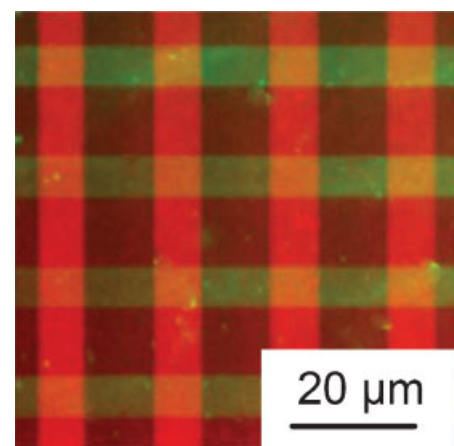


Direct Transfer of Preformed Patterned Bio-Nanocomposite Films on Polyelectrolyte Multilayer Templates

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Microarrays containing multiple, nanostructured layers of biological materials would enable high-throughput screening of drug candidates, investigation of protein-mediated cell adhesion, and fabrication of novel biosensors. In this paper, we have examined in detail an approach that allows high-quality microarrays of layered, bionanocomposite films to be deposited on virtually any substrate. The approach uses LBL self-assembly to pre-establish a multi-layered structure on an elastomeric stamp, and then uses μ CP to transfer the 3-D structure intact to the target surface. For examples, different 3-D patterns containing dendrimers, polyelectrolyte multilayers and two proteins, sADH and sDH, have been fabricated. For the first time, the approach was also extended to create overlaid bionanocomposite patterns and multiple proteins containing patterns. The approach overcomes a problem encountered when using μ CP to establish a pattern on the target surface and then building sequential layers on the pattern via LBL self-assembly. Amphiphilic molecules such as proteins and dendrimers tend to adsorb both to the patterned features as well as the underlying substrate, resulting in low-quality patterns. By circumventing this problem, this research significantly extends the range of surfaces and layering constituents that can be used to fabricate 3-D, patterned, bionanocomposite structures.



Introduction

Functional, three-dimensional (3-D) nano and microstructures composed of amphiphilic molecules, such as proteins, lipids, polyelectrolytes and dendrimers, have potential applications in drug screening devices, biosensors, biocatalysis, optoelectronics, and other devices.^[1–3] Conven-

tionally, such 3-D structures can be created by using microcontact printing (μ CP),^[4] where an elastomeric polydimethylsiloxane (PDMS) stamp having the desired topographic pattern is coated with a molecular ink and then brought into contact with the surface. Removal of the stamp leaves behind a thin layer of ink having the same pattern as the stamp. Based on the chemical contrast between the inked features and the ink-free background, additional layers can then be selectively deposited on either the features or the background using thin film deposition methods such as layer-by-layer (LBL) assembly.^[1]

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During the LBL assembly process, biological activity can be imparted to these micro-structures or arrays by incorporating proteins or other biomolecules. Several techniques have been examined for fabricating micron-sized arrays of proteins, including photolithography, μ CP and photochemistry.^[3,5–8] While each of these methods may be useful for certain applications, each has its own limitations, particularly in the area of non-specific binding.^[3] Microfluidic networks have also been extended to create sub-micron patterns on which proteins were subsequently adsorbed.^[9–11]

The biological activity of these 3-D structures can further be augmented by co-immobilization of other macromolecules, such as dendrimers and polyelectrolyte multilayers (PEMs), both of which allow the local environment of the protein to be customized. Dendrimers represent a class of macromolecules, that, unlike conventional polymers, have well defined, highly branched, compartmentalized structure in the nanometer size range.^[12–15] Dendrimers can have a highly functionalized outer surface, making them promising candidates for applications in drug delivery,^[16] imaging^[17] and sensing.^[18] They can also serve as functional frames to encapsulate small molecules needed by proteins, including enzyme cofactors and electron mediators. PEMs, introduced by Decher,^[19] are thin films formed by sequential deposition of oppositely charged polyelectrolyte species. PEMs are robust, easy to fabricate, and have tunable architectures (i.e., film composition and physical and chemical microstructure).^[20,21] Polyelectrolytes can be used to immobilize hydrophobic membrane proteins onto hydrophilic substrates, entrap ionic or polar small molecules needed by the proteins, and act as an ion-selective barrier to screen out interfering molecules.^[22] PEM applications have further been extended to functional polymers,^[23] colloids,^[2,24,25] biomaterials^[22] and selective electroless metal depositions.^[26,27]

In some cases, LBL assembly of 3-D structures onto patterns deposited by μ CP is hindered by the lack of sufficient chemical contrast between the features and background (i.e., the continuous layer on which the pattern is deposited), making it difficult to deposit additional layers cleanly on only the features or the background. This problem could be particularly challenging when a layer of amphiphilic molecules (e.g., proteins and some dendrimers) is to be deposited, because amphiphilic molecules can adsorb to both hydrophilic and hydrophobic surfaces. PEMs also present challenges, because the alternating anionic and cationic layers could adsorb to either positive or negative charges on the background surface.

