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Microfluidic Protein Patterning on Silicon Nitride Using Solvent Extracted Poly(dimethylsiloxane) Channels

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Abstract

Biomolecular patterning is essential for the creation of sensing motifs that rely on receptor-ligand binding for selectivity. Microfluidic devices have the potential to aid in the development of simple, robust methods for biomolecular patterning and therefore contribute to the generation of protein, DNA, and cell microarrays. In microfluidic patterning, the choice of both substrate and microfluidic channel material is essential for control of both the receptor binding for maximal signal generation as well as non-specific adsorption that acts as chemical noise. In this study, polystyrene, glass, silicon nitride, and poly(dimethylsiloxane) (PDMS) were evaluated as substrates for protein patterning using two types of PDMS microchannels for patterning, native PDMS and solvent-extracted PDMS (E-PDMS). E-PDMS microfluidic channels resulted in better patterning characteristics than native PDMS channels as determined by a higher fluorescence intensity of immobilized protein on all substrate types tested. Microfluidic patterning was then applied to perform two- and four-layer immunoassays.

Keywords

Microfluidic; protein patterning; PDMS; immunoassay

1. Introduction

Understanding and controlling the interaction of proteins with solid surfaces is important for many applications, including fabrication of protein microarrays, development of affinity binding biosensors, cell patterning, and synthesis of biocompatible materials [1]. Because of this significance, many approaches have been developed for protein patterning and the study protein adsorption / desorption kinetics on solid surfaces [2]. The emergence of microfluidic devices and microfluidic channels provides an intriguing way to study these interactions in a simple low-volume approach [3]. The small channel dimensions (from micron to submicron scale) ensure laminar flow conditions in the channels. The surface-to-volume ratio is also much higher in microfluidic channels than in traditional benchtop patterning systems, and this effect makes bulk-surface interactions more efficient. The reagent requirements for microfluidic patterning are typically no more than a few microliters, making the approach cost-effective when dealing with expensive antibody or DNA systems and limited sample volumes [4]. Through use of a microfluidic channel, a well-defined region on a surface can be patterned for

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constructing adsorbed protein layers. This approach allows specific control of surface patterning with minute sample size.

PDMS is the most frequently used polymer for microfluidic patterning due to its ease of fabrication, low cost and desirable physical properties [5]. The geometry of PDMS microchannels can be easily tailored through soft lithography and, as a result, these devices have been employed in research activities ranging from genomics and proteomics to microarrays, separation devices, biosensors, and immunoassays [4,6–9]. PDMS is not without problems, however. The hydrophobicity of PDMS makes wetting difficult with aqueous solutions and the cross-linked material is known to both adsorb and absorb hydrophobic molecules. Finally, one of the most significant problems with PDMS is hydrophobic recovery, whereby, linear oligomers diffuse to the surface, making the surface of the material unstable over time. Recently, a method of recovering the oligomers through solvent extraction was reported [6,10]. The resulting surface has been reported to be much more stable but has never been used in microfluidic patterning experiments.

Silicon nitride (SN) is a material with excellent chemical, electrical and optical properties that is widely used in the semiconductor industry as a dielectric, and in advanced optical materials such as waveguides [11–15]. It is the goal of the present work to ultimately develop label-free biosensors fabricated from planar, silicon nitride waveguides. To achieve this goal, we must pattern selective binding agents on the surface of silicon nitride. There is previous work demonstrating application of silicon nitride as part of a biosensor using either immobilized proteins or cells [16–18]. These papers describe the protein or cell immobilization efficiency but not a quantitative evaluation of protein binding. As a first step toward the development of optimized, integrated optical waveguide biosensors, protein adsorption on silicon nitride was compared with other common materials used for immunoassays, in addition to studying the effect of solvent extraction on the PDMS stamp.

