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Modification of fibrous poly(L-lactic acid) scaffolds with self-assembling triblock molecules

John C. Stendahl[a], Leiming Li[a], Randal C. Claussen[b], Samuel I. Stupp[a,b,c,*]

[a] Department of Materials Science and Engineering, Northwestern University, 2220 Campus Dr., Evanston, IL 60208-3108 USA
[b] Department of Chemistry, Northwestern University, 2145 Sheridan Rd., Evanston, IL 60208-3113 USA
[c] Feinberg School of Medicine, Northwestern University, 303 E. Chicago Ave., Chicago, IL 60611-3008 USA

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Abstract

Molecular self-assembly offers an effective method to modify the surface properties of common biomaterials by presenting biologically relevant chemistry in a controlled, ordered fashion. This work reports on self-assembling triblock molecules containing rigid cholesteryl segments followed by flexible oligomers of L-(lactic acid) and second generation L-lysine dendrons. Second harmonic generation and small angle X-ray scattering indicate these molecules self-assemble into multilayer polar structures when cast from ethyl acetate solutions and segregate into polar polydomains when annealed. These self-assembled layers significantly improve water wettability when coated onto poly(L-lactic acid) fibers. Scaffolds formed from fibers modified by self-assembly enhance adhesion of 3T3 mouse calvaria cells and produce greater population growth rates. These results demonstrate the use of self-assembly to present biologically relevant chemistry on surfaces of biomaterials. Applications of this technology include the modification of substrates for cell culture, tissue engineering, and cell transplantation.

Keywords: Cell adhesion; Polylactic acid; Scaffold; Self-assembly; Surface modification

1. Introduction

The preparation of scaffolds for tissue regeneration in vitro or in vivo requires better understanding of chemical and physical interactions at cell/biomaterial interfaces. We address here this fundamental issue by utilizing self-assembling triblock molecules containing cholesterol and lysine moieties to modify poly(L-lactic acid) (PLLA) tissue engineering scaffolds. These amphiphilic molecules are designed to present biologically relevant chemistry in a controlled, ordered fashion that improves cellular affinity for scaffold materials and helps invoke a specific cellular response.

Cellular responses to common synthetic biomaterials such as PLLA are heavily influenced by surface chemistry. These surfaces acquire biological activity in vivo and in vitro only after a layer of protein is deposited from the surrounding medium [1]. This adsorption of proteins, which is rapid and largely irreversible, creates the primary surface with which cells interact. As a structure that is analogous to the extracellular matrix (ECM) in natural tissue, the adsorbed protein layer furnishes anchorage sites for cells and regulates their microenvironment by providing nutrients as well as signaling epitopes that influence multiple aspects of cell physiology, including metabolism, proliferation, migration, differentiation, and macromolecular synthesis [2]. In addition, there is evidence that cells are capable of interacting directly with biomaterial surfaces, either via pseudopodia that extend through the protein layer or by the consumption of adsorbed proteins to establish direct contacts [3]. For these reasons, it is believed that the chemical properties of a biomaterial surface can influence cellular behavior *directly*, via plasma membrane contact, and also *indirectly*, by regulating the composition and conformation of adsorbed proteins.

Tissue engineering scaffolds are intended to substitute for ECM, providing temporary structure for cell

*Corresponding author. Department of Materials Science and Engineering, Northwestern University, 2220 Campus Dr., Evanston, IL 60208-3108 USA. Tel.: +1-847-491-3002; fax: +1-847-491-3010.
E-mail address: s-stupp@northwestern.edu (S.I. Stupp).

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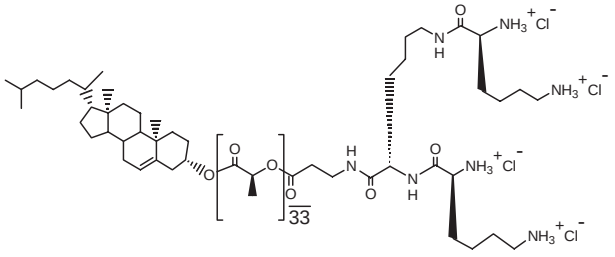
attachment and proliferation, and aiding in the maintenance of differentiated function. Although natural ECM polymers such as collagen and laminin are attractive scaffold candidates because they contain numerous molecular signaling domains that regulate cell function, they can be difficult to process and are often immunogenic and capable of producing unpredictable biological responses [4].

In this study, scaffolds were formed from biodegradable PLLA fibers bonded by mechanical compression. Despite its excellent biocompatibility, PLLA is hydrophobic and devoid of chemical constituents that interact favorably with cells. The objective of this work was to study the self-assembly of amphiphilic triblock molecules on PLLA fiber surfaces to modify them noncovalently. This methodology could be important to introduce bioactivity or drugs on biodegradable polyester surfaces in scaffolds for regenerative medicine and also in other materials, such as sutures to aid healing processes. As shown below, the triblock molecule studied here contains a rigid cholesteryl segment followed by a flexible oligomer of L-lactic acid and a second generation L-lysine dendron. The two hydrophobic segments should have affinity for the hydrophobic surfaces of biodegradable polyester materials so that the hydrophilic dendron will be displayed on the outer surfaces. If desired, the dendron can then be used to anchor proteins, peptides, sugars, and other molecules. The synthesis and characterization of similar molecules was previously reported by our laboratory [5–7]. We discuss below more details of the rationale behind the specific chemical structure of these molecules.



