PLGA nanometer surface features manipulate fibronectin interactions for improved vascular cell adhesion

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Abstract: The largest cause of mortality in the Western world is atherosclerotic vascular disease. Many of these diseases require synthetic vascular grafts; however, their patency rate is only 30% in small (<6 mm) diameter vascular grafts after 5 years of implantation. In an effort to increase small diameter vascular graft success, researchers have been designing random nanostructured surface features which enhance vascular cell functions. However, for the present study, highly-controllable, nanostructured features on poly(lactic-*co*-glycolic acid) (PLGA) surfaces were formulated. To create ordered nanostructured roughness on PLGA surfaces, either 500, 200, or 100 nm polystyrene nanospheres were separately placed onto mica. These were then used as a template for creating an inverse poly(dimethylsiloxane) mold which was utilized to cast PLGA. Compared to all other PLGA films formulated, AFM results demonstrated greater initial fibronectin spreading on PLGA which possessed spherical 200 nm features. Compared to smooth PLGA, PLGA with 500 or 100 nm surface features, results further showed that PLGA with 200 nm spherical features promoted vascular cell (specifically, endothelial, and smooth muscle cell) adhesion. In this manner, the present study demonstrated a specific nanometer surface feature size that promoted fibronectin spreading and subsequent vascular cell adhesion; criteria critical to vascular graft success. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 81A: 678–684, 2007

Key words: vascular; PLGA; nanometer; fibronectin; endothelial cells; vascular smooth muscle cells

INTRODUCTION

The largest cause of mortality in the Western world is atherosclerotic vascular disease.[1] This comes in the form of coronary artery and peripheral vascular disease. Current treatment methods involve bypassing occluded arteries with either autologous veins or biocompatible synthetic materials. To date, the first material of choice for small diameter vascular grafts is an autologous vein.[2] However, some 30% of patients do not have sufficient autologous bypass material due to disease or previous use.[2,3] In these cases, a necessary alternative can be a synthetic vascular graft.

Polymers have been used as vascular implants since 1952 when Vinyon-N was first introduced as a graft material.[4] Other materials such as Dacron, polyurethane, and polytetrafluoroethylene (PTFE) soon followed, giving the vascular prosthesis community a wide range of physical and mechanical properties to choose from. While these materials, namely Dacron and PTFE, have had success in replacing large arteries, grafts for replacing smaller diameter arteries (namely those under 6 mm) have seen very limited success.[5] Thrombosis and intimal hyperplasia have plagued small diameter vascular grafts causing a substantial drop in patency rate, from 95% for large diameter vascular grafts to 30% in small (<6 mm) diameter vascular grafts only after 5 years.[5] Clearly, due to these low patency rates and subsequent high need for those suffering from vascular diseases, new synthetic material formulations for small diameter vascular grafts are required.

Novel classes of polymers that are under intense investigation to become the next-generation of small diameter vascular grafts with increased efficacy are biodegradable polymers such as poly(lactic acid), poly (glycolic acid), and their copolymers.[6,7] For example, it has been shown that tubular scaffolds of poly(glycolic acid) can support vascular smooth muscle and endothelial cell growth *in vitro*.[7–9] However, these materials are composed of micron sized fibers and consequently possess surface topographies that are dissimilar to the natural nanodimensional components (i.e., proteins) of the tissue being replaced.[10,11] Since vascular tissue is

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primarily composed of collagen and elastin (proteins with a nanometer structure), it is clear that cells of the vascular tissue are accustomed to interacting with such nanostructured architectures. The natural nanostructure of vascular tissue has been demonstrated in prior reports.[3,8] In contrast, most synthetic materials currently being used as vascular grafts are not designed to have nanometer surface features.[11]

The latest trend in vascular biomaterial research has therefore focused on improving vascular cell function by designing materials with nanoscale surface features.[12–16] Although under debate, 'nano' usually refers to surface features less than 1 micron, or more specifically less than or equal to 100 nm. Recently, Miller et al.[12] showed that increasing the nanometer surface roughness on poly-lactic acid/poly-glycolic acid (PLGA) (50/50 wt % mix) films improved vascular smooth muscle cell adhesion and proliferation. Random, uncontrollable, nanometer surface features were made on PLGA in this study through chemical (NaOH) etching techniques.[12] After further investigation, enhanced vascular cell function was determined to be a result of topographical (not chemical) changes during the etching process[12]; namely, after chemistry effects were removed utilizing a silastic casting technique, endothelial and vascular smooth muscle cell function increased on PLGA with nanostructured compared to flat surface features.[12]