In the present work, microfluidic channels made from PDMS or solvent-extracted PDMS (E-PDMS) were used for protein patterning on different common organic and inorganic substrate surfaces, specifically, polystyrene, PDMS, E-PDMS, glass and silicon nitride (SN). The patterning effects of native PDMS channels and hydrophilic E-PDMS channels were determined by measuring the fluorescent intensity of immobilized proteins on each substrate. In all cases, E-PDMS microchannels resulted in higher fluorescence intensities for immobilized proteins. Once an optimized system was developed, E-PDMS was used to carry out immunoassays. Both two-layer and four-layer, moderate throughput immunoassays of C-reactive protein (CRP) were conducted on SN surfaces using E-PDMS microfluidic channels for patterning. In the four-layer format, antibodies were immobilized on the surface using the biotin-avidin complex. To our knowledge, these are the first results demonstrating quantitative immunoassays on SN using microfluidic patterning with E-PDMS channels. The present approach for microfluidic patterning has the potential to be applied in biosensing and cell culturing applications.

2. Experimental

2.1 Chemicals and materials

Sodium phosphate monobasic ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), sodium phosphate tribasic ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$), sodium chloride (NaCl), triethylamine (TEA, peptide synthesis grade), ethyl acetate and acetone (ACS certified) were from Fisher Scientific (Fair Lawn, NJ). Tween-20 (SigmaUltra), human serum albumin (HSA, purity 99%, fatty acid free) and bovine serum albumin (BSA, purity 98%, initial fraction by heat shock fraction V) were from Sigma (St. Louis, MO). Fluorescein-5-isothiocyanate (FITC, isomer I) was from Invitrogen (Eugene, OR). Dimethyl sulfoxide anhydrous (DMSO) was from Acros (Geel, Belgium). Avidin (from egg white) and

streptavidin were from Calbiochem (La Jolla, CA). NeutrAvidin™ Biotin-Binding Protein (NABBP) and Sulfo-NHS-Biotin were from Pierce (Rockford, IL). Nanosep 3K (3,000 MWCO) Omega centrifuge tubes were from Pall (Ann Arbor, MI). Slide-A-Lyzer® mini dialysis units with a 3,500 MWCO were from Pierce (Rockford, IL). Purified water (18 MΩ cm⁻¹) from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare all solutions.

Silicon wafers were from Silicon Inc. (Boise, ID). SN-coated wafers were donated by BioStar Inc. (Louisville, CO). The SN film was deposited by reactive ion sputtering on a thermally oxidized silicon wafer using a Nordiko 7000 with a silicon target and NH₃ as the nitrogen source. This sputtering method resulted in a 50 nm SN film with an average superficial roughness of 2 nm. The SN-coated wafer was cut into 2 cm × 2 cm pieces to match the dimensions of the microfluidic channel stamps. BD Falcon™ standard style polystyrene bacteriological Petri Dishes (three sizes: 60 × 15 mm, 100 × 15 mm and 150 × 15 mm) were from Becton Dickinson Labware (Franklin Lakes, NJ). Plain glass micro slides (No.2947, thickness: 0.96 to 1.06 mm) were from Corning Glass Works (Corning, NY). Sylgard®184 Silicone Elastomer Base and Sylgard®184 Silicone Elastomer Curing Agent were from Dow Corning Corporation (Midland, MI). Micro-punches (i.d. 0.51mm and 1.5mm) were from Technical Innovations (Brazoria, TX).

