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Fabrication of 3-D Gelatin-Patterned Glass Substrates With Layer-by-Layer and Lift-Off (LbL–LO) Technology

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Abstract—The assembly of multilayer films of gelatin onto glass substrates using layer-by-layer and lift-off (LbL–LO) technology to modify the surface topography and chemistry properties of in vitro cell culture scaffolds is described. The ability to generate such nanoscale systems containing cell-adhesive materials on optically transparent substrates with microscale lateral dimensions, nanoscale vertical dimensions, molecular vertical precision, and flexibility in material selection has important implications for tissue engineering, drug discovery, and basic research in cell biology. Toward this goal, a systematic study on the electrostatic adsorption properties of fluorescein 5-isothiocyanate-gelatin B (FITC-gelatin) was completed. In addition, the integration of protein nanoassembly with microlithographic feature definition was used to pattern three-dimensional FITC-gelatin nanofilms on planar glass substrates. The experimental results indicate that FITC-gelatin is negatively charged at pH 9 and can be alternately assembled with a positively charged polion, poly(diallyldimethylammonium chloride) (PDDA), to form multilayer films on solid templates with thickness of 5–10 nm per bilayer. Furthermore, images of protein/polymer nanocomposites indicate that LbL–LO is an efficient way to realize the designed substrates. These findings will benefit future research on cell culture and tissue engineering that require methods of generating protein patterns to fabricate novel in vitro cell culture systems.

Index Terms—Cell culture, layer-by-layer (LBL) self-assembly, micropatterning, polyelectrolytes, protein adsorption.

I. INTRODUCTION

It is widely recognized that proteins and extracellular matrix (ECM) molecules play a vital role in biomaterial interactions. Because protein adsorption is apparently the major mediating factor in host response to foreign materials, and because cells typically interact with materials through molecular signals, interest in the basic processes involved in the adsorption of biomacromolecules onto surfaces has grown tremendously [1]–[6]. In addition to understanding the fundamental surface activity properties of important biomolecules, the precise control over interactions of cells and biological molecules with nonnatural or hybrid materials—biosurface engineering—has bloomed into a very active field due to the implications for understanding basic cell biology as well as technological applications in medical devices. Examples of methods developed toward surface micro/nanoengineering include micro-contact printing (μ-CP) [7], [8], self-assembly techniques (e.g., self-assembled monolayers, SAMs [9]–[11]), chemical vapor deposition (CVD) [12], [13], plasma treatment [13], [14], and radio-frequency glow discharge (RFGD) [15].

One of the self-assembly techniques, electrostatic layer-by-layer (LbL) self-assembly, has recently been applied to the deposition of ultrathin protein films for purposes of chemical and biomedical engineering [16]–[22]. The advantage of this approach is the versatility (any charged molecules, any combination in alternating charge), precision (few nanometers per layer, normal to surface), and mild conditions (aqueous solutions, near-neutral conditions, low ionic strength) with which multilayer films with complex architecture may be engineered [16]. Pioneering work in developing LbL methods for protein immobilization demonstrated the assembly of simple architectures of multicomponent protein films by alternating adsorption of oppositely charged macromolecules with LbL [16], [17]. Since those early reports, the technique has been widely used to form various polymer/protein assemblies of different materials with increasing levels of vertical complexity, and this work has recently been thoroughly reviewed [18]. Despite the large amount of work performed on protein nanoassemblies, however, little of this has been directed toward understanding the cell–material interactions of these novel hybrid biomaterials.

Some examples of investigations toward using polymeric—and polymer/inorganic nanoparticle—composite LbL nanofilms for surface modification of polymers, metals, and ceramics to control cell adhesion are available [19], [20], [23]. In addition, some basic studies with common polyelectrolyte materials have recently given further insight into cellular interactions with polymer ultrathin films, and the dependence of these interactions on the underlying architecture [21], [23], [24]. Recent interesting work on the interactions between cells...
and signal proteins embedded in polymer films has also been presented [25]. These reports, however, explore the interaction of cells with assemblies of “standard” polyelectrolyte materials and nanoparticles on surfaces, and have not attempted to employ specific materials with cell-adhesive properties to control cell-surface interactions.