However, through the creation of random, uncontrollable, nanostructured surface roughness, it is not possible to ascertain specific feature sizes important for promoting vascular cell functions. Some researchers have indeed created controllable nanometer surface features through polymer demixing techniques and have measured increased vascular cell spreading.[16] However, in such studies, it is unclear what mechanism is increasing vascular cell responses.[16] To create highly controllable nanometer features, the present study used polystyrene colloidal nanoparticles placed on mica to cast PLGA. To elucidate a possible mechanism that may enhance vascular cell functions on nanostructured surfaces, initial protein and subsequent vascular cell interactions were further investigated on these PLGA constructs. In this manner, the objective of the present study was to understand a specific size range at which vascular cell adhesion may be enhanced on PLGA and the initial interactions between PLGA and proteins that may be causing such responses.

MATERIALS AND METHODS

Materials

To create ordered nanostructured roughness on poly(lactic-*co*-glycolic acid) (PLGA) surfaces, 500, 200, and 100 nm polystyrene spheres (Nanobead NIST traceable standards, coefficient of variance between 2 and 4%; Polysciences, PS nanospheres) were first separately placed onto mica. These were used as a template for creating an inverse poly(dimethylsiloxane) (PDMS; Dow Corning) mold, which was then used to cast PLGA. Specifically, 50 mL of the colloidal PS nanospheres were dispensed onto freshly cleaved mica substrates (Hi-Grade Mica(Grade V2); Ted Pella).[17] The solution was then allowed to air dry at room temperature for 48 h.

A well established PDMS casting process was used to transfer the roughness from the PS nanospheres to PLGA.[12,13] Specifically, SYLGARD 184 Silicone Elastomer® (Dow Corning) was mixed (10 : 1 wt/wt %) with a silicone resin curing agent (Dow Corning) and exposed to vacuum pressure (25 in Hg) for 10–15 min at room temperature. The elastomer was then poured into a petri dish containing the above mentioned substrates. The elastomer was allowed to cure for 48 h at room temperature. After the prescribed time period, the resulting negative elastomeric mold was peeled off, inverted, and placed in another petri dish. The final mold was then allowed to cure at 100°C for 1 h.

A PLGA-containing solution was prepared by dissolving (at 50–60°C) 0.5 g of PLGA (50/50 wt %; 12–16.5 $\times$ 10^3 MW, poly (lactic acid)/poly(glycolic acid); Polysciences) in 8 mL of chloroform (Mallinckroft) for 40 min. PLGA was then cast into the mold and allowed to sit for 24 h. The cast polymer and mold were then placed into a vacuum oven for 2 days at 15 in Hg at room temperature. The cast PLGA of varying nanometer surface features was then removed from the mold. Smooth PLGA films (control) were created by allowing the PLGA to cure on flat petri dish surfaces according to standard procedures.[12] All of the resulting PLGA films were cut into 1 $\times$ 1 $\times$ 1 cm^3 strips and sterilized under UV light for 24 h until needed for experiments.

Borosilicate glass cover slips (reference material; 18 mm diameter; Fisher) were soaked in acetone (Mallinckroft) for 10 min, sonicated in acetone for 10 min, soaked in 70% ethanol for 10 min, sonicated in 70% ethanol for 10 min, etched in 1*N* NaOH (Sigma) for 1 h, rinsed in distilled water, and dried in an oven at 70°C. The cleaned cover slips were then sterilized in an autoclave at 250°C for 20 min and stored at room temperature until needed for experiments.

Materials characterization

Atomic force microscopy (AFM; NanoScope IIIa Atomic Force Microscope; Digital Instruments) was used to evaluate the surface topography of the original three types of PS nanospheres (500, 200, and 100 nm) placed on mica and the resulting three types of cast PLGA surfaces. For this purpose, height images of each sample were captured in ambient air at 15–20% humidity at a tapping frequency of $\sim$300 kHz, and at a frequency near the resonance of the cantilever. NanoScope imaging software (version 4.31, Digital Instruments) and ImagePro were used to analyze the resulting images. WSxM software was utilized in the analysis of AFM surface profiles.

