Soft-Lithography-Mediated Submicrometer Patterning of Self-Assembled Monolayer of Hemoglobin on ITO Surfaces

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The recent direct fabrication of micropatterned arrays of active biomolecules has received overwhelming attention due to its potential applications in biosensors and medical diagnostics. Here, we report an indirect soft-lithography-mediated method of fabricating hemoglobin (Hb) patterns in the submicrometer length scales region on indium-tin-oxide (ITO). Scanning electron microscopy, quartz crystal microbalance, and cyclic voltammetry studies reveal the formation of a self-assembled monolayer of Hb on an ITO surface. The present approach provides an easy and reliable means for simultaneous and defectless passivation and submicrometer patterning of proteins, thus indicating its potential application in biological assay.

Introduction

The idea of achieving high-throughput direct patterning of biomolecules onto surfaces is both interesting and challenging. The patterning of arrays of biomolecules in the submicrometer to nanometer length scales would serve as an important means for fabricating devices for applications in miniaturized biosensors and advanced medical diagnostics.[1] Much work has been done in these areas, and potential applications have been demonstrated.[2]

Among the biomolecules studied, proteins emerge as the ones that are actively pursued in such patterning due primarily to their biological importance. Current methods employed for protein immobilization commonly involve modification of the substrate surface properties and/or the protein adsorbates prior to immobilization.

Two general methods for immobilization of proteins have been reported. The first involves a physical approach, for example, direct spraying of proteins onto mica and platinum/carbon-coated mica.[3] The second involves derivatizations of proteins, for instance, thiolation using Traut's reagent or water-soluble carbodiimide followed by chemisorption onto bare or chemically modified gold surfaces.[3]

To improve adhesion of proteins onto these surfaces, biotin/streptavidin interaction has been utilized also.[4] A similar approach has also been reported by Droz et al.[5] Other immobilization schemes along the same line as the latter approach include attachment of proteins onto a photoactive self-assembled monolayer (SAM) on gold[6] and antigen/antibody binding onto alkanethiol passivated gold surfaces.[7] Although these approaches have been successfully demonstrated to achieve protein immobilization, they have inherent shortcomings. While direct spray methods allow an achievable fast turnover time compared to immobilization methods, they suffer the disadvantage of not being able to produce submicrometer length scale patterns. As for the second approach, besides being a time-consuming process, the involvement of the covalent binding could possibly hinder the activation of the studied proteins. Thus, to simplify the immobilization methods, microcontact printing (μCP) of proteins has been adopted.[8,9] While one method[8] employed a micromolded poly(dimethylsiloxane) (PDMS) stamp to directly print proteins onto a pretreated receiving surface after immersion of the stamp into the protein solution of interest, the other[9] used a hydrogel stamp loaded with antibody to directly ink proteins onto an aminosilylated receiving surface. Both methods resulted in successful attachment of proteins in a few seconds and formation of patterns with submicrometer resolution and control. Common to both methods, pretreated substrates were used. However, the PDMS stamp used was required to be immersed in the solution for a substantial period of time for equilibration, while the hydrogel stamp required rather careful preparation and had to be maintained at a low temperature. Thus, an easier but efficient method is urgently needed to pattern protein with submicrometer resolution directly onto suitable substrates.

Due to its transparent and conductive properties, indium-tin-oxide (ITO) has been widely used as an electrode for electrochemistry of biomolecules.[10] Recently, studies have also shown the existence of specific adsorption of phosphonic acids, carboxylic acids, and amines on ITO surfaces,[11] which may facilitate the potential application of this material in protein immobilization.

Hemoglobin (Hb), a tetramer heme protein, is widely used as a model protein for fundamental studies in electrochemistry, structural elucidation, and bioactive

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functionalities. Hook et al.[12] recently studied the adsorption of Hb to a hydrophobic self-assembled methyl-terminated thiol monolayer on a gold surface by a quartz crystal microbalance (QCM). Here, we report a simple and yet efficient method for achieving submicrometer patterning of Hb on an ITO surface, i.e., through a soft-lithography-mediated approach. A QCM was used to determine the optimal deposition conditions. Surface coverage was then calculated from the results of QCM and cyclic voltammetry (CV), which shows the formation of a self-assembled monolayer of Hb on ITO surfaces. Possible mechanisms for such adsorption were also discussed.