Studies of LbL self-assembled polyelectrolyte films have been completed on proteins such as albumin, IgG, myoglobin, pepsin, and gelatin [1], [2], [17], [18] for the express purpose of creating biofunctional materials, though these studies did not investigate cell–material interactions. Of these, gelatin is particularly important for engineering cell-surface properties because of its form as denatured collagen, one of the most commonly occurring proteins in the human body, and one that plays a central role in the formation of extracellular matrix with which cells have extensive communication [26]–[33]. For this reason, the adsorption properties of gelatin have been assessed with an eye toward biomedical applications. The structure and properties of chitosan–gelatin bilayer scaffolds have been shown to provide a permissive environment for cell growth, and this is therefore a promising matrix for tissue engineering [26]. It was demonstrated that the gelatin-peptide-coated surface had better affinity for endothelial cells and nerve cells compared to the control surface without gelatin [29], [33]. From these investigations, it is clear that gelatin is a useful material for surface modification of tissue engineering scaffolds and implanted devices.

Depositing gelatin onto the surface of solid substrates is a useful technology to modify the surface properties of in vitro cell culture scaffolds, presenting a suitable adhesion signal for many cell types. For example, one study presented a dip-coat method to adsorb gelatin onto substrate surfaces [1]. This technique is simple and straightforward and may be efficient; however, the uniformity and density gelatin adsorption with such a process depends greatly on the surface hydrophobicity of the base substrate, and our experience has shown that it is difficult to form a uniform gelatin film on hydrophobic base substrates. Electrostatic adsorption is a more attractive approach to improve gelatin deposition due to monolayer formation with simple processing, mild conditions, excellent repeatability, and general applicability to any charged substrate.

The LbL electrostatic self-assembly process, while useful for uniform coating of entire surfaces, is less useful for generating two-dimensional (2-D) and three-dimensional (3-D) patterns. Techniques have recently been developed for producing single-layer micropatterns using elastomeric stamps [34], [35], but generations of 3-D structures with this approach are also technically challenging and labor intensive. Thus, a novel method has recently been developed to combine photolithography, electrostatic LbL self-assembly, and lift-off steps to fabricate micro- and nanostructures [36]–[39]. This combination, called LbL plus lift-off (LbL–LO), exploits the advantages of each technique: a 2-D pattern with microscale lateral dimensions can be easily generated using photolithography; ultrathin films with nanometer precision in thickness can be deposited on and around patterned resist with LbL self-assembly; and the corresponding film pattern can then be obtained after liftoff.

The LbL–LO method presents a promising approach to cell patterning on many surfaces, as it is likely that micro/nanoscale structures comprising multilayer films of gelatin and other proteins can be patterned on many base substrates to provide specific cell-adhesive properties. The resulting structures are expected to have more uniform surface compared to dip-coating methods, low cost compared to other physical methods of surface modification, and wide selection of materials, architectures, and substrates with which to work. To date, however, the demonstrations of LbL–LO have been limited to organic and inorganic materials, mainly applied toward applications in microelectronics–biomolecules have not yet been integrated into this process, and it is unknown whether the process is sufficiently mild and the adsorption forces binding gelatin are adequate to maintain surface integrity during liftoff.

Therefore, this paper reports on the electrostatic self-assembly properties of gelatin, followed by an investigation of the integration of gelatin into the LbL–LO process. Specifically, mass adsorption and surface charge properties of gelatin were assessed using quartz crystal microbalance, spectroscopic, and zeta-potential measurements. FITC-gelatin was employed in the fabrication of 3-D polyelectrolyte/FTTC-gelatin patterned to facilitate microscopic observation of resulting patterns, allowing assessment of pattern resolution and uniformity. These studies were designed to determine whether LbL–LO with gelatin is a useful tool to fabricate microscale patterns of thin films, specifically for gelatin-presenting micropatterns, which provides a basis for future research on designed cell culture scaffolds and basic research using these engineered systems.