Electron spectroscopy for chemical analysis (ESCA) was performed on all substrates of interest to the present study using a Surface Science Instruments (SSI) X-Probe instrument. An aluminum $K_{\alpha 1,2}$ monochromatized X-ray source was used to stimulate photoemission of the inner shell electrons of the sample. The energy of this electron was then recorded and analyzed for identification purposes.

All material characterization studies were completed in triplicate at least three separate times.

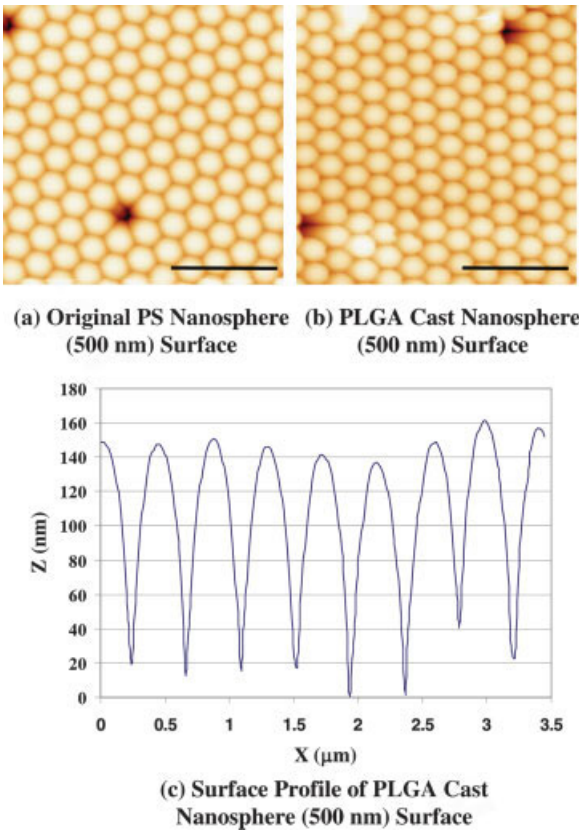

Figure 1.

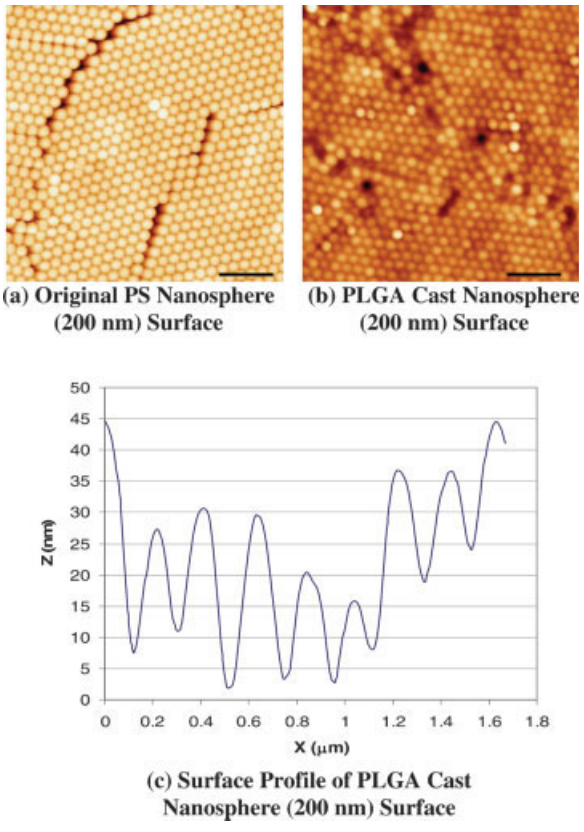

Figure 2.