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Cholesterol was selected because of its physiological nature as well as its mesogenic [8] properties that trigger self-assembly of the molecules on the polyester fibers. Ordered molecular layers not only allow for greater spatial control of bioactive ligands attached to the dendron, but potentially provide self-renewing surfaces as levels are biodegraded by cells. Moreover, as an essential component of eukaryotic cells, cholesterol serves as a chemical precursor in the biosynthesis of important compounds such as steroid hormones, bile acids, and lipoproteins [9]. Cholesterol has also been shown to stimulate RNA and protein synthesis [10,11] and controls many aspects of plasma membrane function. Most importantly, cholesterol modulates membrane stiffness [10–14] and permeability [12] and plays significant roles in regulating other essential membrane functions such as signaling [12,15–17], protein transport [12], receptor function [12–15,17], and immune responses [12,15]. Many of these functions of cholesterol are now attributed to its role in the assembly and operation of lipid rafts, the sphingolipid-rich domains of the exoplasmic surface that regulate protein and receptor activity. By utilizing cholesterol we not only provide a thermodynamic driving force to create ordered assemblies of bioactive molecules on the biodegradable fibers, but also incorporate chemistry that has a high thermodynamic affinity for plasma membranes and is central to cell survival and function.

Lysine residues in the dendrons are well suited for cell adhesion because they are moderately hydrophilic and carry positive charges on their side chains. Basic amino acids such as lysine and arginine are prevalent in heparin binding domains of extracellular adhesion proteins and are believed to mediate cell adhesion via electrostatic interactions with negatively charged proteoglycans on cell exteriors [18,19]. These interactions facilitate the organization of actin stress fibers and, along with integrin binding, are essential to the formation of focal adhesion complexes [20–22].

The hydrophilicity of lysine is also potentially beneficial for cellular interaction in an indirect nature because it may help to limit the denaturation of adsorbing proteins. Proteins that adsorb to hydrophobic surfaces tend to adopt unnatural conformations to accommodate the dissimilar chemistry, often reconfiguring to expose hydrophobic regions that are normally internalized [23–25]. By allowing adsorbed proteins to remain in conformations that more closely resemble their natural, chemically functional states, surfaces hydrophilically modified with **1** may be more likely to promote specific signaling interactions with cells.

In this work, we present the physical characterization of **1** and study its ability to influence cellular behavior when coated in thin, self-assembled layers on PLLA fiber scaffolds. By introducing biologically relevant chemistries in an ordered fashion, self-assembling molecules such as **1** could provide effective routes to functional surface modifications in common biomaterials.

2. Materials and methods

2.1. Molecular synthesis

Synthesis of **1** began with the ring opening polymerization of L-lactide by the hydroxyl of cholesterol [6]. Three equivalents of cholesterol were reacted with $AlEt_3$ for 15 min at room temperature to give the trialkoxyl

aluminum initiating species. This initiator was heated at 80°C in toluene for 4 h in the presence of 10 equivalents of L-lactide to yield an oligomer with an average degree of polymerization 33 and a polydispersity (M_w/M_n) of approximately 1.1. The second generation Lys dendron with a β-alanine core was prepared from the benzyl ester of β-alanine and $N\alpha,N\varepsilon$-di-BOC-L-lysine dicyclohexylammonium salt via EDCI/HOBt solution phase coupling, with the intermediate BOC groups cleaved by 4 M HCl in dioxane, and core benzyl ester removed by hydrogenolysis in the last step. The dendron was then coupled to the free hydroxyl of the terminal lactic acid unit by EDCI/DMAP esterification and the terminal BOC-protected amines were converted to their hydrochloride salts by treatment with 4 M HCl in dioxane. A more detailed account of the synthesis will be reported elsewhere [26].

2.2. Differential scanning calorimetry (DSC)

Data were collected with a DSC instrument with ramp speed 10°C/min. Glass transitions were determined from the second heating cycle.

2.3. Small angle X-ray scattering (SAXS)

Films were cast onto glass slides from an 11.3 mg/ml solution of **1** in chloroform. Before film deposition, slides were precleaned in 1.0 M NaOH in isopropanol for 5 min and rinsed successively in Millipore water and acetone. The solution of **1** was run through a 0.22 mm filter and added dropwise via syringe to slides and allowed to dry in a chamber saturated with chloroform vapors. Once dry, films were placed in vacuum at room temperature for 48 h to remove residual solvent. A portion of films were annealed at 75°C in N_2 atmosphere for 1.5 h. Finished films were scraped by razor into 0.7 mm glass capillary tubes, which were subsequently sealed by flame. Powder diffraction patterns were collected with a small angle X-ray scattering machine with a rotating anode generator. A high-resolution small-angle camera with an area detector at a distance of 630 nm was used to record data. Diffraction rings were integrated over 360° to yield the scans presented below. Sodium behenate was used to calibrate the system.