II. MATERIALS AND EXPERIMENTAL METHODS

A. Materials and Chemicals

The following chemicals and materials are commercial products that were purchased for general use in the experiments. Anionic poly(sodium 4-styrenesulfonate) (PSS, MW 70,000), cationic poly(diallyldimethylammonium chloride) (PDDA, MW 400,000) and poly(allylamine hydrochloride) (PAH, MW 15,000), fluorescein 5-isothiocyanate (FITC, MW 389.4), and gelatin B from bovine skin (Bloom 225) were purchased from Sigma–Aldrich. Bis(2,2’-bipyridine)-4’-methyl-4-carboxybibpyridine-ruthenium N-succinimidyl ester-bis (hexafluorophosphate (Ru(bpy)2(mcbpy-O-Su-ester) (PF6)2, MW 1,014) was purchased from BioChemika. Silica particles, 400 nm in diameter, were purchased from Bangs Laboratories, Inc, Fishers, IN.

B. Instrumentation

1) A quartz crystal microbalance (QCM, Iwatsu, SC-7201) was used to monitor the mass change of the material deposited on the electrode during the assembly process. The resonance frequency of the silver electrode is 9 MHz and QCM frequency shifts are proportional to the amount of mass deposited on the electrode. The relationship between frequency and film thickness can be expressed by the modified Sauerbrey equation: 

\[ d(\text{nm}) = -0.022 \Delta F(\text{Hz}). \]
2) A UV-Vis spectrometer (Agilent 8453) was applied to measure the concentration change of gelatin dissolved in buffered solution. Quartz cells with 1-cm pathlength were used.

3) A zeta-potential analyzer (Brookhaven Instruments Corporation, software version 4.0) was used to identify the polarity and density of the surface charge of materials layered onto nanoparticles.

4) A fluorescence spectrometer (QM-4, Photon Technology International) was also utilized to monitor the intensity of fluorescence on the solid substrate.

5) An inverted epifluorescence microscope (Nikon ECLIPSE TS100/TS100-F) with digital camera (Nikon COOLPIX 995) was used for observing and recording the results of substrate fabrication.

C. Procedures

1) Labeling Gelatin B With FITC: Gelatin was conjugated with a fluorescent dye to facilitate inspection of nanoscale patterns comprising polycation/protein films. A protein-labeling procedure for tagging gelatin B (gelatin) with FITC was adopted from the guidelines provided by Molecular Probes and SigmaAldrich. Briefly, 0.1 M sodium bicarbonate buffer was prepared and adjusted to pH 9. Next, 20 mg of gelatin was dissolved in 2 mL of 0.1 M sodium bicarbonate (NaHCO₃) buffer. Then, 0.2 mg FITC powder was dissolved in 200 μL dimethylformamide (DMF). While stirring the gelatin solution slowly, the FITC solution was added. The reaction was incubated at room temperature with continuous stirring for 1 hour. Finally, the conjugate was separated from unreacted labeling reagent with an Amersham Pharmacia Biotech PD-10 desalting column.

2) Electrostatic LbL Self-Assembly Process: The deposition of nanocomposite polymer/protein films was achieved using electrostatic self-assembly. The standard layering process was as follows (details of specific experiments are given in Section III): individual aqueous solutions of polycation and protein at concentrations of 2–3 mg/mL were prepared and adjusted to the appropriate pH, which varied depending on the purposes of the experiments as noted below. For each case, a substrate was pretreated with incubation in H₂SO₄:H₂O₂ (7:3) solution at 70 °C for about one hour to introduce negative surface charges such that the initial layering step can be readily started. The substrate was alternately immersed in polycation solutions for 10 min or protein solution for 20 min, respectively, with an intermediate water rinse for 1 min in all cases. For some experiments, such as QCM analysis, it was necessary to perform a drying step (fluxing with nitrogen) to arrive at an accurate measurement. The removal of water adsorbed on the electrode helped avoid the fluctuation of mass by evaporation during measurement. Using the basic LbL self-assembly method described above, a systematic study of adsorption properties of gelatin was completed.

3) LbL–LO Process: Patterns of polymer/protein nanofilms with microscale lateral dimensions were realized using a combination of conventional photolithography, LbL self-assembly, and lift-off technologies [37], which makes the fabrication of three-dimensional micro- and nano-scale cell culture scaffolds possible. With LbL–LO, the composition, architecture, and surface properties can be modified easily to investigate the cell behavior on a variety of materials. For this particular investigation, the effectiveness of LbL–LO for generating micropatterned scaffolds with nanometer thicknesses and gelatin outer layers was studied.