In a recent communication, we described an approach that overcomes abovementioned difficulties in establishing well-defined, 3-D bionanocomposite patterns.^[28] The approach, intact transfer printing (ITP) entails using LBL assembly to pre-establish a multilayered structure on an

elastomeric stamp, and then using μ CP to transfer the 3-D structure intact to the target surface. While μ CP was recently used to transfer preformed PEMs to a substrate,^[29] in the communication we provided conclusive evidence of the formation of bionanocomposite layered structures on a micropatterned stamp and subsequent transfer of the structures intact to a target substrate. Here, we examine in more detail the fabrication and characterization of some other novel 3-D bionanocomposite architectures (or structures) possible using the ITP approach. In the present study, generation four (G 4) (PAMAM) dendrimers and two different enzymes, secondary alcohol dehydrogenase (sADH) and sorbitol dehydrogenase (sDH), were entrapped in PEM based multilayered structures and then transferred intact to create high-quality micropatterns of bionanocomposite material.

Experimental Part

Materials

G4 amine-terminated PAMAM dendrimer (10 wt-% solution in methanol, $\overline{M}_w \approx 14\,215$), sulfonated polystyrene (SPS) ($\overline{M}_w \approx 70\,000$), sDH, poly(diallyldimethylammonium chloride) (PDAC) ($\overline{M}_w \approx 100\,000$) were obtained from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC) and Alexa Fluor 568 were obtained from Invitrogen (Carlsbad, CA). sADH was expressed and purified according to published procedures.^[30] Sylgard 184 silicone elastomer kit (Dow Corning, Midland, MI) was used to prepare the PDMS stamps for μ CP. Ultrapure water (18.2 M Ω) was supplied by a Nanopure-UV four-stage purifier (Barnstead International, Dubuque, IA); the purifier was equipped with a UV source and a final 0.2 μ m filter.

Preparation of Stamps

The PDMS stamps were made by pouring a 10:1 solution of elastomer and initiator over a pretreated silicon master, which acts as a mold, to allow surface morphology of the stamp to form a negative replica of the master. The silicon master was pretreated with fluorosilanes to facilitate the removal of the PDMS stamps. The mixture was allowed to cure overnight at 60 °C and then peeled off. The masters were prepared in the Keck Microfabrication Facility at Michigan State University (MSU) and consisted of features (parallel lines and circles) from 1 to 20 μ m.

Preparation of PEMs

A computer-controlled Carl Zeiss slide stainer equipped with a custom-designed ultrasonic bath was used to perform LBL assembly.^[1,2,27] For PDAC/SPS multilayers, the concentrations of SPS and PDAC were 0.01 M and 0.02 M, respectively, as based on the molecular repeat units. All polyelectrolyte solutions contained 0.1 M NaCl and were at a pH of 7.0. To deposit PEMs on glass slides, the slides were cleaned with Alconox precision cleaner (Alconox Inc.,

New York, NY) in a sonicator. These slides were then washed with water, dried under N_2 gas and further cleaned using oxygen in a Harrick plasma cleaner (Harrick Scientific Corporation, Brooding Ossining, NY) for 10 min at 20 Pa. To form the first bilayer, the slides were immersed for 20 min in a PDAC solution. Following two sets of 5 min water rinses with agitation, the slides were subsequently placed in a SPS solution for 20 min. They were rinsed again with water, and this process was repeated to build multiple layers.

Fluorescent Labeling of PAMAM Dendrimers, sADH and sDH

FITC was predissolved in acetone and added to an aqueous solution of PAMAM dendrimers. The resulting solution was allowed to stand overnight with occasional stirring. The total amount of dye was adjusted to label just one amino group per PAMAM molecule on average, assuming perfect reaction efficiency. The PAMAM solutions were then dialyzed against pure water (pH 7.4) using sterilized and rinsed membrane tubing (MW cut-off 2 000) to dialyze out unreacted FITC.

sADH aliquots were labeled using FITC (green dye) or Alexa Fluor (red dye). A 5.5 mg sample of sADH, in 2 mL Tris buffer was dialyzed against a 0.1 M sodium bicarbonate solution, pH 8.5, for 24 h. The bicarbonate solution was changed every 6 h during the dialysis process. FITC (or Alexa Fluor) was then added to the protein solution in 1:1 mol ratio and continuously stirred for 10 h. The protein solution was then dialyzed against phosphate buffer (pH 7.4) for 24 h, changing the buffer every 6 h, to remove excess FITC (or Alexa Fluor).

Fabrication of 3-D Structures or Architectures

Figure 1 depicts the different 3-D architectures that were fabricated and studied.