2.4. Second harmonic generation (SHG)

Films of **1** were prepared using the same procedure for those in SAXS characterization. Film thickness was varied by the number of drops added and measured by profilometry. A *p*-polarized *Q*-switched Nd:YAG laser (1.064 μm, 10 Hz, 5 ns) was focused onto each sample with a 45° fixed incident angle. The 1.064 μm fundamental beam was split into two paths, with one beam passing through the sample and the other through a quartz reference to normalize readings against laser power fluctuations. The *p*-polarized component of the second harmonic light generated by each sample was collected after passing through an infrared filter. A second set of measurements was made after annealing the same samples at 75°C in N_2 atmosphere for 1.5 h.

2.5. Contact angle measurements

Contact angles of water on 0.1 mm diameter PLLA fibers were measured by the Wilhelmy technique. After cleaning by sonication in Millipore water, fibers were cut into ~1 cm sections and dipped into a 1.5 mg/ml solution of **1** in ethyl acetate and allowed to dry. A separate set of fibers was dipped in neat ethyl acetate as a control. Individual treated fibers were suspended vertically from a microbalance and static force measurements were recorded before and after immersion into Millipore water. Equilibrium forces on the fiber in this arrangement are related by the Young equation [27]

$$\gamma_{sv} - \gamma_{sl} = \gamma_{lv} \cos\theta,$$

where γ_{sv}, γ_{sl}, and γ_{lv} are surface tensions at the fiber–air, fiber–water, and air–water interfaces, respectively, and θ is the contact angle of water on the fiber. Since the change in force on the fiber upon immersion, ΔF, is equal to $(\gamma_{sv} - \gamma_{sl})$ multiplied by the fiber perimeter p, contact angles can be calculated as follows,

$$\Delta F = p\gamma_{lv} \cos\theta.$$

Due to similarity in the densities of water and PLLA, buoyant forces on fibers were considered negligible. Mean contact angles of the coated and control fibers are presented with uncertainties of one standard deviation.

2.6. Fiber settling

PLLA fibers with diameters of approximately 12 μm were cut into ~1.5 mm lengths and dip coated in a 1.5 mg/ml solution of **1** in ethyl acetate. Fibers dipped in neat ethyl acetate were used as controls. Equal weights of ethyl acetate dipped control fibers and fibers coated in **1** were placed in vials containing Millipore water. Slight manual agitation was used to disperse fibers, and then vials were permitted to settle over 30 min.

2.7. Scaffold construction

In acellular work, 10 mg of coated fibers prepared as described above were loaded into a 0.25″ cylindrical die in the presence of Millipore water and compressed at 700 kPa for 10 s. For work involving cells, 10 mg of the coated fibers were added to the die along with 2.4×10^5 MC 3T3-E1 mouse calvaria cells (generously donated by Lonnie Shea) suspended in 190 μl minimum

essential alpha medium (MEM-α) with 10% fetal bovine serum (FBS). Cell-fiber suspensions were compressed into scaffolds as described above and transferred to 24 well polystyrene culture plates. After 2 h of incubation, the seeded scaffolds were suspended in 2 ml MEM-α with 10% FBS. The 2 h delay before addition of MEM-α was instituted to provide cells with opportunities to attach without being rinsed off scaffolds.

2.8. Hoechst 33258 DNA content assay

At intervals of 1, 7, and 14 days after seeding, scaffolds formed by the above procedure were removed from culture and stored at −20°C. Frozen scaffolds were lyophilized and weighed, and then submersed in 0.5 ml of 0.125 mg/ml papain in phosphate buffer with EDTA. Specimens were incubated for 15 h in a 60°C water bath to dissolve proteinaceous matter and release DNA into solution. Fifty microliters of post-digestion supernatant were added to poly(methyl methacrylate) cuvettes containing 2 ml of 0.01 mg/ml buffered Hoechst 33258 solution. DNA content of samples was quantified by measuring emission intensities at 450 nm with a spectrofluorometer with exciting wavelength 355 nm. Intensity measurements were converted to DNA concentrations by a standard curve constructed from measurements of prepared calf-thymus DNA standards. Cell densities were extrapolated from total DNA contents per dry mass scaffold by applying the standard of 7.7 pg DNA per cell. Average cell contents of scaffolds are presented with *t*-based confidence intervals of 95%.

2.9. Scanning electron microscopy (SEM)

Immediately after removal from culture, scaffolds were fixed for 1 h with 2% glutaraldehyde in sodium cacodylate buffer and then post-fixed for an additional hour in 1% OsO_4. Fixed specimens were dehydrated in ethanol, critical point dried, mounted onto Al stubs with carbon tape, and sputter coated with 8 nm Au/Pd. All images were collected with a Hitachi 4500 SEM operating at low accelerating voltage.

