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Comparison of Selective Attachment and Growth of Smooth Muscle Cells on Gelatin- and Fibronectin-Coated Micropatterns

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Tissue engineering research has been on going for many years, people are making all the effort to explore the cell functions in cellular level and even in molecular level. Make the cells functional in an *in vitro* environment is a preliminary goal for the implantation and repair of complicated tissues/organs. Fabricating artificial ECM to mimic the *in vivo* environment is an essential approach in tissue engineering. The work in this paper is to study how rat aorta smooth muscle cells (RASMCs) behave in two engineered cell culture scaffolds: gelatin- and fibronectin (FN)-coated micropatterns. The investigation on the initial attachment and further growth of SMCs cultured on gelatin- and FN-coated micropatterns was addressed. This study focused on both the characterization of gelatin and fibronectin assembly properties and cell responses to these two protein-coated micropatterns. Thin film patterns with gelatin and fibronectin coatings were fabricated on microscope glass slides using photolithography, electrostatic layer-by-layer self-assembly and lift-off (LbL-LO) technologies. In this work, the scaffolds were built up by commonly used polyelectrolyte materials and proteins through LbL process, containing cationic poly(diallyldimethylammonium chloride) (PDDA), poly(allylamine hydrochloride) (PAH), anionic poly(sodium 4-styrenesulfonate) (PSS), gelatin and fibronectin. The resulting polyelectrolyte thin films were characterized by contact angle (CA), quartz crystal microbalance (QCM), atomic force microscopy (AFM), and fluorescence microscopy. CA measurement shows the consistent hydrophylicity of gelatin surfaces in different number of layers with LbL deposition method. Different from our previous QCM measurement of gelatin, fibronectin does not show highly electrostatic attraction to either positively or negatively charged polyelectrolytes, although it can be weakly assembled to both polyelectrolyte surfaces. AFM images show Gelatin- and FN-coated micropatterns are around 50–60 nm thick. RASMCs were cultured on these gelatin- and FN-coated micropatterns. It was observed that, for the cells cultured on gelatin-coated micropatterns, they initially landed on the gelatin-coated surface, not on the PDDA-coated surface in between. But further growth of the cells was affected by the shape of the patterns: strip pattern limited cell growth beyond the patterns, but square patterns could not. While, it was found interestingly, for the cells cultured on FN-coated micropatterns, SMCs initially landed on PDDA-coated surface, and then migrated to FN-coated both square and strip patterns. These findings indicate that both gelatin and fibronectin are adhesive proteins, but they have different effects on the initial attachment and later growth for SMCs.

Keywords: Smooth Muscle Cells (SMCs), Attachment, Layer-By-Layer Self-Assembly, Polyelectrolyte, Gelatin, Fibronectin (FN).

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1. INTRODUCTION

Control *in vitro* cell behavior over the methods of surface patterning and surface modification has been developed for several decades. There are many approaches to pattern cells, usually by the way to pattern adhesive and nonadhesive regions on a surface, such that cells attach to the adhesive regions and are repelled by or unable to attach to the nonadhesive regions.[1] Cells adhere preferentially to the patterns that are coated with adhesion molecules, such as collagen[2,3] and fibronectin[4] but not to the control regions on which nonadhesive polymer films are deposited.

There has been an increasing interest in developing new biomaterials to improve biocompatibility of substrate surfaces that can resist or enhance cellular adhesion by mimicking extracellular matrix (ECM) *in vivo*.[5,6] Studies suggest that physical and chemical material surface properties[7,8] from ECM, including surface topography, surface hydrophilicity/hydrophobicity, and surface charge polarity and density in engineered cell culture substrates, may provide important regulatory information to control cell adhesion, spreading, and growth.[9–11] The use of thin films of ECM proteins by applying contact printing and electrostatic layer-by-layer self-assembly has been applied in studies of cell–cell/cell-matrix interactions.[12–15] ECM controls many aspects of cell behavior, including attachment, growth, alignment, migration, and differentiation.[16,17] Artificial ECMs and related biomaterials have been being explored as microstructured scaffolds for tissue engineering applications.[18–20]