Case 1

In Case 1, a PDMS stamp was dipped in a solution of FITC labeled sADH ($1 \text{ mg} \cdot \text{mL}^{-1}$) or Alexa Fluor labeled sDH solution ($1 \text{ mg} \cdot \text{mL}^{-1}$) in $50 \times 10^{-3} \text{ M}$ phosphate buffer (pH 7.4) for 2 h. The stamp was washed thoroughly with water and then either 20 or 40 PDAC/SPS multilayers were deposited on it according to above mentioned procedure. The modified PDMS stamp was then washed with water, dried under nitrogen and brought into contact with a glass slide coated with 10.5 PDAC/SPS bilayers. The stamp was removed after 20 min, and the resulting patterned substrate was washed thoroughly with water to remove loosely bound molecules.

Case 2

In Case 2, a PDMS stamp was dipped in a ($1 \text{ mg} \cdot \text{mL}^{-1}$) solution of sADH (either FITC labeled or unlabeled) in $50 \times 10^{-3} \text{ M}$ phosphate buffer (pH 7.4) for 2 h. The stamp was washed with water and dipped in $10 \times 10^{-6} \text{ M}$ aqueous solution of PAMAM dendrimers (pH 7.4) (FITC labeled or unlabeled) for 30 min. The stamp was rinsed with water, and 30 PDAC/SPS multilayers were deposited. The modified PDMS stamp was washed with water, dried under nitrogen and brought into contact with a glass slide coated with 10.5 PDAC/SPS bilayers. The stamp was removed after 20 min, and the resulting patterned substrate was washed thoroughly with water.

Case 3

In Case 3, a plasma cleaned PDMS stamp was dipped in FITC labeled sADH solution ($1 \text{ mg} \cdot \text{mL}^{-1}$, pH 7.4 in phosphate buffer) for 2h. The stamp was rinsed with water, and one PDAC/SPS bilayer was grown in it. The stamp was then washed with water and dipped in Alexa Fluor labeled sADH solution ($1 \text{ mg} \cdot \text{mL}^{-1}$, pH 7.4 in phosphate buffer) for 2 h. The modified PDMS stamp was then washed with water, dried under nitrogen and brought into contact with a glass slide coated with 10.5 PDAC/SPS bilayers. The stamp was removed after 20 min and the resulting patterned substrate was washed with water.

Microscopy

All the fluorescence images were obtained with a Nikon Eclipse E 400 microscope (Nikon, Melville, NY) using two filter sets, one for FITC (Ex: 465–495/DM: 505/Em: 515–555), and the other for Alexa

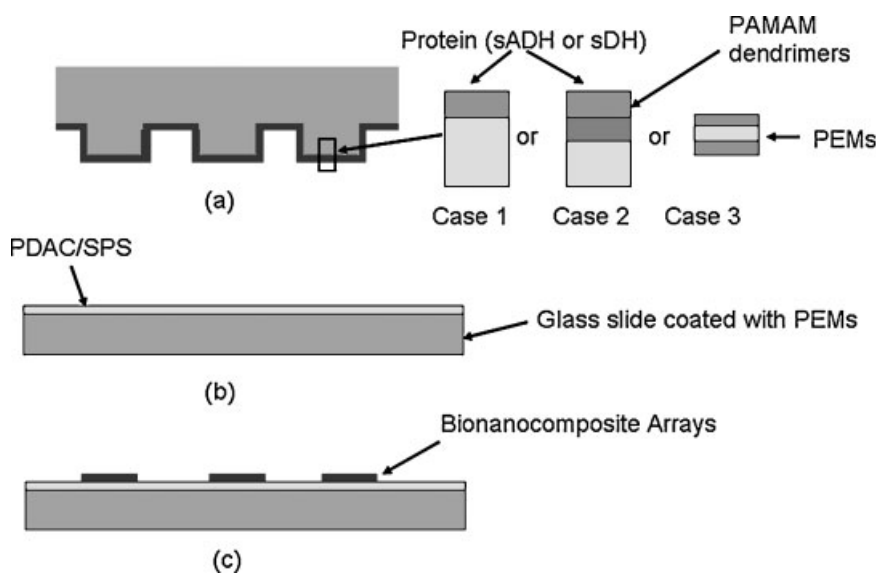


Figure 1. Schematic representation of the procedure used for printing: (a) stamp coated with a layer of proteins (sADH or sDH) and then PEMs (PDAC/SPS bilayers) (Case 1); a layer of sADH, a layer of G4 PAMAM dendrimers and then PEMs (Case 2); and a PDAC/SPS bilayer sandwiched between sADH layers (Case 3). (b) Glass slide coated with PEMs (10.5 bilayers). (c) Patterned substrate.

Fluor (Ex: 510–560/DM: 565/Em: 590–690). AFM images were obtained with a Nanoscope IV multimode scope (Digital Instruments, Santa Barbara, CA). The microscope was equipped with tapping-mode etched silicon probes. The thickness analysis of the patterns was determined using cross-sectional analysis of the AFM images.