3. Results and discussion

3.1. Characterization of self-assembly via SAXS and SHG

The self-assembling nature of **1** is demonstrated in powder SAXS scans shown in Fig. 1, where the (0 0 1) *d*-spacings for as-cast material and material annealed at 75°C for 1.5 h were determined to be 13.3 and 14.0 nm, respectively. The as-cast material revealed a (0 0 2) peak as well, confirming the layered structure. With $T_g =$ 54°C in the dry, bulk state, **1** exhibits glassy properties at physiological temperature. This measurement was primarily used to determine annealing temperature, but does provide some insight into the molecule's properties at physiological conditions. Flexibility is desirable for biological interactions, and one can expect dendron mobility to be enhanced by hydration. The width of the diffraction peaks is likely due to packing imperfections or the short coherence length of the layered structure. These imperfections could be ascribed to the polydispersity of oligo(L-lactic acid) segments. However, the decrease in diffraction intensity upon annealing suggests heating serves to decrease the level of order.

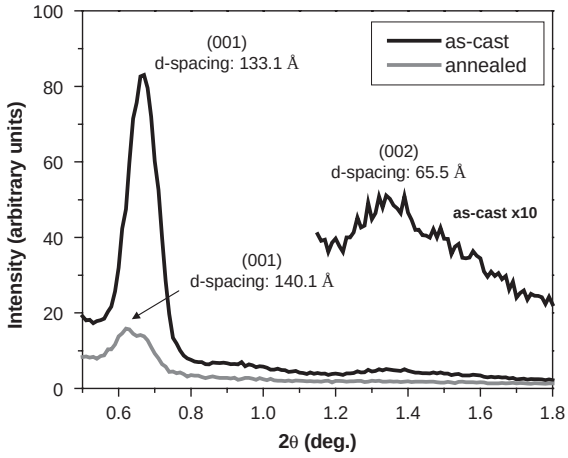


Fig. 1. SAXS scans versus 2θ for annealed and as-cast films.

The *d*-spacings observed in the diffraction patterns closely match the predicted length of **1**. The lengths of cholesterol and the G2 lysine dendron segment are ~12 Å and ~10 Å, respectively.[1] Assuming each lactic acid unit has a length of ~2.9 Å in a 10_3 helical conformation [28], the entire length of an average molecule can be estimated to be 11.8 nm, but this value should increase with hydration of the lysine dendron. Because the periodicity demonstrated by SAXS is very close to one molecular length for both samples, the molecules could be packed into monolayer lamellae without significant interdigitation.

SHG intensities indicate that films of **1** have polar character when cast from a chloroform solution (Fig. 2). The growth in signal strength with increasing film thickness suggests the polar order is a bulk property of the as-cast films and that this signal does not merely correspond to an interfacial effect of broken symmetry [29]. Given the periodicity revealed by SAXS, it is likely that a portion of the molecules in as-cast samples are packed head-to-tail into noncentrosymmetric monolayers (Fig. 3). Since stacked or interdigitated bilayers and randomly oriented monolayers are centrosymmetric and would not contribute significantly to SHG, they are

[1] calculated by Cerius2 molecular modeling software.

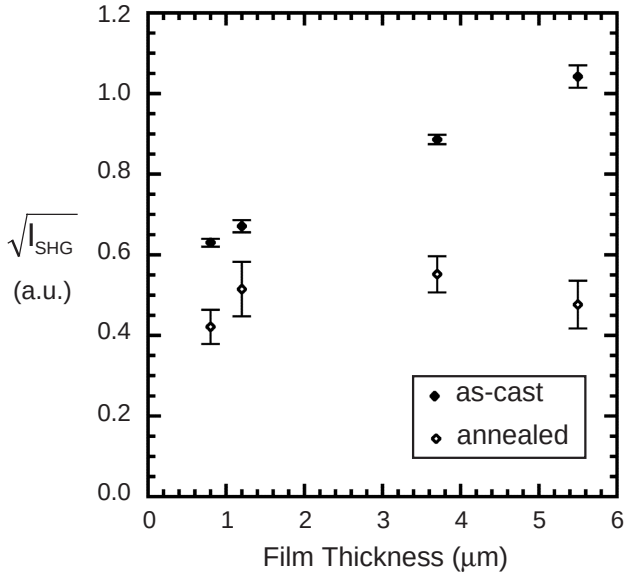


Fig. 2. $\sqrt{I_{SHG}}$ vs. film thickness for as-cast and annealed films of **1**.