Experimental Section

QCM. The QCM system used was the same as previously reported.[13] Figure 1 shows the process flow for realizing Hb passivation on an ITO/Au electrode surface on a piezoelectric (Pz) crystal. First, a thin film of ITO (~10 nm) was sputtered onto a designated area of a clean gold electrode (with an original frequency of F_0) through a shadow mask using a magnetron sputtering system (Discovery-18 deposition system, Denton Vacuum, Inc.). The resultant decrease in frequency to F_1 allows us to calculate the mass of the deposited ITO.[13] This modified electrode was then used to study the adsorption behavior of Hb on an ITO surface. Hb (human) was obtained from Sigma and dissolved with Milli-Q water to obtain solutions with different concentrations and pH value of 5.3, which were in the pH range of native Hb.[14] After the ITO surface was equilibrated with Hb solution, followed by rinsing with Milli-Q water and drying under a stream of purified N_2 gas, the frequency was recorded as F_2. The surface coverage of Hb was then calculated from the frequency change, $\Delta F = (F_1 - F_2)$, according to the Sauerbrey equation.[13] For in situ studies, the ITO/Au electrode was directly immersed into the Hb solutions. The frequency changes with immersion time were recorded using Labview software (v. 5.0, National Instruments). By comparison of the results obtained using different experimental conditions, an optimal Hb adsorption condition was obtained.

Submicrometer Patterning of Protein. To identify an efficient method for submicrometer patterning of Hb, we used two different process flows to prepare the substrates (see Figure 2). In process flow A, after the spin coating of a uniform layer of photoresist (PR) (thickness ca. 2 μm) on a clean silicon (001) wafer, the PR was exposed via photolithographic method either using a 248 nm UV-exposure system (ASML, USA) followed by development or subjected to a μCP approach with a PDMS mold. The mold was prepared by mixing 10:1 (w/w) of the siloxane base oligomers and its platinum-based curing agent (Dow Corning Sylgard Elastomer 184) and pouring this mixture onto a Si master with submicrometer patterns. The final mixture was cured at 100 °C for 4 h. Subsequently, the cured PDMS mold was cut and peeled with care after cooling to room temperature. In μCP, the mold was pressed (10 psi) against the PR and hardbaked at 100 °C for approximately 10 min to cure the PR. When it cooled to room temperature, the PDMS mold was removed. Both methods allowed submicrometer patterning of PR on the Si(001) surface. To obtain a submicrometer pattern of ITO, such Si(001) wafer was first placed into the sputtering chamber to deposit ITO with ~5 nm thickness and then immersed into acetone (HPLC grade) for thorough liftoff of the spin-coated PR. The Hb solution was then dropped onto the surface of the wafer with submicrometer patterns of ITO until equilibrium was obtained. Followed by rinsing and drying, the ITO-coated wafer was ready for further detection assays. In process flow B, we first sputtered ITO on an entire surface of a clean silicon wafer followed by spin coating of PR and μCP with PDMS mold. After the passivation of Hb, rinsing, and liftoff of the deposited PR as mentioned above, we obtained submicrometer patterns of Hb across the entire ITO surface.