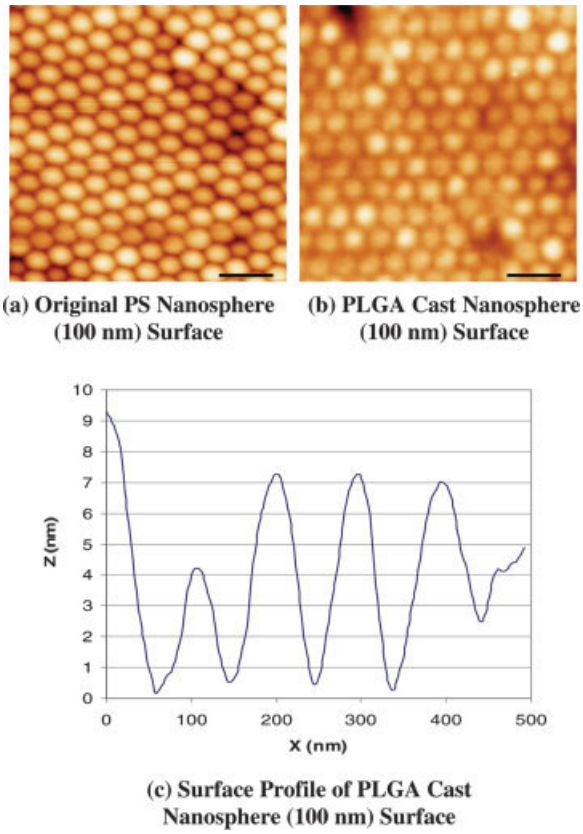

Figure 3. AFM images of the (a) original PS nanosphere (100 nm) surface and (b) PLGA cast nanosphere (100 nm) surface. (c) Surface profile of PLGA cast nanosphere (100 nm) surface. Scale bar (in black on bottom right) is 150 nm. PLGA surface features (150 nm) were measured to be 95 nm in diameter, as obtained by WSxM software analysis. PS, polystyrene; PLGA, poly-lactic-*co*-glycolic acid.

Fibronectin interactions

Because of the importance of initial fibronectin adsorption towards promoting vascular cell function on implant surfaces,[18] the characteristics of fibronectin adsorption to each PLGA substrate was characterized qualitatively and quantitatively by AFM. Briefly, 1 mL of either a 0.5 or 5 μg/mL fibronectin solution (in PBS; bovine plasma; Sigma) was placed

Figure 1. AFM images of the (a) original PS nanosphere (500 nm) surface and (b) PLGA cast nanosphere (500 nm) surface. (c) Surface profile of PLGA cast nanosphere (500 nm) surface. Scale bar (in black on bottom right) is 2 μm. PLGA surface features (500 nm) were measured to be 430 nm in diameter, as obtained by WSxM software analysis. PS, polystyrene; PLGA, poly-lactic-*co*-glycolic acid.

Figure 2. AFM images of the (a) original PS nanosphere (200 nm) surface and (b) PLGA cast nanosphere (200 nm) surface. (c) Surface profile of PLGA cast nanosphere (200 nm) surface. Scale bar (in black on bottom right) is 1 μm. PLGA surface features (200 nm) were measured to be 195 nm in diameter, as obtained by WSxM software analysis. PS, polystyrene; PLGA, poly-lactic-*co*-glycolic acid.

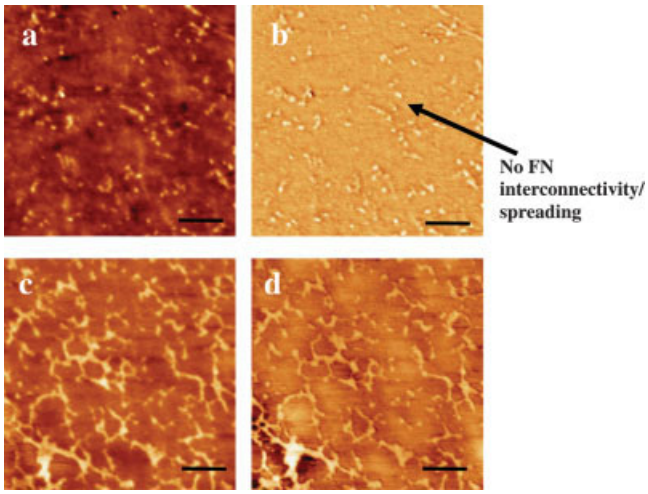

Figure 4. AFM images of FN-coated smooth PLGA surfaces. Representative (a) height and (b) phase AFM images of FN-coated (0.5 μg/mL) smooth PLGA surfaces showed discrete FN adsorption and no interconnectivity between FN molecules. Additionally, (c) height and (d) phase images of FN-coated (5 μg/mL) smooth PLGA surfaces showed some interconnectivity between FN molecules. Images are 750 × 750 nm^2. Scale bar (in black on bottom right) is 150 nm. FN, fibronectin.