Fig. 1 is a schematic illustration of the general LbL–LO fabrication procedure used to construct the microtextured scaffolds, which has been described in detail elsewhere [37]. A glass substrate was pretreated in aqueous H₂SO₄:H₂O₂ (7:3) solution to induce negative charges on the glass surface. PR 1813 positive photoresist (Shipley) was spun on the glass substrate, exposed through a photomask, and developed in MF 319 developer to obtain ~1.5 μm thick photoresist pattern. After the completion of photolithography, the LbL self-assembly process was performed, as described, to deposit polyelectrolyte and protein nanocomposite films on the glass substrate with the predesigned photoresist. The patterned substrate was then soaked and sonicated in acetone to remove the unexposed photoresist patterns ("lift-off"), leaving the patterns of polyelectrolyte/protein films. Finally, the polyelectrolyte and protein patterns were inspected with an optical/fluorescence microscope to assess the quality of the resulting structures.

4) Coating Particles With Polyelectrolyte and Gelatin: The self-assembly properties of FITC-gelatin were studied in order to assess the conditions under which multilayer films containing this molecule could be formed. To determine the charge properties of the labeled protein using measurements of zeta potential, nanoparticles were coated with alternating layers of polycations and proteins. PDDA, PSS and gelatin were dissolved in tris(hydroxymethyl)aminomethane (TRIS) buffer, adjusted to pH 4.0 or 10.0, and DI water (about pH 5.6), with a concentration of about 2 mg/mL. The selection of pH 4.0 and 10.0 buffers was
to test the charge polarities of gelatin at pH values away from its isoelectric point. Next, 400 nm silica particles were coated with PDDA/PSS and PDDA/gelatin through a process of alternate exposure, centrifugation, and water rinsing [18]. During the layering process, the particles were soaked in PDDA, PSS, and gelatin solution for 20 min for each layer, and rinsed with pH 4 or pH 10 TRIS buffers, or DI water, twice prior to zeta-potential measurement. In addition, the UV-Vis spectra of the original gelatin solution and gelatin solution remaining were measured after layering silica particles.

To further confirm the charge polarity of FITC-gelatin, 400 nm silica particles coated with FITC-gelatin were scanned by fluorescence spectroscopy. In one case, the silica particles were assembled with films of PDDA/PSS/FITC-gelatin, and in a second case, particles were coated with PDDA/FITC-gelatin. In both cases, PDDA and PSS were dissolved in pH 5–6 DI water with a concentration of 2 mg/mL; FITC-gelatin was made in pH 9 NaHCO₃ buffer at a concentration of about 10mg/mL. Using the same PDDA, PSS, and FITC-gelatin solutions, silica particles were layered in the order of PDDA/PSS/FITC-gelatin/PDDA/FITC-gelatin for zeta-potential measurement.

5) Layering Polyelectrolytes and Gelatin on QCM Silver Electrodes: The thickness of gelatin/polymer films formed using LbL was assessed to provide an estimate of nanostructure height. By assembling films on silver electrodes, mass changes were performed to provide an approximate measure of film thickness of FITC-gelatin as well as further information regarding the charge properties of the protein. For this phase of the work, two architectures were studied. The first method involved layering the electrode with PDDA and PSS solutions dissolved in deionized (DI) water at a concentration of 2 mg/mL (no salt added), and FITC-gelatin solution in the order of (PDDA/PSS)₂/(PDDA/FITC-gelatin)₄. The second method was to layer the electrode with PDDA and PSS solutions dissolved in DI water at a concentration of 2 mg/mL, with 0.5 M NaCl, and FITC-gelatin solution in the order of (PDDA/PSS)₃/(FITC-gelatin/PSS)₄. The reasons for using these two architectures were: 1) to determine the polarity of gelatin, providing a point of comparison for the zeta-potential analysis and 2) to compare the influence of salt on the thickness of layering films, which is believed to be important to the stability of polyelectrolyte patterns on the substrate.