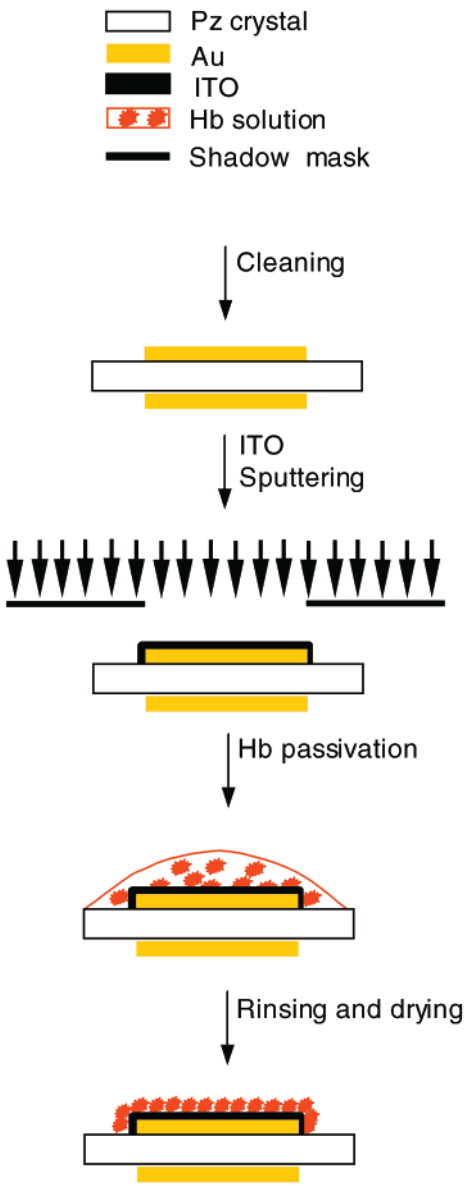

Figure 1. Process flow for preparation of an ultrathin layer of ITO on Au electrode of a Pz crystal for Hb passivation.

Characterization. SEM images were acquired using a FEG XL 30 Philips scanning electron microscope at 10 kV accelerating voltage. Cyclic voltammetric experiments were carried out on a scanning potentiostat (model 362, EG&G, PARC, USA). A standard three-electrode system composing with a SCE reference electrode, a platinum counter electrode, and an ITO sputtered (~10 nm thickness) silicon wafer (Si(001)) as a working electrode (WE) was used. All the electrochemical experiments were conducted at room temperature.

Results and Discussion

We calculated the frequency changes ($F_1 - F_0$) for different batches after ITO sputtering, and an average mass change of ~3780 ng was obtained with the CV of 7% ($n = 10$) for 10 nm thickness. The consistency in frequency changes ensures the uniformity and reproducibility of the deposited ultrathin ITO on the Au electrodes. To determine the optimum condition for the passivation of Hb on ITO