Results and Discussion

In the ITP procedure depicted in Figure 1, a nanocomposite structure consisting of proteins, polyelectrolytes or dendrimers is assembled on top of an elastometric stamp using LBL assembly, and then transferred intact to a target substrate. Successful transfer depends on the relative strengths of binding force between nanocomposite top layer and the target surface, and that between the nanocomposite base layer and the stamp. The base layer should strongly and evenly adsorb on the stamp; non-uniform pattern transfer can occur without a smooth base layer. However, the attractive interactions between the base layer and stamp should be weak enough to allow easy detachment from the stamp during printing. To achieve this balance, we exploited hydrophobic interactions between the PDMS stamp and the sADH (or sDH) base layer in the fabrication of all 3-D structures. The adsorption of this base layer using non-electrostatic interactions provided a charged surface for the assembly of other layers. The ITP process was not reproducible and non-uniform when other macromolecules such as PDAC or SPS were used as base layers.

Figure 2 (a and b) shows the top-view and cross-sectional AFM images, respectively, when a single layer of sADH was transferred. The light regions in Figure 2a

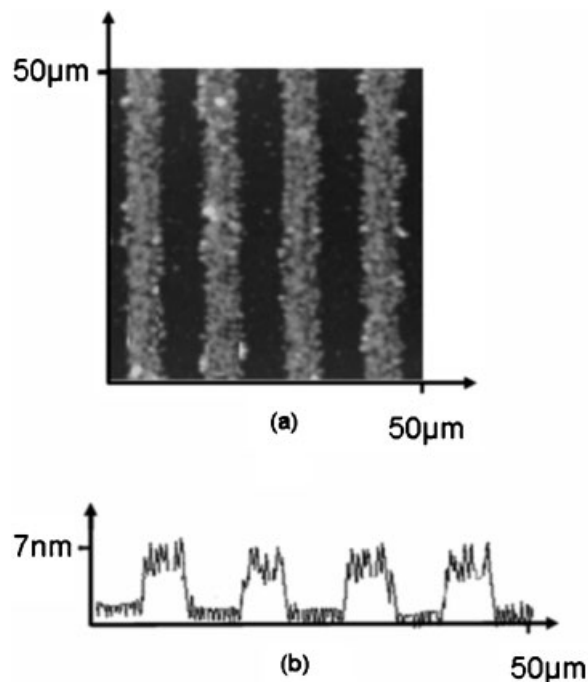


Figure 2. (a) 2-D AFM image of sADH patterns on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate. (b) Cross-sectional AFM image of sADH patterns on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate.

indicate the deposited sADH patterns, while the dark regions depict the underlying substrate. The sADH patterns had an average height of 7 ± 2 nm. Figure 3 (a and b) shows the top-view and cross-sectional images, respectively, when a multilayer film (see Figure 1, Case 1) consisting of sADH base layer and 20 PDAC/SPS bilayers, $(\text{sADH})_1(\text{PDAC/SPS})_{20}$, was transferred onto a PEM coated glass

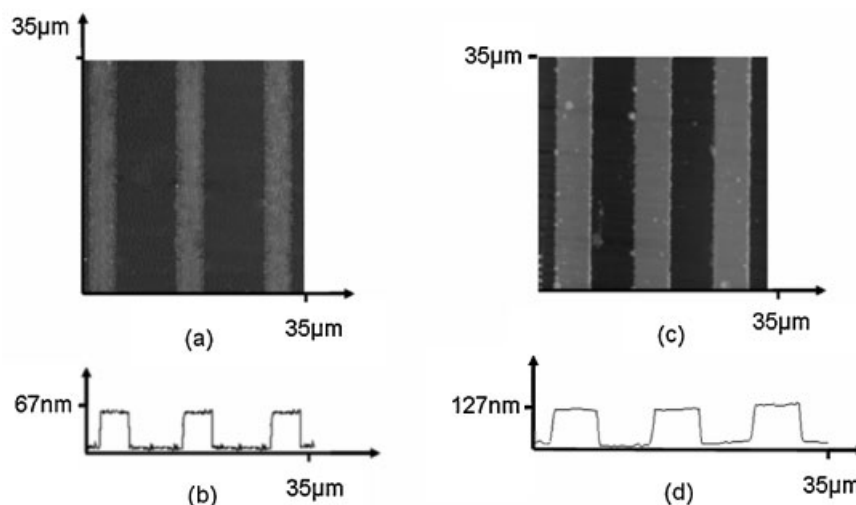


Figure 3. 2-D and cross-sectional AFM images (a, b) when a $(\text{sADH})_1(\text{PDAC/SPS})_{20}$ multilayer film was transferred to a PEM (10.5 PDAC/SPS bilayers) coated glass substrate; (c, d) when a $(\text{sADH})_1(\text{PDAC/SPS})_{40}$ multilayer film was transferred to a PEM (10.5 PDAC/SPS bilayer) coated glass substrate.