2.2 Preparation of FITC-labeled protein

Antigen and antibody samples containing Tris or NaN₃ were dialyzed against 25 mM phosphate buffer (pH 7.4) at 4°C prior to labeling with FITC. Freshly made 1 mg/mL FITC-DMSO solution was added to the protein solution at the ratio of 20:1 (protein / FITC, w/w) and mixed overnight at 4°C. The labeled products were centrifuged five times at 9,000 g using Nanosep 3K tubes to remove free FITC. The CRP/anti-CRP interaction is pH dependent and has its highest binding strength near the physiological pH value (7.4) [19]. Thus, the residue was reconstituted in 25 mM phosphate buffer, pH 7.4, and used directly. Using centrifugation, the majority of unbound FITC was removed with minor loss of protein. The purity of FITC-CRP was evaluated by capillary electrophoresis (CE) using a Beckman PACE/MDQ CE instrument equipped with a UV detector with a detection wavelength of 214 nm. The separation was performed using fused silica capillary (inner diameter of 50 μm, effective length 44.3 cm, total length 54.6 cm), and a background electrolyte consisting of 50 mM phosphate, pH 8.5. Injection was performed at 0.8 psi for 4 s and separations were carried out using an applied voltage of +12 kV. Concentrations of purified FITC-protein were measured using the Pierce Micro BCA™ protein assay protocol. The purified FITC-labeled protein was divided into 5 μL aliquots, stored at -20°C and isolated from light.

2.3 Preparation of biotinylated protein

Sulfo-NHS-biotin was used as the labeling reagent according to the protocol from Pierce for EZ-link® Sulfo-NHS-Biotin Reagents. The labeled product was centrifuged five times at 9,000 g using Nanosep 3K tubes to remove unreacted biotin. The purified biotinylated protein was divided into 5 μL aliquots and stored at -20°C.

2.4 Preparation of PDMS microfluidic channels

PDMS stamps containing microfluidic channels were prepared by pouring a mixture of silicone elastomer and curing agent at a ratio of 10:1 (w / w) onto a prefabricated mold [5]. After stirring thoroughly for 5 min to homogenize the mixture, the plastic container was put into a vacuum chamber to degas. When all the air bubbles were removed, the polymer mixture was poured into the mold and cured in an oven at 65°C for at least 2 h. For E-PDMS fabrication, PDMS stamps were immersed in stirred triethylamine for 2 h. The solution was replaced with fresh triethylamine after 1 h of extraction. The PDMS stamps were then transferred into ethyl acetate

and treated for 2 h, followed by 2 h of acetone extraction, each with stirring. The extracted PDMS stamps were dried in an air stream and placed in an oven at 65°C for at least 2 h following the acetone extraction step [6]. PDMS and E-PDMS were used directly as substrates. The stamp was then placed channel-down onto the substrate surface, where it adhered non-covalently due to conformal contact to create a finished microfluidic device [20].

2.5 Modification of the substrate and stamp surfaces

Prior to protein patterning, PDMS and E-PDMS microfluidic channels were oxidized in an air plasma (Harrick Scientific, PDC-32G) for 45 s with a power of 18 W to make the microfluidic channels hydrophilic. The surface treatment of different substrates was subsequently evaluated. When used as the substrate, untreated glass formed a strong, irreversible bond with oxidized PDMS. In most cases the stamp could not be removed from the substrate without damaging one or both. When the glass was sonicated in methanol prior to patterning, the stamp could be readily removed after patterning. For the polystyrene substrate, the methanol treatment yielded lower leakage from the channel area than the untreated polystyrene. Both PDMS and E-PDMS could be used as substrates without pretreatment. SN was rinsed with deionized water and methanol to remove the organic contamination and / or dust.

2.6 Protein patterning protocol using PDMS / E-PDMS microfluidic channels

The patterning of FITC-HSA was conducted through PDMS / E-PDMS microfluidic channels (50 μm) on different substrates. A 1 μL volume of sample solution was pipetted into each onboard inlet reservoir, with rapid subsequent filling of the microchannels due to capillary action. The device was placed in a humidified enclosure to minimize evaporation and incubated for 45 min. The entire device was then immersed in phosphate buffered saline (PBS, 50 mM phosphate, 0.9% NaCl, pH 7.4) and the stamp was peeled off quickly to prevent the sample from spreading to the adjacent areas on the chip [3]. The substrate was rinsed with 3 mL of 0.05% Tween-20 (in PBS) 3 times to reduce non-specific binding, followed by copious rinsing with de-ionized water, dried by air stream, and then analyzed using an inverted fluorescence microscope. Then, the fluorescence intensities of different patterning regimes (stamp / substrate) could be compared. For the two-layer immunoassay, the first layer (anti-CRP) was patterned through a 5-channel array and incubated for 40 min. The unmodified surface was blocked using 1% BSA in PBS. The second layer (FITC-CRP) was then patterned using a second 5-channel array in a similar manner but with the microchannels oriented perpendicularly to the first layer. After a 45 min incubation of the FITC-CRP, the stamp was peeled off and the substrate was vigorously rinsed with 0.05% Tween-20 PBS buffer and water, leaving behind a 5×5 mosaic pattern (Fig. 1).