The fabrication of polyelectrolyte multilayer thin films on substrate surfaces is crucial for scientific and biomedical fields in order to modify and improve the substantial characteristics of these surfaces and has received much attention recently as a simple yet versatile technique for assembling various nanostructured thin film coatings.[12,15,21–24] The effective deposition of biomaterials with polymers thus results in a potentially great change in biological affinity.[25–28] Self-assembled monolayers (SAMs) has attracted more and more attention for mechanistic studies of the interactions of proteins and cells with surfaces and for developing methods to pattern the adhesion of cells,[13,29–31] to prepare surfaces that present ligands for selective interactions with proteins,[32–34] and to design electrostatic dynamic substrates that can modulate the selective recognition of proteins.[35,36] It was reported that multilayer thin films containing several protein species were built up by SAMs.[37–39] Nanostructured polyelectrolyte multilayer thin films electrostatically assembled alternately from such polyelectrolytes were investigated for the *in vitro* cell interactions, which indicated by manipulating the multilayer pH or ionic strength assembly conditions or both, the molecular architecture of the thin films may powerfully direct a single multilayer combination to be either cell adhesive or cell resistant.[13,40,41] Layer-by-layer self-assembly and lift-off (LbL-LO) technique provides an alternative method to produce adhesive patterns that are useful for spatially positioning cells and controlling their growth, which involves the combination of general photolithography and basic layer-by-layer self-assembly techniques.[42–44]

Attachment is always an important factor to control cell growth in an *in vitro* environment, and it is assumed that cells would like to grow on the region where they prefer to land on. It is true in some cases, but not always. As known, collagen acts as scaffolding for human bodies and controls cell shape and differentiation and it is the most important building block in the entire animal world. While gelatin, essentially denatured collagen isolated from animal skin and bones, is very cheap and widely used in many scientific and industrial applications.[45–49] The extracellular matrix also contains noncollagenous adhesive proteins, such as fibronectin, which play critical roles in organizing the matrix and in enabling cells to attach to it. In this work, the initial attachment and further growth of SMCs were investigated by two, gelatin and fibronectin, protein-coated micropatterns. It was found that gelatin and fibronectin controlled cell growth in different manners from each other.

Gelatin charge properties and cell cultured on gelatin- and FN-coated flat surfaces have been reported in our previous work.[50,51] In this paper, the charge properties of fibronectin and hydrophobicity of assembled gelatin films were studied. The surface topography of gelatin- and FN-coated micropatterns was shown by AFM images. Cell behaviors on both protein-coated patterns were observed by optical/fluorescence microscope, which revealed that these SMCs initially attached on gelatin-coated pattern surface, but did not landed on FN-coated surface. However, further growth of the cells is much different from those on gelatin-coated scaffolds to FN-coated scaffolds. The cells initially landed on gelatin-coated strip patterns still continued to grow on these patterns, but cells landed on gelatin-coated square patterns may grow on the PDDA-coated surface also. While, although SMCs did not landed on FN-coated surface, finally, all the cells migrated and only grew on the FN-coated patterns, no matter square patterns or strip patterns.

2. MATERIALS AND METHODS

All polyelectrolytes were dissolved in deionized (DI) water at pH 5.5 at the concentration of 2 mg/mL with 0.5 M KCl, and the adsorption experiments were performed at the same pH except specified. Gelatin (Bloom 225, Type B, Sigma-Aldrich) and poly(sodium 4-styrenesulfonate) (PSS, MW 70,000, Sigma-Aldrich) were used as the negatively charged polyelectrolytes, and poly(diallyldimethylammonium chloride) (PDDA, MW 400,000, Sigma-Aldrich) and poly(allylamine hydrochloride) (PAH, MW 15,000, Aldrich Chem. Co.) were used as positively charged electrolytes. Fibronectin