substrate. The average pattern height was approximately 67 ± 3 nm. On the other hand, the average pattern height (see Figure 3, c and d) was approximately 127 ± 4 nm when a $(sADH)_1(PDAC/SPS)_{40}$ multilayer film was transferred. Taking into account the contribution from the sADH layer (approximately 7 nm, Figure 2), the pattern height was found to linearly increase with the number of PDAC/SPS bilayers. From these values, the average height of a PDAC/SPS bilayer was determined to be 3.0 ± 0.15 nm, which agrees well with the literature value of 3.4 nm for similar deposition conditions.^[1]

Fluorescence microscopy was used to characterize the quality of multilayer film transfer. Figure 4a is a fluorescence image of the circular patterns obtained on the transfer of a multilayer film consisting of a layer of fluorescently labeled sADH and 20 PDAC/SPS bilayers onto a PEM-coated glass. The well-defined circular fluorescent

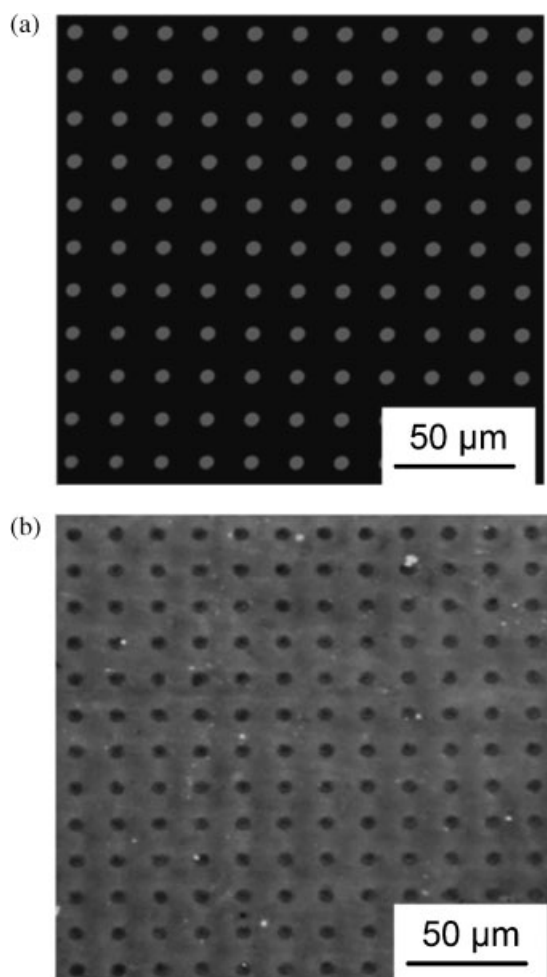
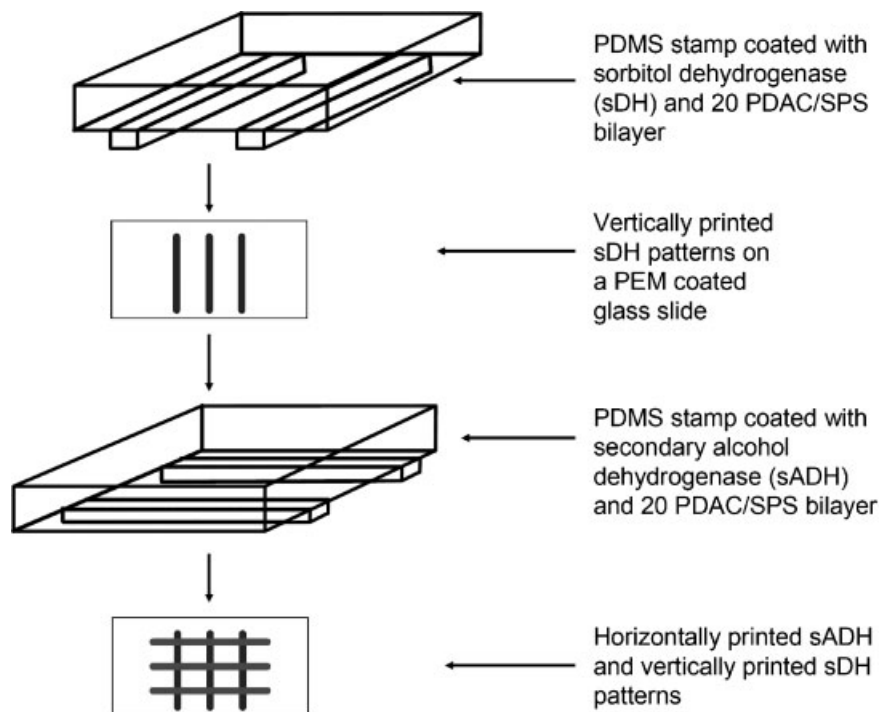


Figure 4. Fluorescence images of: (a) the circular patterns obtained via ITP of a multilayer film consisting of a layer of fluorescently labeled sADH and 20 PDAC/SPS bilayers, on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate; (b) the PDMS stamp surface after printing.