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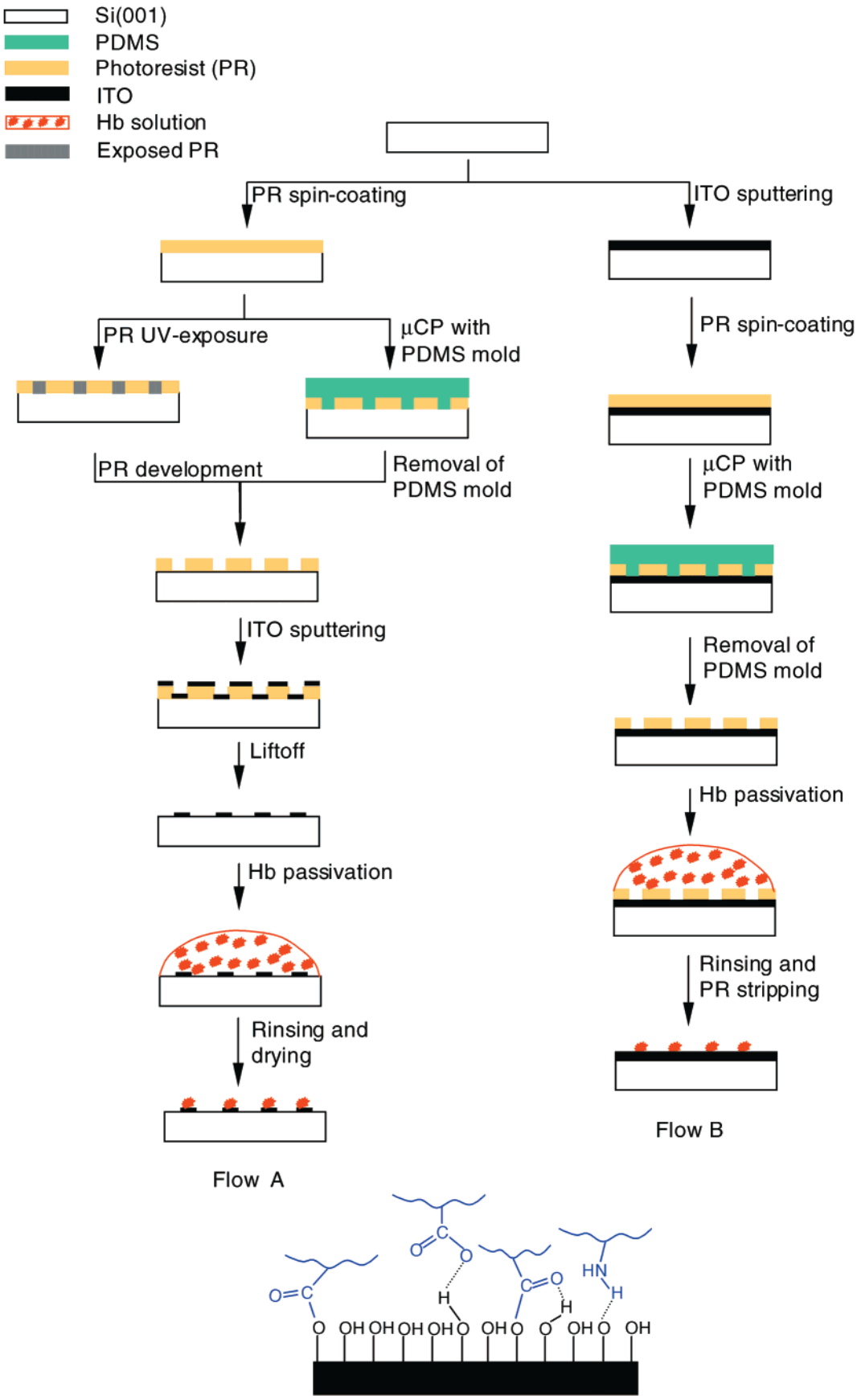

Figure 2. Two process flows for submicrometer patterning of Hb SAM on ITO/Si(001) surfaces. Schematic representations of proposed ester bonds and hydrogen bonding are also shown.

surface, we first used a QCM deposited with ITO to perform in situ measurements.

Figure 3A shows the frequency changes with immersion time for different concentrations of Hb solutions. In