(Sigma Chemical Co.) was used for the QCM measurement and weakly assembled to polyelectrolyte surface. Fluorescein 5-isothiocyanate (FITC, MW 389.4, Sigma-Aldrich) and Texas-Red (TR, MW 796.74, Molecular Probes) were labeled to PAH forming FITC-PAH and TR-PAH for pattern recognition. Potassium chloride (KCl) and Nanostrip were also purchased from Sigma. Glass substrates (Fisher Scientific) were the general precleaned microscope slides as base solid supports for the fabrication of thin film patterns. Positive photoresist 1813 and MF 319 developer used in the lithography process were ordered from Shipley. RPMI 1640 cell culture medium (HyQ® RPMI-1640 Medium cell culture reagents, HyClone) and Fetal Bovine Serum (FBS, Atlanta Biology Co.) were used as basic nutrition for SMCs. Vybrant® Apoptosis Assay Kit #5 Hoechst 33342/propidium iodide and Alexa Fluor® 488 phalloidin purchased from Molecular Probes were used to stain nuclei and F-actin of SMCs.

Polyelectrolytes, gelatin and fibronectin solutions were prepared and glass substrates were pretreated with Nanostrip at room temperature. Photolithography technology is a widely used technique in microelectronic and MEMS industry. The approach employed in the self-assembly adsorption experiments to build up polyelectrolyte thin films is similar to those reported in the publications.[43, 50] A schematic illustration of LbL-LO process for this work is given in Figure 1.

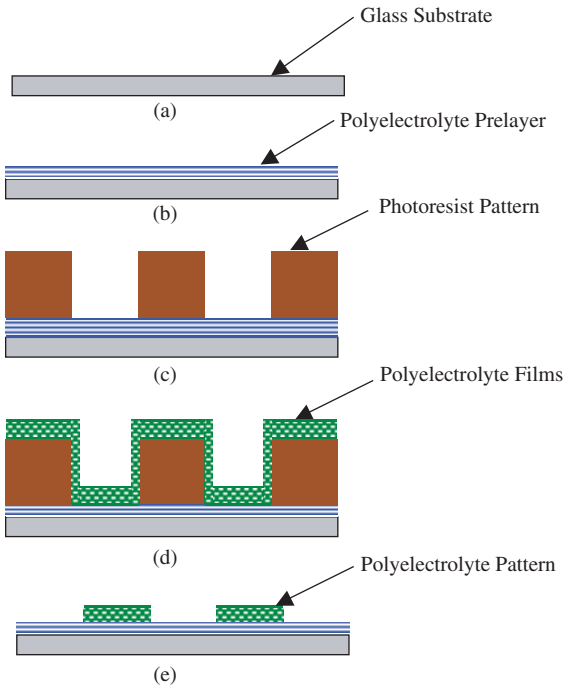

Fig. 1. Schematic illustration of LbL-LO process. (a) Plain glass substrate after Nanostrip pretreatment. (b) Self-assembled polyelectrolyte prelayer on glass substrate. (c) Photoresist pattern after lithography. (d) Polyelectrolyte films forming on the photoresist pattern by layer-by-layer self-assembly process. (e) Polyelectrolyte pattern after Lift-off process.

The charge property of fibronectin was tested with a Quartz Crystal Microbalance (QCM, Iwatsu, SC-7201, Universal Counter). The hydrophilicity/hydrophobicity and stability of polyelectrolyte multilayer thin films were measured with a contact angle system (OCA, Dataphysics, Germany). Gelatin- and Fibronectin-coated polyelectrolyte patterns were inspected and characterized with an inverted epifluorescence microscope (Nikon ECLIPSE TS100/TS100-F) and an atomic force microscope (AFM, Q-Scope™ 350, Quesant Instrument Corporation).

Monolayer cell culture technology applied in the experiments is same as in our previous report.[51] All cells used were RASMCs, which were obtained from Louisiana State University Medical Center at Shreveport. Cells were observed with the inverted epifluorescence microscope for a period of up to one week. RPMI 1640 complete cell culture medium was usually changed every other day over the course of the culture to supply necessary nutrition required by SMCs.