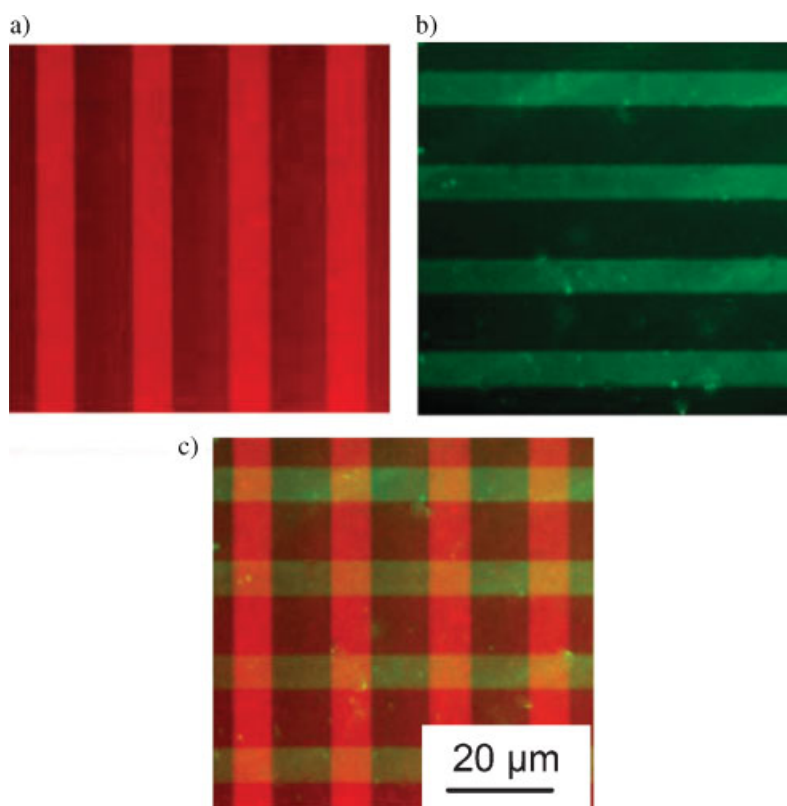
features and clean background regions indicate high-quality pattern transfer. The fluorescence image of the stamp (Figure 4b) is essentially a negative replica of Figure 4a. The clean circular features and fluorescent background suggest the multilayered film was completely transferred from the circular features and remained completely intact on the background regions. The ITP process was reproducible over an area of at least 1 cm^2 .

The ITP approach can also be used to create overlaid bionanocomposite micropatterns. Two different nanocomposite line patterns were sequentially stamped on a surface (Figure 5). The first pattern, consisting of red Alexa Fluor labeled sDH and 20 PDAC/SPS bilayers, was stamped on a PEM (10.5 PDAC/SPS bilayers) coated glass surface. The second pattern, consisting of green FITC labeled sADH and 20 PDAC/SPS bilayers, was deposited on top of the first, with the direction of the two patterns aligned perpendicularly. Figure 6a shows the red fluorescence from vertically printed $(sDH)_1(PDAC/SPS)_{20}$ lines, and Figure 6b shows green fluorescence from horizontally printed $(sADH)_1(PDAC/SPS)_{20}$ lines. Figure 6c shows a digitally combined image showing both the red and green fluorescence. The pattern heights in the crossed and uncrossed regions were found, using the AFM cross-sectional analysis, to be approximately 125–130 nm and 65–70 nm, respectively. These results verify the overlapping of multilayer films at the crossover regions and intact transfer elsewhere. To our knowledge, this is the first demonstration that the ITP approach can be used to overlay multiple bionanocomposite patterns. This ability to overlay multiple patterns, each of which contains a set of amphiphilic molecules such as proteins, dendrimers and PEMs, has potential applications in biocatalysis and fabrication of microarrays for high throughput screening.

The versatility of ITP was further demonstrated by the transfer of a nanocomposite film (see Figure 1, Case 2) consisting of sADH, G4 PAMAM dendrimers and 30 PDAC/SPS bilayers, $(sADH)_1(PAMAM)_1(PDAC/SPS)_{30}$. Fluorescence microscopy was used to establish the existence of alternating protein and dendrimer layers in the multilayered films after deposition. In one case (Figure 7a), only the protein was fluorescently labeled. In the other case (Figure 7b), only the dendrimer was labeled. Fluorescence was observed in both figures, confirming the presence of both protein and dendrimer layers. AFM was then used to confirm the incorporation of PEM bilayers into the multilayered films. Figure 8(a and b) shows the AFM images of a patterned film containing sequential layers of protein, dendrimers and PEMs (30 PDAC/SPS bilayers) on a PEM coated substrate. Figure 8(c and d) shows the AFM images of a patterned film containing sequential layers of protein and dendrimers without the PEMs. The height of patterns was approximately 101 ± 4 nm with PEMs, and approximately 9 ± 2 nm. Based on these data, the average height



■ *Figure 5.* Schematic representation of the process used for multilevel and multicomponent stamping.