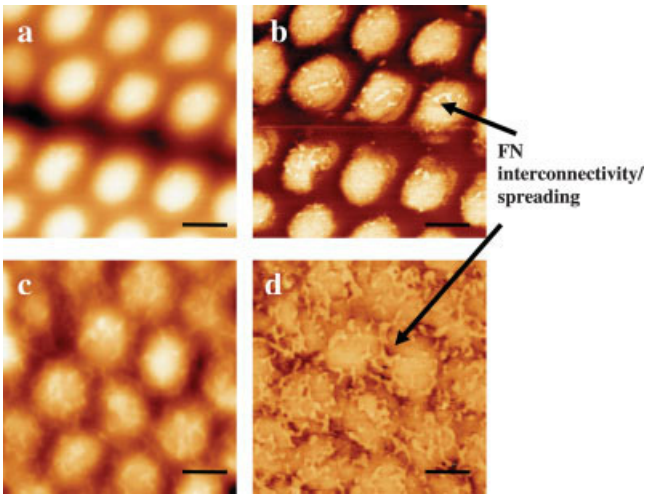

Figure 6. AFM images of FN-coated 200 nm PLGA surfaces. Representative (a) height and (b) phase AFM images of FN-coated (0.5 μg/mL) 200 nm PLGA surfaces showed FN adsorption with interconnectivity between FN molecules. Additionally, (c) height and (d) phase images of FN-coated (5 μg/mL) 200 nm PLGA surfaces showed interconnectivity between FN molecules. Images are 750 × 750 nm^2. Scale bar (in black on bottom right) is 150 nm. FN, fibronectin.

onto each PLGA surface for 10 min. Human plasma fibronectin has a radius of gyration of 15.3 nm in physiological solution and a hydrodynamic radius/radius of gyration of 0.75 (whereas spherical shaped molecules have 1.3).[19] These substrates were rinsed with deionized water and immediately imaged. Fibronectin adsorption was imaged using tapping mode AFM. Height images of each sample were captured in ambient air at 15–20% humidity at a tapping frequency of ~300 kHz, and at a frequency near the resonance of the cantilever. NanoScope imaging software (version 4.31, Digital Instruments) and ImagePro were used to analyze the resulting images. Specifically, to ascertain differences in spreading once adsorbed, fibronectin aspect ratios were calculated by measuring the length and width of fibronectin molecules on each surface. Measurements were recorded and averaged from 20 random fibronectin molecules per substrate. Experiments were completed in triplicate at least three separate times.

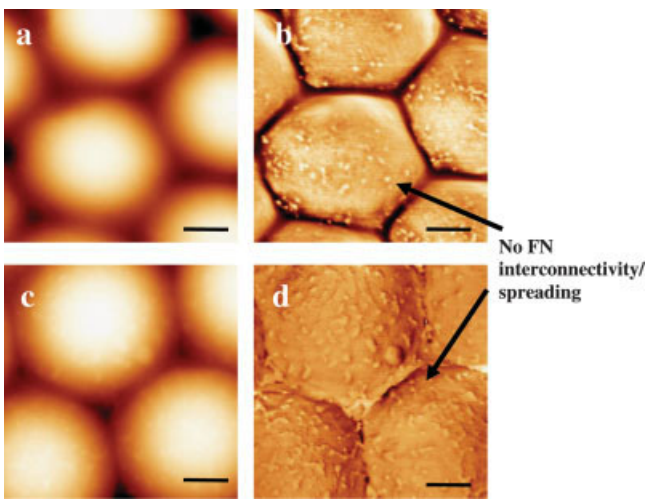

Figure 5. AFM images of FN-coated 500 nm PLGA surfaces. Representative (a) height and (b) phase AFM images of FN-coated (0.5 μg/mL) 500 nm PLGA surfaces showed discrete FN adsorption with no interconnectivity between FN molecules. Additionally, (c) height and (d) phase images of FN-coated (5 μg/mL) 500 nm PLGA surfaces showed no interconnectivity between FN molecules. Images are 750 × 750 nm^2. Scale bar (in black on bottom right) is 150 nm. FN, fibronectin.