6) Layering FITC-Gelatin Onto Planar and Structured Solid Glass Substrates: It has been shown that deposition of gelatin on the surface of cell culture scaffolds can increase cell attachment [20], [29], [33]. This step of the series of studies was designed to demonstrate patterning of polyelectrolyte/protein multilayer films into structures with microscale lateral dimensions, nanoscale vertical dimensions, molecular vertical precision, and flexibility in material selection. This is a desirable capability for cell research and tissue engineering, but one that is difficult to achieve. Based on the study of gelatin properties, FITC-gelatin was known to be negatively charged. After the pretreatment and photolithography, a glass substrate was layered with PDDA and PSS (2 mg/mL in DI water at pH 5.6), and FITC-gelatin to achieve an architecture of (PDDA/PSS)₃/(PDDA/FITC-gelatin)₃. The designed microstructures had feature size arranged from 50 to 120 μm.

III. Results and Discussions

A. Zeta-Potential and UV-Vis Measurements of Gelatin B at Different pH Values

Table I shows the zeta-potential measurement results for silica particles before and after coating with films of different outermost layers at pH 4 and pH 10. These data demonstrate that pH significantly affects the surface potential of silica particles layered with different coating materials, which agrees with previous results [18]. Gelatin B has an isoelectric point of 4.8–5.1 (as quoted by Great Lakes Gelatin), resulting in varying charge polarities and densities when placed in a solution with pH different from its isoelectric point. All of these data match expected values—the carboxylate-modified silica particles maintain negative charge, which shows slight increase with pH; the PDDA coat layer shared a decreasing positive charge as pH increases; and gelatin exhibited a reversed polarity from positive to negative as pH rose above the isoelectric point. The measured potentials of gelatin in pH 4 TRIS buffer were positive, while the potentials of gelatin in pH 10 TRIS buffer were negative.

Table II contains peak absorbance values (∼215 nm) for gelatin solutions before and after layering silica particles with two different layering architectures. These data directly indicate the consumption of gelatin after layering silica particles and allow estimation of mass adsorbed to surface with Beer’s Law in each case. As indicated by the zeta-potential measurements, plain silica particles are negatively charged and PDDA is positively charged at pH 7.6, but both exhibit some shift in charge densities at pH 4 and pH 10. The data in Table II show that, at pH 4, more gelatin is adsorbed by plain silica particles than PDDA-coated particles. Conversely, at pH 10, more gelatin is adsorbed by PDDA-coated particles than plain silica particles. It can be inferred from these data that gelatin is positively charged at pH 4 and negatively charged at pH 10, which agrees with the isoelectric point of gelatin. Furthermore, it can also be concluded that there exists an additional attractive force between gelatin and other materials since gelatin is absorbed by both positively and negatively charged particles. Thus, spontaneous adsorption of gelatin despite electrostatic repulsion was observed from the UV-Vis spectra of original gelatin solutions compared to gelatin solutions after layering the silica particles at pH 4 and pH 10. These data demonstrate the adsorption
of gelatin on silica particles during the suspension process, regardless of surface charge and pH.

B. QCM, Zeta-Potential, and Fluorescence Spectrum Measurement of FITC-Gelatin

FITC, a fluorescent dye with pH sensitive properties, was used to allow ease of visualization of the gelatin in nanofilms. FITC also served as a model for other fluorescent probes, which can be conjugated to polyions or proteins assembled into multilayer films to be used as in situ indicators. Thus, a fluorescent tag may provide a label for analyzing structures as well as a probe for future sensing of cell culture conditions in vitro microenvironment. QCM, zeta-potential, and fluorescence spectroscopy measurements were performed after labeling gelatin with FITC to study the charge properties of the FITC-gelatin conjugate.

1) QCM Measurement: The QCM measurements are shown in Fig. 2. It was evident that FITC-gelatin could be alternately adsorbed onto the electrode with PDDA, as shown in Fig. 2(a). This was anticipated, as the amine-labeling process was expected to reduce the number of positively charge residues available on the protein. In contrast, FITC-gelatin could not be efficiently deposited in multilayer films with PSS, as shown in Fig. 2(b). It is known that PDDA (a strong polyelectrolyte, always charged regardless of pH) is positively charged in DI water while PSS (isoelectric point: pH < 1) is negatively charged in DI water [18]. Thus, the QCM measurements confirm that FITC-gelatin is negatively charged.