■ *Figure 6.* (a) Red fluorescence from vertically printed (Alexa Fluor labeled-sDH)₁(PDAC/SPS)₂₀ lines on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate. (b) Green fluorescence from horizontally printed (FITC labeled-sADH)₁(PDAC/SPS)₂₀ lines, on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate. (c) Digitally combined fluorescence image showing both red and green fluorescence.

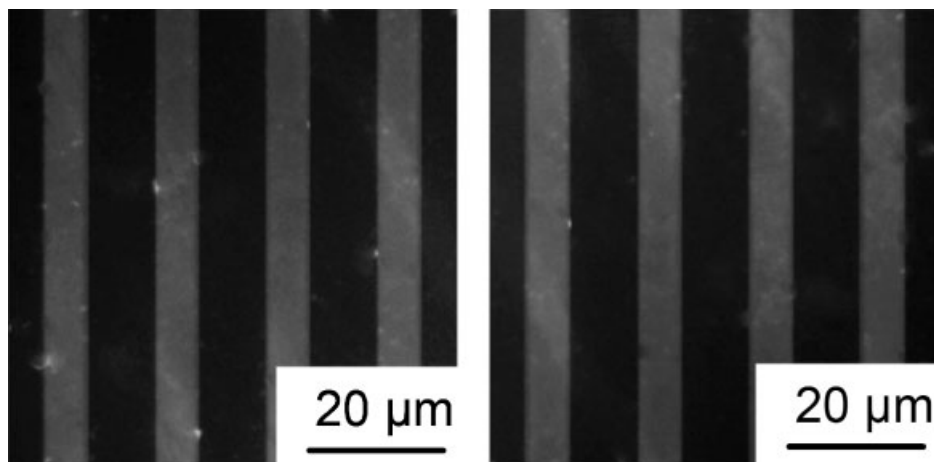


Figure 7. (a) Fluorescence image of the line patterns of sADH, G4 PAMAM dendrimers and PEMs (30 PDAC/SPS bilayers) with fluorescently labeled sADH as the topmost layer, on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate. (b) Fluorescence image of the line patterns of fluorescently labeled dendrimers sandwiched between patterned sADH layer and PEMs (30 PDAC/SPS), on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate.

of each PDAC/SPS bilayer was estimated to be about 3.07 ± 0.14 nm, which is in agreement with published values.^[1]

Potential advantages of ITP were further demonstrated by stamping a multilayer film consisting of a PDAC/SPS bilayer sandwiched between two sADH layers (Figure 1, Case 3). Fluorescence microscopy was again used to confirm the presence of the different protein layers. The base

height of the PDAC/SPS bilayer can be estimated to be 3 ± 1 nm, which is consistent with our previous results.^[1] Figure 9d shows the topographical image of a patterned film consisting of $(\text{sADH})_1(\text{PDAC/SPS})_1(\text{sADH})_1$. The average height of the patterns was 17 ± 2 nm. Based on these data, the height of the topmost sADH layer was estimated to be 7 ± 2 nm. To our knowledge, this is the first time it has been shown that the ITP approach can be used to

transfer of patterns containing multiple layers of proteins. Such patterns can be used to study protein-protein interactions and to develop novel biosensors.

We believe electrostatic interactions between the enzymes, dendrimers and PEMs to be responsible for stabilizing these multilayered structures. Weak polyelectrolytes change their conformation or charge density with pH.^[20,21] Thus, the shape and stability of the resulting 3-D structures formed with weak polyelectrolytes often vary with pH. To avoid such effects, we used strong polyelectrolytes, such as SPS and PDAC, whose charge density is relatively unaffected by pH.

Our novel approach, in which bionanocomposite arrays are pre-established on a stamp and then transferred intact to the target substrate, is based on *topographical contrast* between the feature and