Formation of the multilayer immunoassay included four steps: (1) the entire SN surface was first incubated with 2.5 mg/mL biotin-BSA for 90 min; (2) an aliquot of 1 mg/mL NABBP was patterned through a 5-microchannel array in an E-PDMS stamp using a 30 min incubation; (3) the remaining solution in the channels was flushed out by pipetting PBS buffer through the system. An aliquot of 2.5 mg/mL biotin-anti-CRP was flowed through the channel array and allowed to incubate for 30 min; (4) the stamp was removed. For the patterning of FITC-CRP at different concentrations, a second stamp containing a 5-microchannel array was oriented perpendicular to the channels in steps 2 and 3. The FITC-CRP solutions were incubated in the microchannels for 30 min. The second stamp was then removed and the substrate was rinsed 3 times with 0.05% Tween-20 buffer.

2.7 Measurement of the fluorescence intensity on patterned substrates

Images of patterned substrates were captured using a CoolSNAP^{cf} or CoolSNAP HQ² charge-coupled device (CCD) camera (Photometrics, Tucson, AZ) mounted on a TE2000-U inverted fluorescence microscope (Nikon, Melville, NY) with a Bright line® filter (Semrock, Rochester,

NY). The exposure time was set as 600 ms and bin 2×2 unless otherwise noted. The fluorescence intensity was acquired using the linescan function in Metamorph 7.0 (Molecular Devices, Downingtown, PA) with scanning width of 20 pixels. Signal was measured as the difference in fluorescence (ΔF) between patterned and unpatterned substrate regions.

3. Results and discussion

3.1 Comparison of HSA adsorption through different patterning regimes

Different substrates, polystyrene, glass, PDMS, E-PDMS and SN were used to evaluate the patterning of FITC-labeled HSA. Two types of microfluidic channels were used for patterning, PDMS and E-PDMS. Fig. 2 shows fluorescent micrographs of patterned layers on polystyrene, glass, SN, PDMS and E-PDMS substrates. The fluorescence intensity is proportional to the amount of immobilized FITC-labeled HSA per unit area on each substrate. Fig. 3 shows the average fluorescence intensities acquired from linescans of the images in Fig. 2. Among the five substrates, glass substrate showed the best patterning in term of fluorescence intensity (average ΔF was 133), using PDMS and E-PDMS microchannels. PDMS, E-PDMS, and SN substrates showed a moderate response. Polystyrene gave the lowest fluorescent intensity (average ΔF was 27). Due to the wettability of E-PDMS versus PDMS, the E-PDMS microchannels afforded a better patterning efficiency in terms of fluorescence intensity for each substrate employed in the experiments. Specifically, the patterning using E-PDMS channels resulted in 31.3% higher fluorescence intensity than PDMS on glass, 23.0% higher on PDMS, 17.2% higher on E-PDMS, 10.1% higher on polystyrene and 4.5% higher on SN. It has been shown that in the PDMS microchannels, molecular adsorption alters the composition of bulk solution [21]. The less hydrophobic character of E-PDMS makes it adsorb less protein than PDMS. This can be seen in the data for substrates made of PDMS and E-PDMS (Fig. 3) leading to a higher protein concentration in solution, respectively. Therefore more protein is adsorbed to the substrate when E-PDMS microchannels are used. Because SN showed an intermediate fluorescent signal and our subsequent work will be performed on a SN waveguide, SN was chosen as the substrate for the remaining immunoassay experiments.