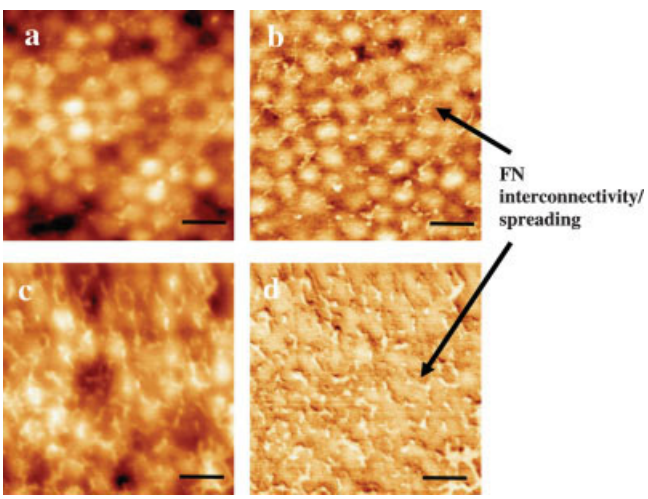

Figure 7. AFM images of FN-coated 100 nm PLGA surfaces. Representative (a) height and (b) phase AFM images of FN-coated (0.5 μg/mL) 100 nm PLGA surfaces showed discrete FN adsorption with interconnectivity between FN molecules. Additionally, (c) height and (d) phase images of FN-coated (5 μg/mL) 100 nm PLGA surfaces showed interconnectivity between FN molecules and a masking of the underlying nanometer surface features. Images are 750 × 750 nm^2. Scale bar (in black on bottom right) is 150 nm. FN, fibronectin.

TABLE I
Greater Fibronectin Spreading on 200 nm PLGA Surface

Substrate	Fibronectin Aspect Ratio
Smooth PLGA	1.89 ± 0.05
500 nm	1.67 ± 0.1*
200 nm	3.14 ± 0.19*,**
100 nm	2.10 ± 0.07*

Data = mean ± SEM; $n = 3$.
*$p < 0.01$ (compared to smooth PLGA).
**$p < 0.01$ (compared to 100 nm PLGA surfaces). Data was obtained from AFM images for the adsorption of 5 μg/mL of fibronectin.

Cell assays

To correlate the adsorption of fibronectin to cell adhesion, vascular smooth muscle cell and endothelial cell adhesion assays were performed. Rat aortic smooth muscle cells and rat aortic endothelial cells were purchased from VEC Technologies (Greenville, NY) and used without further characterization. Vascular smooth muscle cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone) supplemented with 10% fetal bovine serum (FBS; Hyclone cat. no. SH30070) and 1% penicillin/streptomycin (P/S; Hyclone), while endothelial cells were cultured in MCDB-131 Complete Medium (VEC Technologies). All cells were incubated under standard cell culture conditions, consisting of a sterile, humidified, 95% air, 5% CO_2, 37°C environment. Population numbers for all cells used in the experiments were between 6 and 10.

To determine their adhesion to the nanostructured PLGA surfaces, vascular smooth muscle and endothelial cells were suspended through enzymatic (Trypsin; Sigma) lifting from cell culture plates, placed separately in respective serum free media after trypsin neutralization, seeded at 3500 cells/cm^2 onto the substrates of interest, and were allowed to adhere for 4 h under standard cell culture conditions. Prior to the cell adhesion assay, some of the substrates were soaked (1 mL) in either 10% FBS in respective cell culture media or fibronectin (at a final concentration of 5 μg/mL; Sigma) overnight at 37°C. At that time, the protein solution was removed, the coated substrates were rinsed in PBS, and used immediately to assess cell adhesion.

After the 4 h adhesion assay, cells were fixed, stained, and counted according to standard procedures.[12] The number of cells in each of five random fields per substrate were counted using a light microscope, averaged, and recorded as cells/cm^2. All experiments were run in triplicate and repeated at least three separate times per substrate. Data was analyzed by ANOVA followed by student *t* tests.

RESULTS

Materials characterization

As can be seen, regardless of nanosphere size, PS particles formed a closed-packed configuration after drying on mica (Figs. 1–3). AFM images provided further evidence of the successful transfer of the closed-packed PS nanosphere topography to the cast PLGA. Importantly, ESCA results did not provide evidence of silicon contamination on the PLGA cast surfaces through the use of the silastic mold process. As expected, the chemistry was similar between all cast PLGA surfaces studied here.