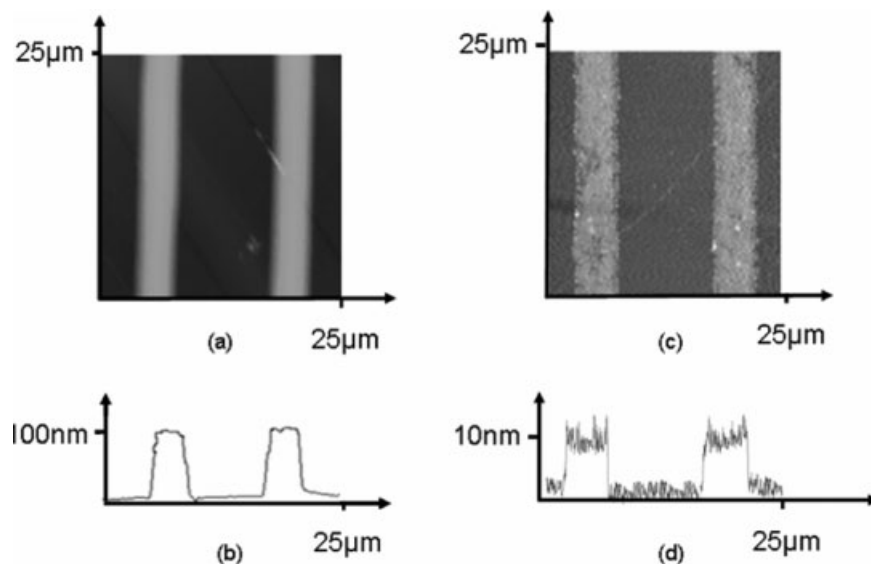


Figure 8. (a, b) 2-D and cross-sectional AFM images of a patterned film containing sequential layers of sADH, PAMAM dendrimers and PEMs (30 PDAC/SPS bilayers), with sADH as the topmost layer, on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate. (c, d) 2-D and cross-sectional AFM images of a patterned film containing sequential layers of sADH and PAMAM dendrimers with sADH as the topmost layer, on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate.

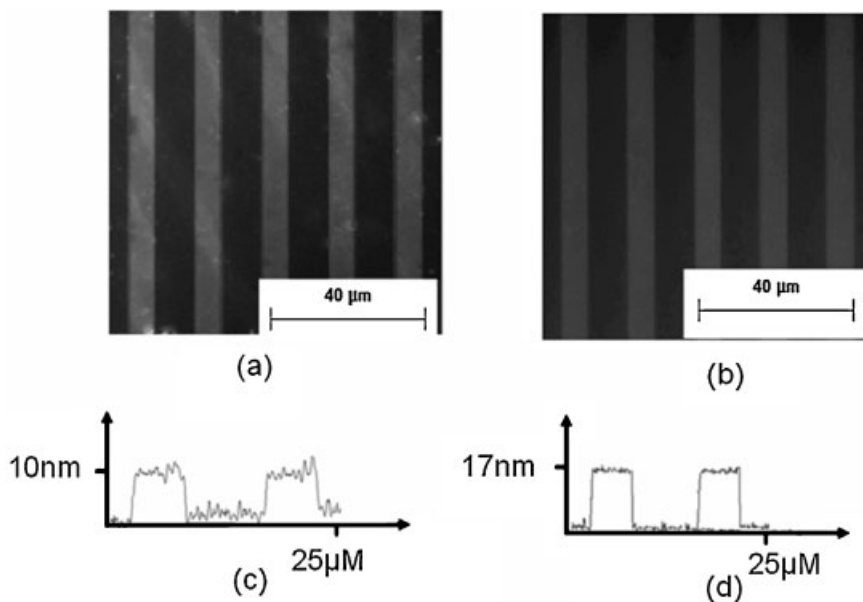


Figure 9. (a, b) Fluorescence images of the patterned films of a PDAC/SPS bilayer sandwiched between FITC labeled sADH base layer and Alexa Fluor labeled sADH topmost layer, on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate. (a) Green fluorescence emanating from FITC labeled sADH base layer, obtained using the filter set Ex: 465–495/DM: 505/Em: 515–555. (b) Red fluorescence emanating from Alexa Fluor labeled sADH top layer, obtained using the filter set Ex: 510–560/DM: 565/Em: 590–690. (c) Cross-sectional topographical image of a patterned film containing sequential layers of sADH and 1 PDAC/SPS bilayer on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate. (d) Topographical AFM image of a patterned film consisting of a PDAC/SPS bilayer sandwiched between two sADH layers on a PEM (10.5 PDAC/SPS bilayers) coated glass substrate.

background regions of the pattern, rather than *chemical contrast*. In a previous communication,^[28] we were able to demonstrate that the ITP approach offers significant advantages over the conventional, directed-self-assembly approach in cases when the chemical contrast is marginal or when amphiphilic or zwitterionic molecules (e.g., proteins) are involved.

This research significantly extends the range of surfaces and layering constituents that can be used to fabricate 3-D, patterned, bionanocomposite structures. Such structures have a broad range of potential applications, including fabricating protein-containing microarrays for screening drug candidates, studying mechanisms of protein-mediated cell adhesion,^[31,32] diagnosing disease states,^[33] constructing biosensors, and investigating interactions between proteins and other molecules.

Conclusion

In this paper, we have examined in detail the ITP approach, which allows high-quality microarrays of layered, bionanocomposite films to be deposited. The approach was extended to allow nanobiocomposite patterns to be overlaid,

and also create patterns containing multiple layers of proteins. The ITP approach uses LBL self-assembly to pre-establish a multilayered structure on an elastomeric stamp, and then uses μ CP to transfer the 3-D structure intact to the target surface. A variety of 3-D nanocomposite architectures were demonstrated, including patterns containing dendrimers, PEM and two proteins (sADH and sDH). Fluorescence microscopy and AFM conclusively demonstrated the feasibility of the approach for making these 3-D bionanocomposite structures.

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