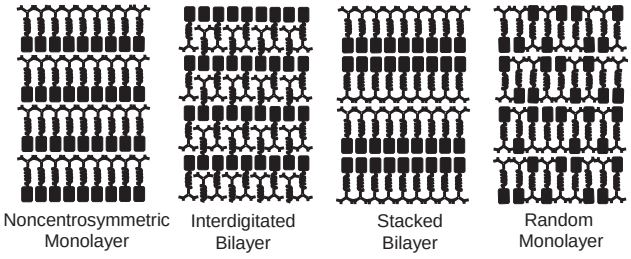


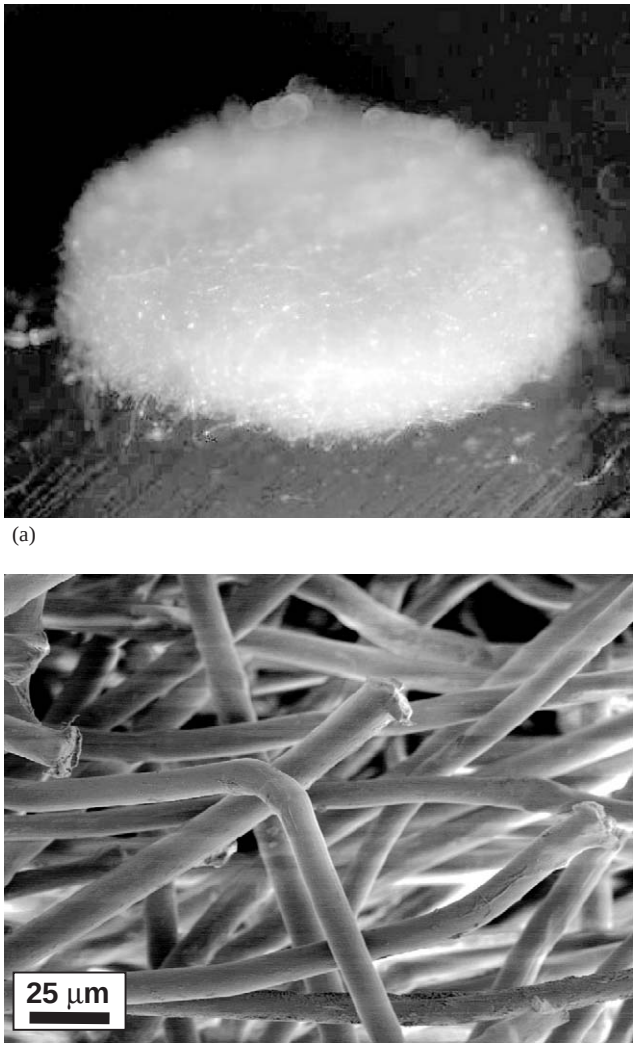
Fig. 3. Possible packing configurations of **1**.

not possible configurations of the polar portions of as-cast films [29,30].

In annealed samples, SHG intensity was lower than as-cast samples and essentially constant across the range of film thickness. Because $\sqrt{I_{SHG}}$ did not scale proportionately with thickness as it did prior to annealing, it is probable that heating caused the films to rearrange into centrosymmetric configurations, for example polar polydomain structures [31,32]. Although it is difficult to determine the extent of polar arrangement in as-cast material from the magnitude of SHG, it is likely that a polydomain structure existed there as well. One would expect that upon annealing, the tendency for polydomain formation increases substantially. The shorter coherence length of the remodeled domains may be related to the SHG and SAXS intensity decrease upon annealing. Hydration may also decrease the polar order parameter of molecular layers assembled on scaffold fibers since it would increase the unfavorable nature of contacts between cholesterol moieties and dendritic segments. The polar monolayer arrangement of **1** should be most beneficial for cellular interactions because the charged, hydrophilic portions of molecules should face outwards in self-renewing layers when assembled on a hydrophobic substrate such as PLLA. Fortunately, the annealing temperature used here and the T_g of **1** lie above body temperature, so the polar configuration would be kinetically trapped as biodegradation proceeds.

3.2. Pressed fiber scaffold morphology

Fiber scaffolds bonded by compression are cylindrical in shape and have the appearance and approximate strength of felt (Fig. 4). Despite the low pressure used in their formation, the pressed fiber scaffolds had enough mechanical integrity to remain intact throughout the study. This fibrous design provides a highly permeable, interconnected structure with a large surface area. Unlike most synthetic polymer scaffolds, however, it avoids the potentially detrimental consequences of harsh solvents and extreme temperatures during processing. In addition, because fibers can be chemically modified via



(a)

(b)

Fig. 4. (a) Pressed fiber PLLA scaffold under low magnification. (b) SEM image of pressed fiber PLLA scaffold.

self-assembly before they are incorporated into scaffolds, this method guarantees uniform coverage of all surfaces, not just those on the scaffold exteriors. Thus, for certain tissue engineering applications, the formation of scaffolds via mechanical compression appears to be a viable alternative to other scaffold processing techniques.