3. RESULTS AND DISCUSSION

1. CA measurement of multilayer polyelectrolytes and gelatin thin films

   CA is a quantitative measure of the wetting of a solid by a liquid. When designing an *in vitro* cell culture system, hydrophilicity/hydrophobicity is an important factor to be considered for cell attachment. Using layer-by-layer self-assembly technology described in the materials and methods section, polyelectrolytes and gelatin were deposited on the planar glass slides with layering structure of PDDA/(PSS/FITC-PAH)$_2$/(gelatin/FITC-PAH)$_4$/gelatin. CA measurement was taken using water drops after 3 hours of completion of layering process.

   Figure 2 shows the CA measurements for different layers of polyelectrolyte thin films. The control glass surface has the smallest CA, almost complete wetting. CAs of PSS-coated surface are around 14 to 17. CAs of FITC-PAH coated surface ranged from 30 to 40 when alternately layered with PSS, while jumped to 40 to 50 when alternately layered with gelatin. Gelatin has a larger CA of 50 to 60. It has been known from our previous investigation, negative charged PSS, gelatin, or Nanostrip pretreated glass surfaces are cell-adhesive (cytophilic) for the attachment of SMCs, while positively charged films, such as PDDA, PAH, are cell-resistant (cytophobic) to SMCs to be attached. These CA measurements demonstrate that there is no direct relationship between hydrophilicity/hydrophobicity and cytophilicity/cytophobicity for these materials. However, Figure 2 also indicates that the wetting property of outmost layer is influenced by the underlying material, which can be seen from the CA difference of FITC-PAH when it is layered

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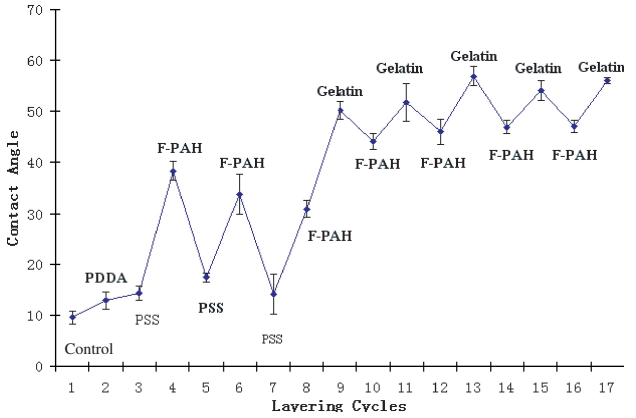


Fig. 2. Contact angle measurement of polyelectrolyte multilayer thin films with architecture of PDDA/(PSS/FITC-PAH)$_2$/(gelatin/FITC-PAH)$_3$/gelatin on glass substrates.

with PSS or gelatin. This idea may be extended to other aspects, such as surface charge polarity/density and roughness, when performing self-assembly process with different materials. Meanwhile, the surface stability of deposited electrolyte thin films with layer-by-layer self-assembly technology could also be seen from Figure 2. The CAs of the same material with different number of deposition layers are relatively consistent. It is very useful to make the surface properties consistent for each of the material when studying cell responses in engineered cell culture systems.

2. QCM Measurement of Fibronectin with polyelectrolytes

Although it is said that every protein has its isoelectric point (IP), the IP of fibronectin was not found during a careful search of the literature and consultation with vendors. QCM measurements were performed to study the charge property of fibronectin, using a similar procedure as for gelatin.[50]

Figure 3(a) shows QCM measurement of fibronectin with an architecture of (PDDA/PSS)$_3$/(PDDA/FN)$_3$/PDDA/(PSS/FN)$_6$ at pH 7.7 using a generic layering procedure. In this layering process, the QCM silver electrode was incubated in polymer solution for 10 minutes and fibronectin solution for 20 minutes for each individual layer. In this figure, it seems that fibronectin can be alternately deposited with PSS instead of PDDA, but the increase of frequency shift is too small to be believable for LbL process compared to previous QCM measurements of PDDA and FITC-gelatin. Although QCM measurements were repeated several times at different pH, as low as 5.76 and 2.0, a satisfactory increment in mass deposition either with PSS or PDDA was not achieved.