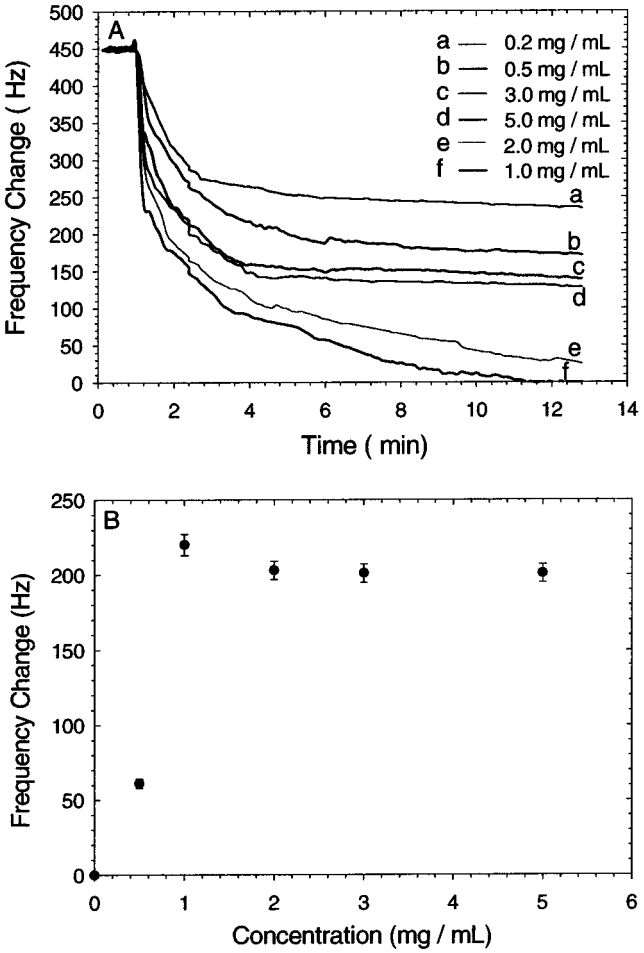

Figure 3. Frequency change vs (A) immersion time for various concentrations of Hb solutions (in situ) and (B) concentrations of Hb solutions (for a total passivation duration of 15min, ex situ).

general, the amount of adsorption was observed to increase with increasing concentrations; 1 mg/mL solution was found to show the maximum absorption during the same immersion time of 15 min, suggesting this as the optimal condition for subsequent submicrometer patterning of Hb on ITO surfaces. However, when the concentration is greater than 1 mg/mL, a reduced adsorption is observed, due probably to the increased mutual interaction between the protein molecules in aqueous media at high concentrations which impedes their binding to the ITO surfaces. Similar trends were obtained when studying the absorption phenomena with an ex-situ approach (see Figure 3B). The differences in the absolute frequency changes for both approaches with respective concentrations could be attributed to the different media of experimental environments (i.e., aqueous solution and air). In the former, the Bruckenstein and Shay and Kanazawa and Gordon's equation[15] was used instead of the Sauerbrey equation[16] to calculate the mass change, which corresponds to the frequency change while taking into consideration the viscosity and density of the liquid medium.

The small variations in the frequency changes for concentrations greater than 1 mg/mL show the possibility of formation of a SAM on ITO surface. The maximum surface coverage was calculated to be $\sim 1.5 \times 10^{-11}$ mol/cm^2 from the corresponding frequency change. Taking into consideration the size of a Hb molecule ($5.5 \times 5.5 \times 7.0$ nm[17]), a closely packed Hb monolayer of the ITO surface should have a surface coverage of $\sim 0.5 \times 10^{-11}$ mol/cm^2, which is three times smaller than the coverage calculated. The difference is reasonable if one considers the surface microroughness of the sputtered ITO.[18]

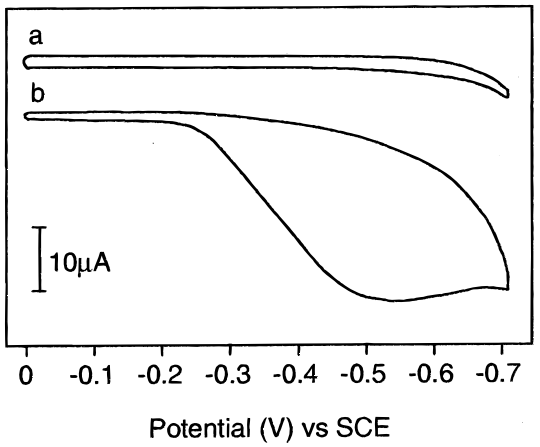

Figure 4. Cyclic voltammograms of (a) non-Hb passivated ITO/Si(001) WE and (b) a Hb SAM passivated ITO/Si(001) WE (with an area of 1.0 cm^2) in 0.2 mol/L acetate solution (pH = 5.0). Scan range was 0 to −0.7 V (vs SCE); scan rate was 50 mV/s.

Since the isoelectric point of ITO is at approximately pH 6, adsorption is expected to work under these conditions with the hydroxyl group playing an active role when adsorption occurs (see below). The fact that ITO was slightly dissolved when immersed into a pH 5.0 acetate solution overnight and a smaller surface coverage was obtained at higher pH further confirmed this hypothesis.