3.3. Influence of self-assembled coatings on interfacial properties of scaffolds

Fig. 5 shows mean contact angles on untreated PLLA fibers, as well as those dipped in neat ethyl acetate and ethyl acetate containing 1.5 mg/ml of **1**. These measurements confirm retention of **1** during the dip coating process and suggest that interactions at the fiber–water interface are significantly modified when PLLA is covered by the self-assembling layers. The lower mean contact angle of coated fibers relative to controls indicates that molecules are oriented with their hydrophilic lysine dendrons facing outwardly and hydrophobic cholesterol blocks contacting the PLLA surface. This orientation is optimal for interactions with cell membranes, as initial cell contacts will likely involve long-range electrostatic interactions between the positively charged substrate and the negatively charged exteriors of cells. Also, this orientation is ideal to establish hydrogen bonds between the cell's polysaccharide shell and the substrate. In fact, the average contact angle measured on fibers coated with **1** was within the range for maximal cell attachment, spreading, and growth determined by Vogler in previous studies [33].

The influence of fiber surface chemistry on aqueous interactions is dramatically evident in the observed settling behavior of loose fibers (Fig. 6). Fibers coated with the self-assembling molecules tend to settle into more compact structures, while ethyl acetate dip controls tend to settle less, forming open networks with greater settling heights. Settling is frequently used in colloid science to quantify degrees of particulate interaction. Generally, the greater the extent of interparticle association, the less compact the structure formed upon settling and thus the greater the settling height [34]. Translating this to the fibrous system studied here, it is apparent that the larger settling height of ethyl acetate dip control fibers is the result of greater interfiber binding interactions. This binding impedes packing and causes the fibers to settle into open networks that do not completely collapse under gravity. In contrast, interfiber association between the hydrophilic coated fibers is apparently less extensive. As a result, there is less resistance to settling and the fibers are able to form more compact structures.

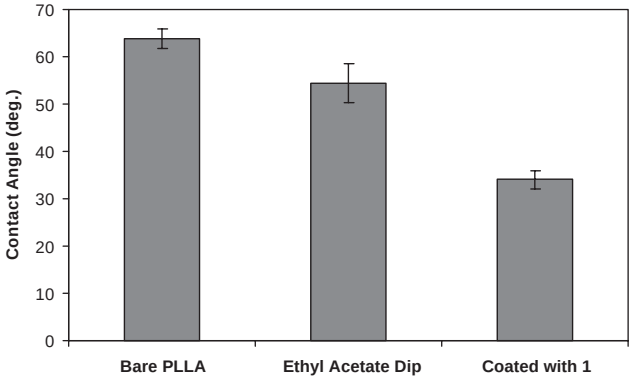


Fig. 5. Water contact angles on PLLA fibers with different surface treatments.

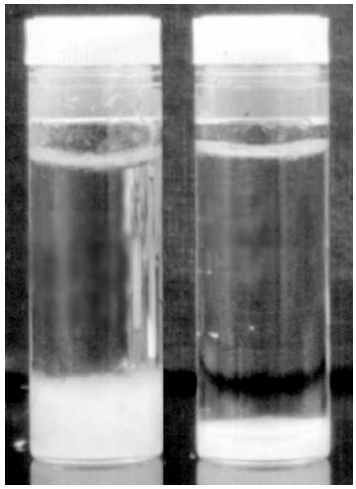


Fig. 6. Settling behavior of loose fibers when immersed in water (left: ethyl acetate dip control PLLA fibers, right: PLLA fibers coated with **1**).

The tendency for network formation in the hydrophobic ethyl acetate control fibers appears to be a manifestation of the long-range hydrophobic interaction. Although the true molecular origin of this interaction in water remains a source of ongoing debate [33,35], recent theoretical and experimental evidence suggest it is related to the depletion of water density at hydrophobic interfaces, leading to the formation of vapor-like layers or even submicroscopic bubbles [36–41]. When two hydrophobic surfaces such as the control fibers are brought into close contact, the depleted areas coalesce and form stabilizing bubbles through capillary vaporization. The growth and bridging of these bubbles is believed to be responsible for the long-range nature of the interaction, which far exceeds the limits of the classical Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloidal stability [41]. Because the settling behavior of the ethyl acetate dipped fibers was observed to be independent of electrolyte content, it is unlikely the interaction is osmotic or electrostatic in origin.

In contrast, the charged, hydrophilic surfaces of coated fibers are likely to be strongly hydrated due to

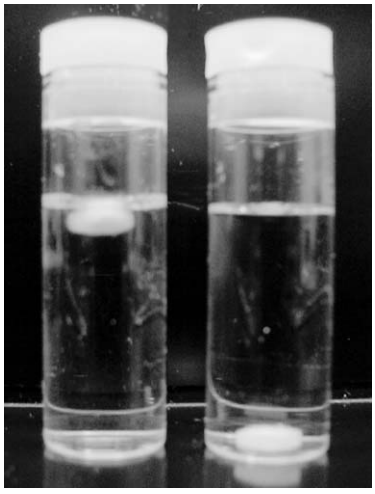


Fig. 7. Typical behavior of pressed fiber scaffolds when immersed in water (left: untreated PLLA fibers, right: PLLA fibers coated with **1**).