3.2 Immunoassay using microfluidic protein patterning

Protein patterning using microfluidic channels facilitates immunoassays because the antibodies can be localized with high resolution, providing for better selectivity and easier implementation of moderate and high-throughput assays [8,22]. A CRP-(anti-CRP) immunoassay system was tested using microfluidic patterning on a SN surface with an E-PDMS stamp. CRP was chosen for this application as a model biomarker because of its known association with a variety of diseases and inflammatory responses [23]. A 5-channel array with 25 μm wide channels was used for the patterning. Anti-CRP was patterned through the five microfluidic channels as the first layer with passive adsorption on the surface. When anti-CRP was patterned, the surrounding area was passivated to reduce non-specific binding and improve detection limits. A variety of blocking solutions were tested to minimize non-specific binding: 1% BSA in PBS, 4% nonfat powdered milk with 0.05% Tween-20 in PBS and Pierce SuperBlock® T20 blocking buffer. 1% BSA afforded the best passivation of the three tested (data not shown) and was chosen as the blocker for subsequent immunoassays. FITC-CRP was patterned after the blocking step to create the second layer. The microchannels for CRP patterning were perpendicular to the channels applied for the anti-CRP layer. FITC-CRP was captured only in regions where it interacted with the antibody, producing the mosaic fluorescent profile shown in Figs. 4A and B. The fluorescence intensities for both channels were obtained by fluorescence microscopy (Figs. 4C and D). The plot of the change in fluorescence intensity (ΔF) versus the $\log[\text{CRP}]$ resulted in a sigmoidal binding curve (Fig. 5). Since the anti-CRP used here was monoclonal, the CRP/anti-CRP interaction can be analyzed as 1:1 with respect to the binding stoichiometry. If the relationship between the signal generated from antigen-antibody binding

and ligand concentration is taken into account, a simple model may be used to quantitatively evaluate the immune interaction:

$$S = \frac{S_{\max} [L]}{K_D + [L]}, \quad (1)$$

where S is the response (fluorescence intensity) generated by the immune interaction, $S_{\max}$ is the response when the ligand feature is saturated, $[L]$ is the ligand concentration applied and K_D is the dissociation constant for the immune interaction [24]. Eq. (1) can be converted into a linear expression:

$$\frac{1}{S} = \frac{K_D}{S_{\max}} \frac{1}{[L]} + \frac{1}{S_{\max}}, \quad (2)$$

Regression of the data shown in Fig. 5 according to Eq. (2) yielded a K_D of 1.7×10^{-7} M. This value is equivalent to previously measured K_D values for CRP/anti-CRP binding [25].

A multilayer immunoassay employing the biotin-avidin complex as binding medium was developed next for the CRP-(anti-CRP) immune system on SN. Fig. 6 shows a schematic of this multilayer immunoassay. The development of a 4-layer system using biotin-avidin chemistry was done to demonstrate the broad applicability of the patterning method. Because BSA strongly adsorbs on SN, the immobilized BSA served as a robust initial binding layer. By linking biotin, avidin and biotinylated-anti-CRP, FITC-labeled CRP was captured at the terminus. The biotin-BSA worked not only as the initial adhesion layer for the immunoassay, but also as a blocker to passivate the surrounding area. Consequently, it was not necessary to use other blocking buffers (data not shown). This multilayer immunoassay resulted in a 5×5 mosaic fluorescent image similar to that described in the previous section, but with higher average signal strength (Fig. 7). For example, the $1.76 \mu\text{M}$ CRP showed a fluorescence intensity of 1828 in the 2-layer format, while in the multilayer format a value of 5916 was observed. Therefore, the use of biotin-avidin conjugation improved the CRP/anti-CRP immunoassay relative to the two-layer immunoassay. Likewise, a sigmoidal binding curve was acquired, as shown in Fig. 8. Regression analysis using Eq. (2) gave a dissociation constant K_D of 1.6×10^{-8} M. The K_D value obtained here is 10-fold stronger than that obtained in the two-layer immunoassay. The difference in K_D is most likely due to surface-induced changes in protein conformation for the two-layer system which reduce the binding strength of anti-CRP for CRP. In the 4-layer format, the BSA modified SN isolates the anti-CRP from the surface and maintains a native conformation which has higher binding strength.

