will result in improved tissue integration.

For this reason, experiments were conducted to test vascular smooth muscle cell, endothelial cell, and fibroblast adhesion to nano-structured PLGA surfaces. Initial cellular adhesion was tested as a key event in the tissue integration/healing of vascular prostheses, since cells must first adhere to a biomaterial surface before subsequent functions such as extra-cellular matrix synthesis/deposition can occur. Results demonstrated that vascular smooth muscle cell adhesion was significantly enhanced on PLGA with increased nanometer surface roughness (compared to untreated conventional PLGA), while fibroblast and endothelial cell adhesion decreased (Figure 2). Additionally, fibroblast density was initially greater than both smooth muscle cell and endothelial cell density (on all PLGA materials tested) after 4 h. These findings are in agreement with previous reports, which have shown selective bladder smooth muscle cell (Thapa et al 2003), osteoblast (Webster et al 2000a and 2000b), and chondrocyte (Park et al 2003) responses to nano-dimensional materials.

Interestingly, trends seen in the 5-day growth experiments mirrored those observed after 4 h in culture. Namely, vascular smooth muscle cell density was significantly enhanced on PLGA with increased nanometer surface roughness (compared to untreated conventional PLGA), while fibroblast and endothelial cell adhesion decreased (Figure 3). An important observation was the decreased fibroblast and increased smooth muscle cell densities (compared to endothelial cells, and also compared to their respective cell numbers at 4 h) on each of the substrates at this longer time point. Specifically, when comparing Figures 2 and 3, the rapid adhesion of fibroblasts followed by their slow growth agrees well with results by Dalby et al (2003); this group also found that after a fast initial cell adhesion, fibroblasts grew less well on nano-topographies compared to flat surfaces. These results suggest that biomaterials with nano-topographies could elicit less fibrous encapsulation while also enhancing tissue integration by selective retention of vascular smooth muscle cells once implanted in the body.

Since both the adhesion and growth studies provided evidence of altered cellular response to nano-topographies, the next goal was to begin to understand the mechanism behind these differences. Importantly, the first response to the exposed surface of an implanted material is protein adsorption. This phenomenon takes place within seconds to minutes, well before cells arrive at and attach to the surface (Ratner and Hoffman 1996). Specifically, the type and concentration of adsorbed protein depends upon the concentration of protein in the surrounding biological fluid,

surface chemistry, and affinity of individual proteins for that particular surface (Ratner and Hoffman 1996). This process is predominantly permanent and proteins are considered immobilized onto the surface after initial adsorption. Not surprisingly, common vascular graft materials (e.g. expanded-polytetrafluoroethylene) have been shown to poorly adsorb serum proteins (Falkenback et al 2000); this may be one indication of why such prostheses fail to integrate successfully with surrounding tissue, for it is the proteins immobilized onto a biomaterial surface that cells recognize and attach to, ultimately resulting in either desirable (tissue integration) or undesirable (thrombosis, fibrous encapsulation) responses.

As a first step in understanding protein adsorption to the nano-dimensional materials tested in the current study, selectively adsorbed serum proteins were analyzed for vitronectin content using ELISA. A variety of proteins likely mediate the observed cellular responses. However, vitronectin was chosen initially based on studies performed by Webster et al (2000b); their findings indicated that vitronectin played an integral part in changing the response of osteoblasts seeded onto nanophase ceramics. Results from the present study showed that treated nano-structured, compared to untreated conventional, PLGA adsorbed significantly more vitronectin after exposure to serum-containing media overnight. These findings are supported by Nikolovski et al who showed that untreated (conventional) PGA and PLGA (75:25) adsorbed vitronectin when exposed to serum-containing media (Nikolovski and Mooney 2000), and provides further evidence of the enhanced potential of nano-dimensional PLGA to adsorb such critical proteins. While direct evidence has been provided to link the effect of vitronectin conformation, etc., on nanophase ceramics to increased osteoblast cell density (Webster et al 2002), the exact mechanism behind the differing responses of vascular smooth muscle cells, endothelial cells, and fibroblasts to nano-structured PLGA remains to be elucidated.

CONCLUSION

Current synthetic materials with micro-structured topographies have failed to successfully replace small diameter blood vessels. A more biologically inspired approach may include mimicking the natural nano-dimensional surface architecture of vascular tissue. Indeed, this study has shown that cellular adhesion and growth can be controlled by changing the nanometer surface characteristics of PLGA films. Specifically, vascular smooth muscle cell functions were significantly enhanced, while competitive cell (e.g. fibroblasts) functions were decreased, on treated nano-structured compared to untreated conventional PLGA. These results may indicate that future materials can enhance tissue integration by simply matching the biomaterial surface characteristics of the surrounding tissue.

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