It was then considered that the adsorption saturation time of deposition from protein solution may be much longer than that for polymer solution. Therefore, an additional QCM measurement was performed, wherein the silver electrode was incubated in

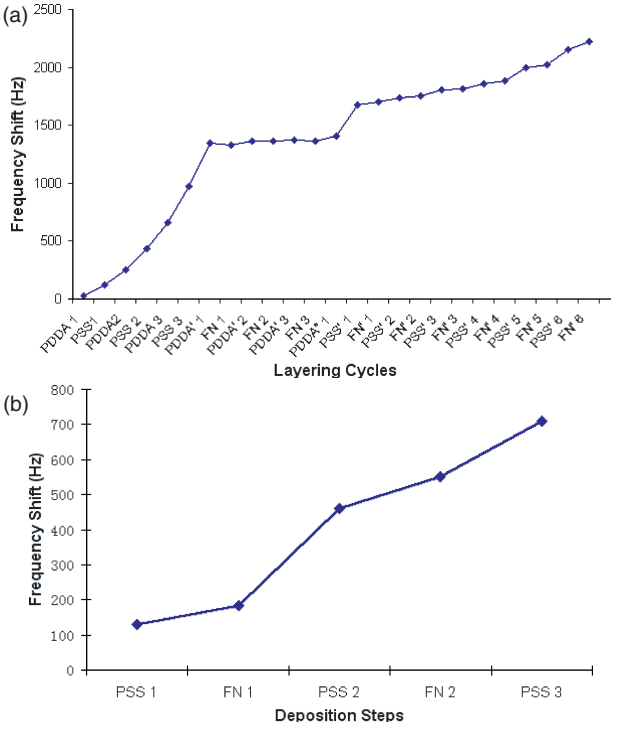


Fig. 3. (a) QCM measurements of (PDDA/PSS)$_3$/(PDDA/FN)$_3$/PDDA/(PSS/FN)$_6$ at pH 7.7; (b) QCM measurement of (PSS/FN)$_2$/PSS at pH 5.76 for overnight incubation for each layer.

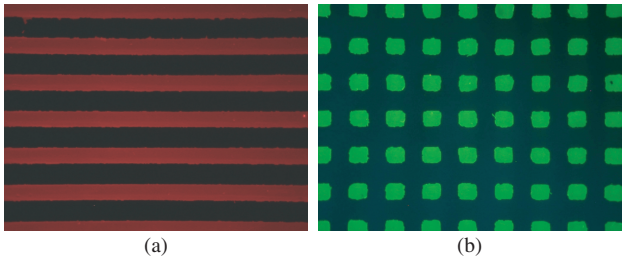
fibronectin solution overnight (around 10–12 hours) for each layer.

Figure 3(b) shows the more typical increase of frequency shift, which seems to indicate that fibronectin has been adsorbed on PSS-coated surface. However, as it is known that most proteins can self-adsorb on almost all the solid surfaces, it is hard to say whether the principal force of the adsorption between fibronectin and PSS is electrostatic or some other attractive force. Therefore, the charge polarity of fibronectin can not be sufficiently demonstrated. Meanwhile, a parallel QCM measurement was also performed to alternately incubate the electrode in fibronectin and PDDA solutions, the expected increment of frequency shift was not achieved in the deposition of PDDA layer. At this point, it is believed that it would have a better fibronectin coating on PSS-coated surface than on PDDA-coated surface.

In addition, in terms of predeposition of cell-adhesive materials, it is not acceptable to incubate the substrates overnight for each layer in LbL process due to the extended time required. Although LbL process was not proven to be applicable for fibronectin, Figure 3(b) does indicate that fibronectin can deposit on PSS-coated surface. This finding is sufficient to allow the contribution of scaffold production using fibronectin as the surface material to study cell attachment. Thus it is reasonable to deposit only one layer

of fibronectin as the outmost layer on PSS-coated surface for saving fabrication time.