It has been demonstrated by some researchers that ionic strength affects the thickness of polyelectrolyte films during the layering process [18], and the same observation can be seen here. In both methods, the precursor layering steps were taken as (PDDA/PSS)₄, and the frequency shift had an approximately linear increase. However, the frequency shift was about 500 Hz (≈11 nm in thickness) per bilayer in method 2 with 0.5 M NaCl added, an increase of ~500% that obtained without salt (only 100 Hz, ~2.2 nm per bilayer), which agrees with previous results. So, adjusting the ionic strength may change the thickness of the layering films.

2) Fluorescence Spectra: The fluorescence spectra of 400-nm silica particles in solution shown in Fig. 3 were collected immediately after the layering and rinsing process. It can be observed from the graph that there is a very weak fluorescence emission peak, barely above the profile due to scattering, for silica particles layered with the order of PDDA/PSS/FITC-gelatin. In contrast, the fluorescence emission peak of silica particles with PDDA/FITC-gelatin layering order is much stronger. It is important to note that spectra of supernatant obtained from these same samples did not show any fluorescence, suggesting that all fluorescence in these data does in fact arise from FITC-gelatin immobilized on particle templates. These data support the findings from QCM and zeta potential measurement experiments that FITC-gelatin is negatively charged. Thus, FITC-gelatin is more strongly attracted to adhere with positively charged PDDA than negatively-charged PSS. However, in absence of electrostatic attraction—in fact, in the presence of repulsive forces—there remains spontaneous attractive force between gelatin and PSS. The emission peak around 520 nm for particles with PDDA/PSS/FITC-gelatin, although very weak, shows some adsorption of gelatin.

3) Zeta-Potential Measurement of Coated Silica Particles With FITC-Gelatin: The zeta-potential measurements of FITC-gelatin are shown in Fig. 4. From this figure, it can be observed that the surface charge changed from a positive value due to PDDA to a negative value for PSS. After assembling FITC-gelatin on the PSS coated particles, the zeta-potential was kept negative, although the value of negative potential changed slightly. UV-Vis and fluorescence measurements confirmed that FITC-gelatin remained in solution, showing that the protein was not completely consumed. The particles...
were then exposed to PDDA solution, and the zeta-potential of FITC-gelatin-coated particles was returned to positive. FITC-gelatin was then layered on the PDDA-coated particles, and the zeta-potential became negative again. These experiments further demonstrate that FITC-gelatin is negatively charged at pH 9, which is consistent with the results from QCM measurements and fluorescence spectra.

C. Fabrication of Cell Culture Scaffolds With FITC-Gelatin on Glass Substrates

Standard microscopy glass slides were used as the solid supports for the fabrication of cell culture scaffolds. Two types of cell culture scaffolds were made as described above: a simple planar substrate with FITC-gelatin layered on the plain glass and a 3-D scaffold fabricated with LbL–LO technology.

1) Layering FITC-Gelatin Film Onto a Planar Glass Substrate: The fluorescence spectra of the planar glass substrate layered with FITC-gelatin, shown in Fig. 5, were taken after adsorbing FITC-gelatin onto the unpatterned glass slide. There was a stronger emission peak after layering four layers of FITC-gelatin than only one layer of FITC-gelatin, as expected. The fluorescence intensity is stronger as more layers of FITC-gelatin are applied to the substrate, indicating that multilayers of FITC-gelatin film are formed on the glass substrate.

It is important to note that, since the LbL self-assembly process is time consuming, it is sometimes advantageous to limit the number of layers employed. For cell culture purposes, it is unnecessary to apply many layers of FITC-gelatin on the surface of a planar substrate, as long as the outermost layer is uniformly assembled with FITC-gelatin. However, for 3-D patterned cell culture scaffolds, micropatterned with nanoscale vertical dimensions, the thickness of the patterns must be considered, which will affect the behavior of cells. Also, a recent report suggested that by manipulating the multiplayer pH or ionic strength assembly conditions, or both, which in turn dictate the molecular architecture of the thin films, one may direct a single multiplayer combination to be either cell adhesive or cell resistant [24].