It is well-known that there exist carboxyl acid groups as well as amine groups on the polypeptide chains of a protein. Typically, the Hb tetramer is a spheroidal molecule, in which the two α and two β subunits are structurally and evolutionarily related to each other.[19] There are extensive interactions between the unlike subunits which are predominantly hydrophobic in character. When dissolved in polar solvents, such hydrophobic groups are deeply buried in the core of the protein molecule while most of the hydrophilic groups extensively interact with the solvent molecules. It is believed that carboxylic acids bind more effectively than the amino groups to the ITO surface.[11d] Since there are 12 and 15 D + E in the α and β subunits of human Hb respectively,[19] it is postulated that there exist accessible carboxylic groups of D and E in Hb solution which can freely interact with the surface hydroxyl groups on the ITO surface in the Hb passivation step. Carboxylic acids are considered to attach to the ITO surface via ester bond formation based on the interactions between surface hydroxyl groups and the carboxylic acid groups.[11a] The abundance of the hydroxyl groups (12−13 OH groups per nm^2)[20] ensures thorough interactions at all possible binding sites. Ester bonds are formed with nearly all the free carboxylic groups from the polypeptide chains of Hb except for those impeded by space and

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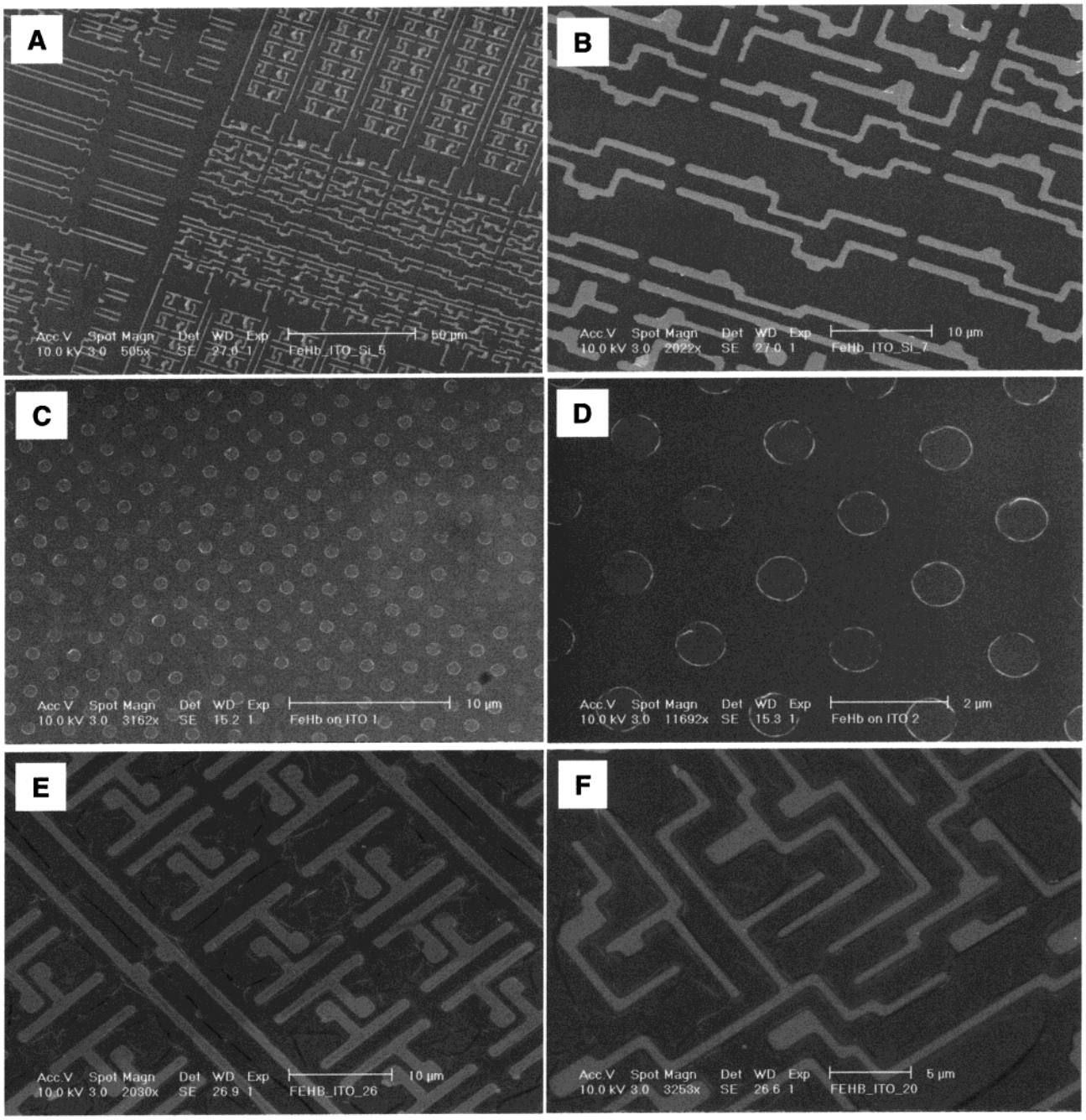