3. Fluorescence microscopy inspection of thin film micropatterns
   Standard microscopy glass slides were used as the solid supports for the fabrication of cell culture scaffolds with gelatin- and FN-coated thin film patterns through LbL-LO. Before layering polyelectrolyte and gelatin/fibronectin nanofilms, the photoresist patterns were inspected in order to control the quality of final polyelectrolyte patterns.
   The characterization of fabrication results for these thin film patterns was facilitated by the inclusion of the fluorescent tags FITC or TR. After layering process, fluorescence images were taken before and after lift-off step. Figure 4 shows the representative fluorescence images of the thin film patterns after lift-off step. The layering architecture in Figure 4(a) is $(PSS/TR\text{-}PAH)_2/(gelatin/TR\text{-}PAH)_4/gelatin$, and in Figure 4(b) is $(PSS/FITC\text{-}PAH)_5/fibronectin$. Both patterns are built on PDDA-coated glass surface. The sizes of these strip and square patterns are ranged from 50 μm to 100 μm. These fluorescence images in Figure 4 demonstrate the success of fabricating polyelectrolyte thin film patterns on PDDA-coated glass substrate with LbL-LO technology.
4. AFM of gelatin- and fibronectin-coated thin film micropatterns
   Using photolithography and layer-by-layer self-assembly, multilayer polyelectrolyte thin film patterns were obtained after lift-off step. AFM scanning was performed for the samples mentioned in the above section. Figure 5 shows 3-D AFM graphs of gelatin- and FN-coated micropatterns. From these 3-D graphs, one can see that photoresist and atop polyelectrolytes are removed from the substrate surface after lift-off. It indicates the thickness of these two gelatin- and FN-coated thin films patterns is around 50 μm to 60 μm.
5. SMCs cultured on gelatin- and FN-coated micropatterns
   To study cell responses to both protein-coated micropatterns, SMCs were seeded onto these engineered substrates, which were sterilized in 1x ABAM.



(a) (b)

Fig. 4. Fluorescence images of thin film 80 μm patterns after lift-off step. (a) $(PSS/TR\text{-}PAH)_2/(gelatin/TR\text{-}PAH)_4/gelatin$ (b) $(PSS/FITC\text{-}PAH)_5/fibronectin$.

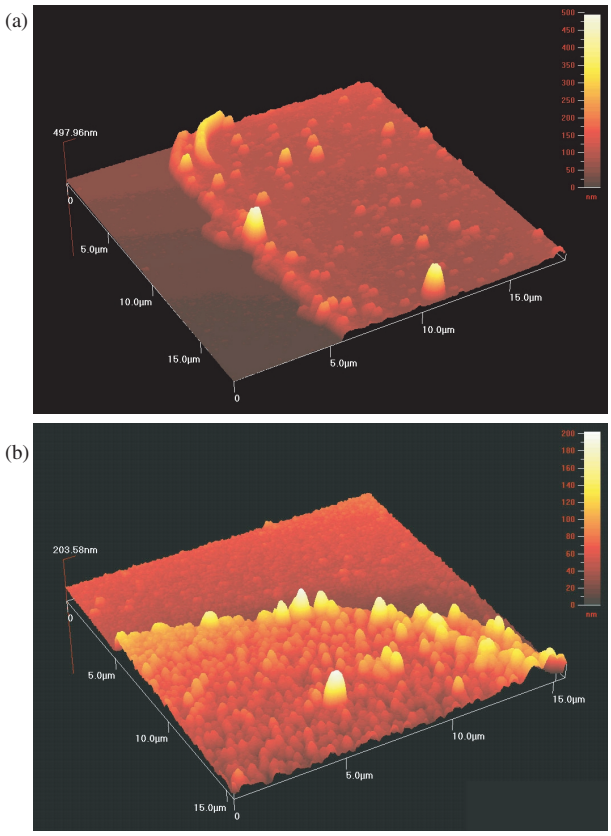
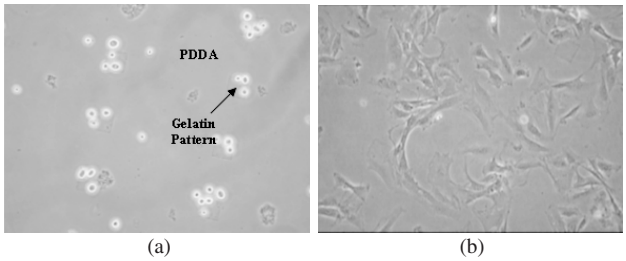


Fig. 5. 3-D AFM graphs of (a) gelatin- and (b) FN-coated patterns.