4. Conclusion

Protein patterning on different substrate surfaces using microfluidic channels made of native PDMS or E-PDMS was investigated. E-PDMS microfluidic channels resulted in better overall patterning characteristics than native PDMS channels. A two-layer mosaic immunoassay for CRP-(anti-CRP) was generated using microfluidic patterning, and the dissociation constant of CRP-(anti-CRP) immune interaction was calculated. Also, a multi-layer immunoassay, which integrated biotin-avidin conjugation and antigen-antibody binding, was performed on a SN surface and the dissociation of constant CRP-(anti-CRP) binding was calculated. To the best of our knowledge, this work represents the first creation of a quantitative immunoassay on a SN surface using microfluidic patterning.

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Charles S. Henry earned his B.S. with Honors from Missouri Southern State College in Joplin, MO in 1994. He then earned a Ph.D. in analytical chemistry from the University of Arkansas in 1998 working under the guidance of Dr. Ingrid Fritsch. Following this, he was an NIH Postdoctoral Fellow in the Department of Pharmaceutical Chemistry at the University of Kansas under the supervision of Dr. Susan Lunte. Dr. Henry joined the faculty of Mississippi State University in 1999. In 2002, Dr. Henry moved to the Department of Chemistry at Colorado State University where he has risen to the rank of associate professor. Dr. Henry's research focuses primarily on the use of miniaturized total analysis systems for the characterization of biological and environmental systems of interest.

David S. Dandy earned his PhD in Chemical Engineering from the California Institute of Technology (1987). After serving as a senior member of technical staff at Sandia National Laboratories, he joined the faculty at Colorado State University (1992), where he is presently a professor and the head of the Department of Chemical and Biological Engineering. He also has a joint appointment in the School of Biomedical Engineering.

Fig. 1.
Procedures for 2-layer immunoassay based on microfluidic patterning.

Fig. 2.

Fluorescence images of microfluidic pattern of HSA on different substrate using PDMS or E-PDMS channels. The numerator indexes (PDMS and E-PDMS) denote the stamp materials. The denominator indexes denote the substrate materials. The FITC-HSA concentration was 1 mg/ml.

Fig. 3.
Comparison of the fluorescence intensities for different patterning regimes as shown in Fig.2.

Fig. 4.

Fluorescent images for CRP-(anti-CRP) immunoassays and the quantitative analysis. A) high CRP concentration range (from left to right: $1.76\ \mu\text{M}$, $0.35\ \mu\text{M}$, $70\ \text{nM}$, $35\ \text{nM}$, $3.5\ \text{nM}$); B) low CRP concentration range (from left to right: $3.5\ \text{nM}$, $0.70\ \text{nM}$, $0.35\ \text{nM}$, $35\ \text{pM}$, $3.5\ \text{pM}$); C) line scan for A; D) line scan for B. The Bio-anti-CRP concentration was $2.5\ \text{mg/ml}$. The $3.5\ \text{nM}$ FITC-CRP in both A and B was used as an internal standard to correlate. The incubation time for each steps were: 40 min for anti-CRP; 36 min for block and 45 min for FITC-CRP.

Fig. 5.
Changes of fluorescence response with increasing CRP concentration for CRP-(anti-CRP) immunoassay.

Fig. 6.
Adlayer scheme for multilayer immunoassay.

Fig. 7. Fluorescent images for multilayer immunoassays and the quantitative analysis. A and B, mosaic image from different CRP concentrations which were as same as in Fig. 4; C and D, line scan for A and B, respectively. The detailed conditions were shown in the text.

Fig. 8. Changes of fluorescence response with increasing CRP concentration in multilayer immunoassay. The line represents a best fit of the data.