Fibronectin interactions

As illustrated in Figures 4–7, fibronectin adsorbed into discernable patterns on all PLGA surfaces of interest. Qualitatively, fibronectin possessed significant amounts of interconnectivity (and, thus, spreading) only when adsorbed onto the PLGA created from the 200 and 100 nm PS nanospheres (Figs. 6 and 7); this was especially true between the nanospherical bumps. However, there was an undesirable masking of the underlying 100 nm surface features through fibronectin coating at high concentrations (5 μg/mL). In contrast, fibronectin adsorption showed no interconnectivity on the smooth PLGA and the 500 nm PLGA (Figs. 4 and 5).

Quantitatively, compared to all other PLGA surfaces, note the increased aspect ratios of fibronectin from its natural conformation in solution (at 5 μg/mL concentrations) when adsorbed on the 200 nm PLGA surfaces; specifically, fibronectin aspect ratios of 1.89, 1.67, 3.14, and 2.10 were measured on PLGA smooth surfaces, 500, 200, and 100 nm surfaces, respectively (Table I). Collectively, information gathered from AFM provided evidence of an optimal interaction between fibronectin on specifically the 200 nm PLGA surface.

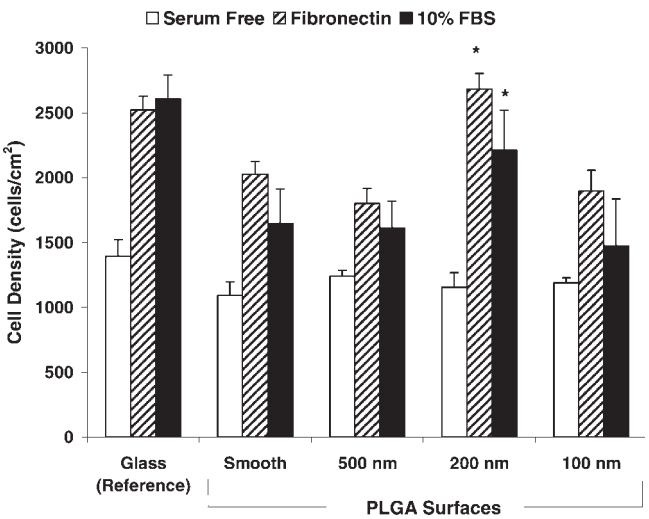

Figure 8. Increased vascular smooth muscle cell adhesion to PLGA surfaces with 200 nm features. Compared to smooth PLGA, vascular smooth muscle cell adhesion was significantly (*$p < 0.05$) enhanced on 200 nm PLGA exposed to fibronectin (5 μg/mL) or 10% FBS. Values are mean ± SEM; $n = 3$; student *t* tests used in statistical analyses.

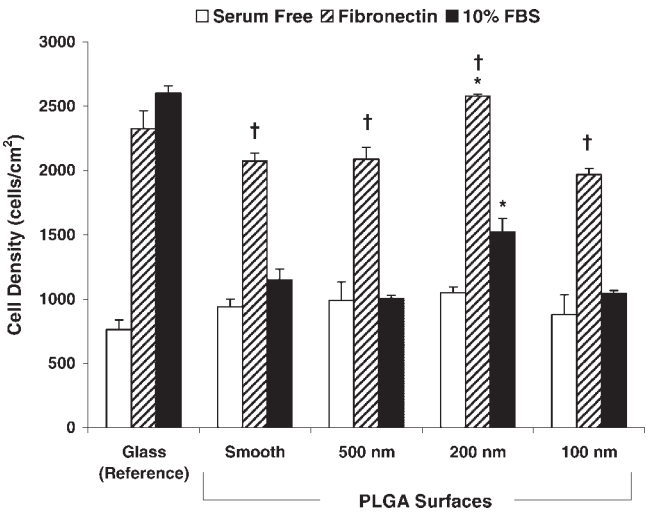

Figure 9. Increased endothelial cell adhesion to PLGA surfaces with 200 nm features. Compared to smooth PLGA, endothelial cell adhesion was significantly (*$p < 0.05$) enhanced on 200 nm PLGA exposed to fibronectin (5 μg/mL) or 10% FBS. Endothelial cell adhesion was also significantly (†$p < 0.05$) enhanced for all substrates coated with fibronectin compared to their respective substrates exposed to 10% FBS. Values are mean ± SEM; $n = 3$; student t tests used in statistical analyses.