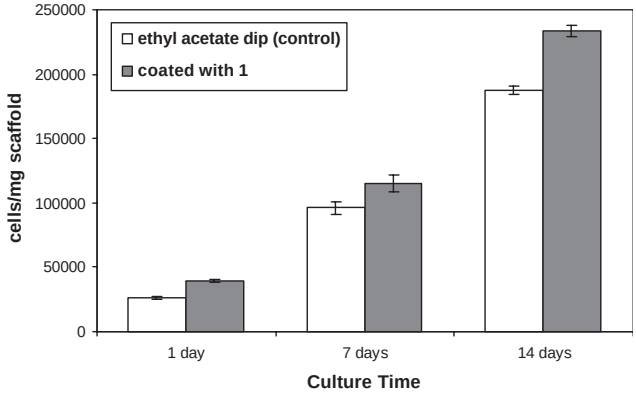


Fig. 8. Cell density versus culture time as determined by Hoechst 33258 assay.

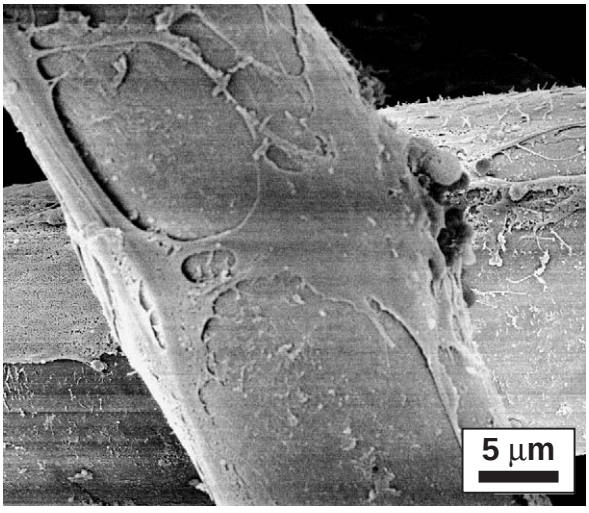
the hydrogen bonding sites they provide and the osmotic pressure generated by their diffuse double layer ions [27]. This hydration shell should discourage the formation of interfiber contacts during settling because this would require the confinement and eventual displacement of interfacial water molecules and double layer ions. As a result, interfiber association is likely to be greatly inhibited until fibers come to rest on the bottom of the vials in compact structures.

Further evidence of altered interactions at the fiber–water interface upon coating with the self-assembling molecules is presented in Fig. 7, which shows typical behavior of pressed scaffolds when introduced to water. Untreated PLLA scaffolds float because water does not wet the interstitial spaces between hydrophobic fibers. In fact, untreated scaffolds usually remain floating for several days, and hydrate only after substantial tapping to release trapped air. In contrast, water completely wets the hydrophilic fiber surfaces in coated scaffolds and causes them to sink soon after exposure. From these collective observations, it is clear that untreated PLLA does not offer enough polarity and hydrogen bonding sites to compete with water's preference for self-association via hydrogen bonding [33]. This behavior is problematic in the aqueous culture environment because it creates significant resistance to cell transport during seeding in porous scaffolds. The incorporation of more hydrophilic surface chemistry via coating with **1** should not only improve cellular access but also provide a more attractive surface on which cells can attach and proliferate.

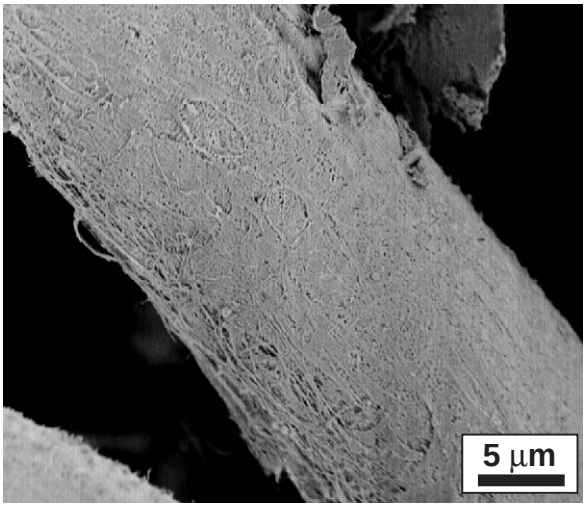
3.4. In vitro evaluation of modified scaffolds

The direct biological advantages of coating fibers with **1** are confirmed by Hoechst 33258 assay data, which indicate that substantially more cells were attached to treated scaffolds than ethyl acetate dipped controls within 24 h and up to 2 weeks of culture (Fig. 8). Population growth rates on both types of scaffolds were linear rather than exponential, indicating moderately high attrition levels. Cell numbers on coated scaffolds increased by an average of approximately 15,000 cells/mg scaffold/day, while rates on ethyl acetate dipped controls were slightly lower at 12,500 cells/mg scaffold/day. The largest difference in cell density between the coated and control scaffolds was after 24 h of culture, at which there were 51% more cells per scaffold weight on coated scaffolds than ethyl acetate dipped controls. This difference in cell density between coated scaffolds and controls decreased to 19% and 24% after 1 and 2 weeks of culture, respectively. The abundance of cells on coated scaffolds is corroborated by SEM images in Fig. 9, which show cells adhered and spread after 24 h, and confluent after 1 week.