The initial attraction of SMCs to gelatin-coated square patterns is clearly shown in Figure 6(a). In 30 minutes of post-seeding, most SMCs landed on the surface of gelatin-coated patterns instead of PDDA-coated surface in between. It indicates that SMCs would like to land on gelatin-coated surface compare to PDDA-coated surface. This phenomenon did not show up for the cells seeded on FN-coated pattern substrate. After 2 days, the cells initially attached on gelatin-coated square patterns began to spread out and grew on the entire surface with both gelatin-coating and PDDA-coating of the substrate shown in Figure 6(b). SMCs seeded on gelatin-coated strip patterns displayed a similar manner of attachment at the beginning and grew on gelatin-coated surface. The



(a) (b)

Fig. 6. SMCs cultured on gelatin-coated 50 μm square patterns. (a) 30 min; (b) 2 days after post-seeding.

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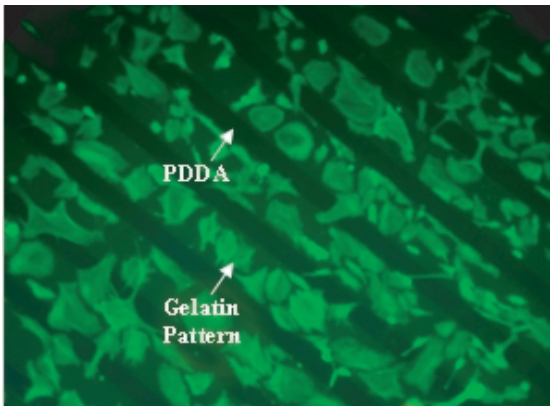

Fig. 7. SMCs cultured on gelatin-coated 100 μm strip patterns, 2 days after passage.

different behavior from that of the cells on square patterns is, after 2 days, they still stick to grow on the gelatin-coated surface of strip patterns; few migrate to the PDDA-coated surface in between. As shown in Figure 7, which is a fluorescence image of cells cultured on gelatin-coated strip patterns after stained by Alexa Fluor® 488 phalloidin.

SMCs cultured on FN-coated micropatterns exhibited totally different behaviors from those cultured on gelatin-coated patterns. As mentioned above, there