Cell adhesion

Compared to any other nanostructured PLGA surface formulated in this study, results showed the best vascular cell adhesion on PLGA with 200 nm surface features when pretreated with either fibronectin or 10% FBS (Fig. 8 for vascular smooth muscle cells and Fig. 9 for endothelial cells). Importantly, the previous results of optimal fibronectin spreading and interconnectivity on the 200 nm PLGA was supported by the present results of greater vascular cell adhesion on the 200 nm PLGA surface pretreated with fibronectin. Additionally, no difference in cell adhesion was found between any substrates exposed to serum free media.

DISCUSSION

This study supports the well known fact that changes in material properties influence initial protein interactions to subsequently change cellular adhesion. It was for this reason that the present study intended to design specific nanometer surface features on PLGA to enhance the interaction with one protein important for vascular cell adhesion: fibronectin.[12] Since chemistry was similar between all PLGA surfaces investigated here, this study provided strong evidence of the influence of one material property (nanometer features) on optimizing fibronectin interactions and subsequently vascular cell adhesion.

Nanotechnology (or the use of nanostructured surface features) plays a key role in this aspect. Alterations in roughness from that of conventional (flat or micron-structured) to nanostructured can influence many properties important for mediating protein interactions (such as energy, charge density, topography, etc.).[20,21] Specifically, as the number of nanometer surface features increase, the charge density at the surface also increases.[20,21] In addition, nanomaterials possess more surface area, altered electron delocalizations, and percentage of atoms at the surface compared to bulk; all of which have been widely exploited in catalytic applications.[20,21]

Improved protein adsorption on nanostructured surfaces important for other implant applications (such as bone, cartilage, bladder, and neural) has been reported in the literature.[12,13,22] For example, it has been shown that ceramic compacts with nanodimensional surface features adsorbed significantly more vitronectin (a protein important for the functions of osteoblasts or bone-forming cells) compared to ceramics with micron-surface features.[22] This was found to occur at specific ceramic surface features with spherical diameters below 49 nm.[23]

As pertaining to vascular applications, the present study demonstrated that the topography of PLGA can be improved through the use of specific nanostructured surface features (i.e., 200 nm spherical diameters) to enhance the adsorption characteristics of fibronectin; an important protein for promoting vascular cell adhesion.[12] Such results can be used by numerous researchers (not only those developing PLGA for vascular applications), since fibronectin is a key cell-adhesive protein for many cell types. But specifically for improving vascular grafts, the present results suggest that perhaps a PLGA small diameter vascular graft composed of 200 nm diameter features would enhance vascular cell surface occupancy to potentially decrease inflammatory cell activation as a result of the exposure of the bare polymer surface. Clearly more research is needed to further test this, but the elucidation of increased endothelial cell adhesion on such surfaces show promise for possible greater endothelialization of nanostructured compared to currently used nanosmooth polymer grafts.

Since the main objective of the present study was to create highly-controllable nanostructured features on PLGA, it is important to mention previous studies which have measured increased fibronectin and vitronectin adsorption on PLGA created to have random nanostructured surfaces.[12,18] In those studies, nanostructured PLGA was created through similar cast-mold techniques utilized here except that the template used was NaOH-treated PLGA.[12,18] For those random nanostructured surfaces, ELISA determined significantly more competitive adsorption of fibronectin and vitronectin from cell culture media supplemented with serum.[18] Moreover, when blocking fibronectin adhesion receptors on vascular cell membranes, smooth muscle and endothelial cell

adhesion was inhibited by 48 and 51%, respectively, on the random nanostructured PLGA coated with fibronectin.[18] Similarly, when blocking vitronectin adhesion receptors on vascular cell membranes, smooth muscle and endothelial cell adhesion was inhibited by 50 and 61%, respectively, on random nanostructured PLGA coated with fibronectin. Coupled with other studies,[18,22] the present study adds evidence that fibronectin interacts significantly different with nanostructured compared to flat or microrough surfaces; criteria which may be useful in explaining greater cell responses on nanomaterials as observed here.

CONCLUSIONS

In this manner, this study provides an important design consideration that may be able to advance the performance of PLGA for vascular tissue engineering applications: creation of 200 nm surface spherical features. Although more studies are needed, the present results of altered initial fibronectin adsorption leading to greater vascular cell (both endothelial and smooth muscle) adhesion on 200 nm compared to either flat, 500 or 100 nm PLGA shows promise for altering nanometer surface features on PLGA to improve vascular applications.

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