Given an attachment time of several hours and a population doubling rate of approximately once every 18 h, it can be reasoned that the 24 h samples primarily reflect initial cell adhesion minus attrition, whereas the 1 and 2 week samples represent proliferation in addition to the initial adhesion and ongoing attrition. The greater net population growth rate on coated scaffolds indicates that cells on these scaffolds reproduced at greater rates and/or died at slower rates than cells on ethyl acetate dipped controls. Because percent differences in cell populations between coated and control scaffolds were largest after 24 h, it is likely that the most significant effect of the self-assembling molecules was to increase initial cell retention. It is not surprising the coatings had their most pronounced influence in the early stages of culture because the deposition of serum proteins and extracellular matrix on scaffolds should eventually lead to screening of the original surfaces. Nevertheless, the outcome of these initial adhesion and growth rate differences was substantial, as there was an average of



(a)



(b)

Fig. 9. (a) SEM micrograph of cells adhered and spread on PLLA fiber scaffolds coated with **1** after 24 h. culture. (b) SEM micrograph of confluent cells on PLLA fiber scaffolds coated with **1** after 1 week culture.

nearly 50,000 more cells/mg scaffold on coated scaffolds than ethyl acetate dipped controls after two weeks in culture.

These results provide evidence for the importance of substrate surface chemistry in tissue engineering. The increased cell retention on coated scaffolds could be attributed to the cationic, hydrophilic character of the lysine dendrons that provides a coulombic complement to anionic charges on cell surfaces and may therefore facilitate the formation of initial cell-substrate contacts [42] as well as the subsequent organization of focal adhesion complexes [20–22]. The hydrophilicity of the lysine dendrons should also help minimize denaturation of adsorbed proteins, [23–25] potentially making ligand presentation more conducive to the formation of integrin mediated adhesive contacts. Signals generated by integrins upon adhesion and assembly of focal adhesion complexes regulate many functions that are critical to the survival of cell populations, including the prevention of apoptosis [43] and initiation of proliferation [44]. Because the time to form integrin contacts and complete a single mitotic cycle may have exceeded the first 24 h of culture, the impact of integrin binding on proliferation should be most evident in the 7 and 14 day specimens, which is consistent with the greater overall population growth rate on coated scaffolds.

The coating of scaffold fibers with the self-assembling molecules studied here offers several advantages over poly(L-lysine). Firstly, **1** is insoluble in water and therefore less likely to erode from surfaces by dissolution. Instead, it can be expected to biodegrade over time into cholesterol, L-(lactic acid), and L-lysine. By using amphiphilic molecules, we are able maintain the hydrophilic, cationic character of L-lysine, yet present it in an insoluble form. The solubility of poly(L-lysine) is problematic not only because it decreases coating stability, but also because various studies have demonstrated that the polymer is cytotoxic in solution [45–48]. Greater configurational freedom makes linear, flexible cationic macromolecules such as poly(L-lysine) more capable of causing cellular membrane disruption and surface charge neutralization than smaller, more rigid cationic molecules [45]. Aside from factors related to cytotoxity, poly(L-lysine) has also been shown in some studies to be potently immunogenic [49,50]. Because L-lysine residues in **1** are presented in the form of very low molar mass dendrons and not as long, linear sequences as they are in poly(L-lysine), it is possible they will not be subject to the same type of recognition by mediators of the immune response.

The cholesteric portion of **1** may also have had an impact on the formation of integrin contacts. Recent studies indicate that increasing the cholesterol content of plasma membranes promotes integrin clustering and focal adhesion complex formation [13]. Evidence suggests that the stimulatory effects that cholesterol and lipid rafts have on integrin activity may be related to cholesterol's influence on the translational mobility of membrane components [13] as well as its ability to induce favorable conformational changes in membrane proteins via specific molecular interactions [17]. It has also been proposed that the vesicular transport and sorting capabilities of lipid rafts may indirectly benefit adhesion through the trafficking of preformed modular adhesion structures to attachment sites in the membrane [51].

In addition to these potential effects on plasma membranes, cholesterol may have other impacts on cell populations by influencing metabolism. Under standard conditions, cells synthesize their own cholesterol and extract it from lipoproteins [12]. Because cholesterol is continuously lost to outside circulation and consumed

for biosynthesis, its intracellular concentration must be closely monitored. By providing an external source of cholesterol, **1** may also help to conserve cellular resources while furnishing extra material for lipid raft incorporation and focal adhesion formation. Although excess influx of cholesterol is toxic [12], there are no indications that this occurred with cells cultured on PLLA coated with **1**.

4. Conclusions

We demonstrated that molecular self-assembly provides an efficient and effective means of creating ordered multilayers on common biomaterials that dramatically alter their surface properties. By presenting biologically relevant chemistry in these interfacial multilayers, a cell response can be triggered which is useful in cell culture, tissue engineering, and cell transplantation.

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