Figure 5. SEM images of submicrometer-patterned Hb SAM on ITO surfaces. (A), (B), (C), and (D) micrographs were obtained from flow A while (E) and (F) micrographs were from flow B as shown in Figure 2. (B) and (D) are zoom-in images of (A) and (C), respectively.

neighboring groups. The underivatized OH groups on the ITO surface may also help to stabilize the adsorption through hydrogen bonding with carboxyl or amine groups from polypeptide chains (see Figure 2). When soaking such ITO surfaces in concentrated salt solutions, only a slight decrease of the peak currents was observed, which indicated the existence of chemical bonding. This phenomenon was ascertained by FT-IR studies which indicated the formation of $COO^{-}M^{+}$ interfacial bonds from adsorption bands[21] observed between 1600 and 1400 cm^{-1}. Such bonding was strong enough to withstand ultrasonication in acetone during PR stripping.

Further evidence of the formation of Hb SAM was confirmed by electrochemical study. Figure 4 shows the cyclic voltammograms in 0.2 mol/L acetate buffer (pH5.0). An irreversible redox peak (curve b) was observed after equilibrating the WE in Hb solution for ca. 15 min. No peak was observed (curve a) when directly using a non-Hb passivated ITO/Si(001) substrate as the WE in the same buffer within the same scan range. As expected, the peak current is observed to be proportional to the scan rate, a characteristic phenomenon of the absence of diffusion.[22] No great increase in peak current was observed when increasing Hb passivation time. By integrating total charge under curve b, a surface coverage of 3.2×10^{-11} mol/cm^2 was calculated, which is close to the QCM value of Hb SAM calculated earlier and thus reinforces the

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observation of formation of the Hb SAM on ITO surfaces. In fact, the adsorption of Hb on ITO surface is so spontaneous that a significant peak was observed by just dipping the WE in Hb solution followed by quick electrode removal and rinse.

Figure 5 shows the SEM[23] images from the different process flows (see Figure 2). Panels A−D of Figure 5 show images from flow A, in which the brighter submicrometer length scale lines and circles correspond to the Hb-passivated ITO. Figure 5E and Figure 5F show images from flow B, in which the brighter lines correspond to SAM Hb on an entire blanket ITO surface. Large consistent and uniform surface coverage of these patterns with no noticeable defects could be fabricated easily with the present approach. However, the irregular lines (cracks) on the ITO surfaces between Hb patterns in panels E and F could be observed and could be attributed to the stress induced by the loading (∼10 psi) during μCP while curing the PR at high temperature. Such cracks or lines were not found in images obtained from flow A which involves only additive ITO patterning. The results obtained so far suggest that both processes are successful methods for highly homogeneous and complex submicrometer patterning of Hb on the ITO surfaces.

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Conclusion

We have described an easy but efficient method for direct submicrometer patterning of Hb SAM on ITO surface. The strong and rather stable bonding of this protein on these ITO surfaces is of great interest as no coupling reagent is involved. This submicrometer patterning via soft lithography or conventional methods may provide attractive means for promising application in the field of biological assay and related fields.

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