2) Fabricating 3-D FITC-Gelatin Patterned Cell Culture Scaffolds: As mentioned in Section III-C, 3-D cell culture scaffolds were fabricated with LbL–LO technology in the order of \((\text{PDDA/PSS})_3/(\text{PDDA/FITC-gelatin})_3\). Before layering polyelectrolyte and FITC-gelatin films, the photoresist pattern was inspected in order to control the quality of the final FITC-gelatin patterns. There were two types of patterns designed for cell culture studies, channel patterns and square patterns, wherein the feature size ranged from 50 to 90 \(\mu m\) in both cases. In Fig. 6, representative images of each type of patterns are shown, from which it can be seen that the patterns have been faithfully transferred from the mask to the photoresist, which is the prerequisite to obtain good polyelectrolyte and FITC-gelatin patterns after the layering process.

The characterization of fabrication results for protein nanofilms was facilitated by the inclusion of the fluorescent tag FITC. Fig. 7 shows fluorescence images of polyelectrolyte
and FITC-gelatin patterns following the lift-off process, for which the layering architecture was (PDDA/PSS)$_3$/(PDDA/FITC-gelatin)$_3$. From the previous QCM measurements, it is estimated that the total thickness of these patterns is about 30 nm. As noted previously, the film thickness might be modulated when using PDDA and PSS solutions by adding salt, typically KCl or NaCl, to the polyanion solutions. The FITC-gelatin patterns shown in Fig. 7 demonstrate the success of layering FITC-gelatin to the glass substrate and fabricating 3-D FITC-gelatin patterns on the glass substrate with LbL–LO technology. The images show excellent lateral resolution, as the fluorescence from FITC-gelatin follows the phase contrast images of photosist from Fig. 6. Beyond the limitations of standard optical photolithography, the resolution achievable for polyelectrolyte thin film patterns produced with LbL–LO is influenced by several other factors, which affect the binding strength of LbL self-assembly. These include properties such as surface roughness and charge density of the base substrate, the charge density and size of the polymers and proteins, the number of layers of polyelectrolyte films, the temperature, pH, and ionic strength. Using the present method, fabricating the thin film patterns on the glass surface at room temperature using PR 1813, features of 10 μm are easily achieved. Structures of this size are adequately small for working with biosurface engineering where cell adhesion is the control parameter of interest, as biological cells are typically 10 to 20 μm in the smallest outer dimension.

While these results show clearly that the gelatin remains immobilized on the surface through the LbL–LO process, it must also be confirmed that the acetone used in the lift-off step does not attack the gelatin molecules and change the cell-adhesive properties. Our preliminary work with smooth muscle cells and neurons [20], [40], [41] shows that use of acetone during lift-off does not significantly affect gelatin. In addition, it has been found that the same substrates may in fact be re-used for multiple experiments with neurons when sterilized with ethanol and UV light.

In summary, the patterning of gelatin on glass surfaces through a combination of LbL electrostatic self-assembly and lift-off methods has been described with a view toward using this capability to pattern cells for basic research and biotechnology applications. This work defines a platform process for producing micro/nanoscale structures with high resolution while simultaneously controlling the composition for specific biomaterial interactions and integrating opto chemical transducers (e.g., absorbing or fluorescent indicators). Future studies will aim to more carefully define the limitations and relative advantages of these micro- and nano manufacturing methods so as to develop an easy-to-use toolkit for generating customized complex biosystems.

IV. Conclusion

As proteins play an important role in the current biomedical engineering, biomaterials, and biosensor fields, and studies on material modification have been developed into the nanometer range, it is necessary to take further steps toward better understanding of protein properties for potential application to cell and tissue culture. In this work, the electrostatic assembly properties of gelatin were studied to determine the potential for patterning cell-adhesive gelatin nanofilms. The experimental results indicate that the charge polarity of gelatin varies as pH shifts away from its isoelectric point, and its charge density changes with both pH and the composition of buffer solution. After labeling gelatin with FITC, the conjugate is still negatively charged and can also be alternately layered with positively charged polycations. Taking advantage of this possibility, FITC-gelatin patterned cell culture scaffolds were successfully fabricated on glass substrates with the novel LbL–LO approach, resulting in uniform, well-defined features on the same order of size as cells. It is believed that this study on gelatin and cell culture scaffold nanofabrication methods lays the groundwork for many future potential applications and will eventually benefit a variety of research and development efforts in tissue engineering and biomaterials.

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