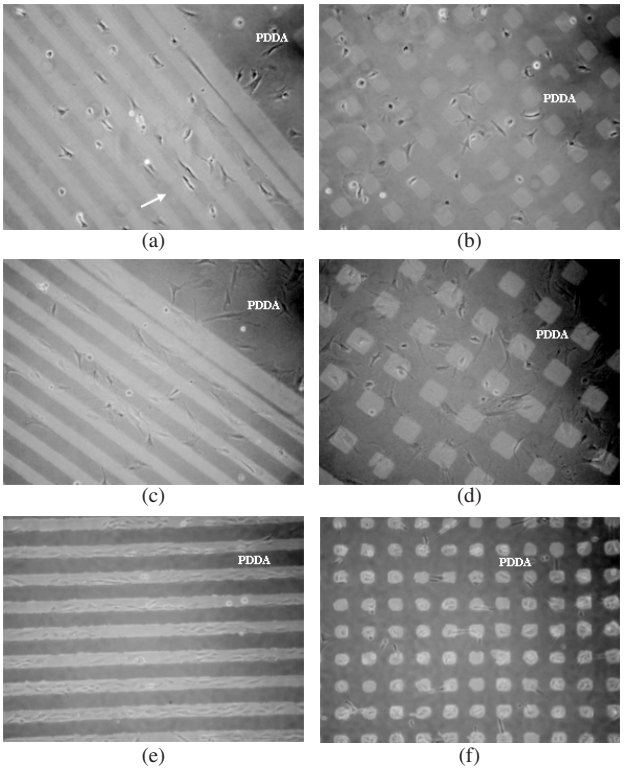

Fig. 8. SMCs cultured on fibronectin-coated 60 μm patterns. (a) Strip patterns, 12 hours; (b) Square patterns, 12 hours; (c) Strip patterns, 24 hours; (d) Square patterns, 24 hours. (e) Strip patterns, 2 days; (f) Square patterns, 2 days. Note: the light strips and squares are fibronectin-coated patterns; the dark regions are PDDA-coated surfaces.

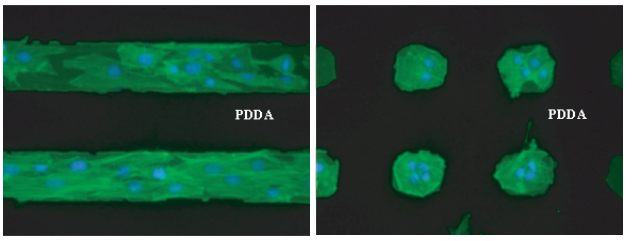

Fig. 9. SMCs cultured on fibronectin-coated 60 μm patterns, 2 days. (a) Strip pattern; (b) Square pattern.

was no significant preference of attraction for the cells to FN-coated surface or PDDA-coated surface in several hours after cell seeding. Then, some of the cells had to land on for surviving. It can be seen in Figures 8(a) and 8(b), at 12 hours, the cells seeded on both strip and square patterns selectively start to attach on the surface with PDDA coating instead of fibronectin coating. It was even observed interestingly that there was a single cell would rather to stay in the narrow PDDA-coated surface between two wider FN-coated strips, see the arrow in Figure 8(a). In Figure 8(b), the cells attached on PDDA-coated surfaces around FN-coated squares. Then, after another 12 hours, something changed that cells were migrating to fibronectin coatings from their original position of PDDA-coated surfaces shown in Figure 8(c) and 8(d). Figure 8(e) and 8(f) are the images taken for these cells of 2 days' post-seeding. It is so unbelievable to be found that almost all the cells have moved onto FN-coated strip and square patterns. For a better inspection, these cells were stained by Hoechst 33342 and Alexa Fluor® 488 phalloidin. The fluorescence images of stained cells in Figures 9(a) and 9(b) clearly show the cell nuclei and F-actins, demonstrating these cells are on the surface of FN-coated micropatterns.

The findings in this work indicate that fibronectin is probably a second mediating adhesive protein, while gelatin is the first modulating protein for the attachment of SMCs. It is known different cell type may have different response to the same material, so the further experiment will probably be focusing on the investigation of responses of other types of cells, endothelia, fibroblast or neurons, to gelatin and fibronectin coatings.

4. CONCLUSION

Fabricating multilayer thin film micropatterns with charged polyelectrolytes and proteins with LbL-LO technique is very useful for the surface modification and topography control, such that it benefit for *in vitro* tissue engineering research work. In this study, RASMCs were cultured on gelatin- and FN-coated strip and square

micropatterns. The experimental results indicate that both gelatin and fibronectin are cell-adhesive proteins, but they affect the attachment and growth of SMCs in different manners. Gelatin seems to be a first cell-adhesive signal, which attract SMCs initially land on gelatin-coated surface. Further growth of these cells depends on the shape of the gelatin patterns: strip patterns have more power to limit the cells to only grow on gelatin-coated surface, while square patterns do not. Fibronectin is much more different from gelatin in the manner to modulate the growth of SMCs. It is not the preferential material for the initial attachment of SMCs, but finally, it attracts all the cells to grow on its surface no matter the shape of the patterns, strip or square. These findings have never been reported at our best knowledge, they may benefit for the researchers working in the areas of tissue engineering and biosensors.

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