LITHOGRAPHIC PATTERNING OF IMMOBILIZED
ENZYMES IN CHITOSAN THIN FILMS FOR MULTI-LAYER,
CHEMICAL/BIOLOGICAL SENSORS

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Abstract — Patterning of immobilized enzyme in spin-cast chitosan thin films will enable the creation of multi-layer biologically-active devices. Bulk immobilized enzymes used in multi-layer devices, in contrast to surface functionalized devices; enable the implementation and characterization of multi-component assays. This paper demonstrates a procedure to immobilize enzymes into an aqueous chitosan solution, spin-cast that solution into a thin-film, and, finally, pattern that thin film using photolithography and oxygen plasma. Enzymes immobilized, deposited, and patterned using this process retain functionality, as shown with the example assay of β-D-galactosidase (β-Gal) and fluorescein di-β-D-galactopyranoside (FDG), a fluorogenic substrate for β-Gal. Hydrogel thicknesses of 200 nm to 1.5 μm (dry) were achieved and line widths down to 2 μm were observed. Finally, a multi-layer stack of chitosan hydrogel is demonstrated using a stopping layer of sputtered silicon dioxide (SiO2).

Keywords — Chitosan, Enzymes, Lithography, Thin Films, Chemical Sensors

I. INTRODUCTION

Chitosan is a β-1,4-linked polysaccharide and is the partially deacetylated form of chitin, the second most abundant polysaccharide in nature after cellulose. Chitosan is one of the most versatile biomaterials and its properties, including biocompatibility, lends itself to a wide array of applications from environmental engineering (ex. flocculation of waste water) to pharmaceuticals (ex. sutures, contact lenses and drug delivery agent) [1]. At the micro and nano-scale, chitosan is a great bioscaffold due to a robust chemical structure with high concentration of primary amino groups (-NH₂) for ionic or covalent bonding binding of biomolecules (e.g. DNA and proteins). Additionally, chitosan is soluble in weak acids and forms a hydrogel, making it an enticing material to work with for microfabrication. By using chitosan and chitin as the main structural material and its hydrogel characteristics and functionalizations, as it is used in nature, a wide variety of biosensors can result.

Table 1 shows just a few relevant sensors that could be realized using proteins immobilized in patterned chitosan microstructures [2]. This research will address the survival of a model enzyme/substrate system in the chitosan matrix during spin casting and lithographic patterning. Future work will focus on the development of electrical protein activity detection methods.

TABLE I. POTENTIAL BIOSENSOR APPLICATIONS AND THEIR RESPECTIVE ENZYME/SUBSTRATE COMBINATIONS [2]

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Application</th>
</tr>
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<tbody>
<tr>
<td>Alcohol Oxidase</td>
<td>Ethanol</td>
<td>Determination of Ethanol Concentration</td>
</tr>
<tr>
<td>Glucose Oxidase</td>
<td>Glucose</td>
<td>Determination of Glucose Concentration (food, medical diagnosis)</td>
</tr>
<tr>
<td>Creatine Deaminase</td>
<td>Creatine</td>
<td>Determination of Creatinine</td>
</tr>
<tr>
<td>Urease</td>
<td>Urea</td>
<td>Determination of Urea (indicator of kidney function)</td>
</tr>
<tr>
<td>Putriscene oxidase</td>
<td>Putriscene</td>
<td>Determination of Meat Freshness</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>Xanthine</td>
<td>Determination of Fish Freshness</td>
</tr>
</tbody>
</table>

Existing methods for patterning hydrogels include soft lithography, electrodeposition, and photolithography with photocrosslinkable chitosan. Soft lithography methods, such as the nanoimprinting method presented by J. C. Cheng et al. [3], are inexpensive, simple, and provide excellent resolution; however, the stamps are difficult to align to the substrate [4], require a uniform surface for efficient pattern transfer, and often require addition of a material plasticizer to maintain feature shapes [5]. Electrodeposition of chitosan has been demonstrated by L. Wu et al. [6]; however, it requires either a conductive substrate or a patterned electrode for deposition and can have dramatic uniformity issues due to variations in the electric field near the deposition surface. Finally, J. M. Karp et al. demonstrated a photocrosslinkable chitosan process for directing growth for cardiac fibroblasts, cardiomyocytes, and osteoblasts (SAOS-2). This process, while simple and inexpensive, suffers from poor feature resolutions around 100 μm and reduces the number of functional groups available to bind biomolecules into the matrix [7].

The current polysaccharide patterning techniques discussed above are accomplished in solution and, as a result, have poor feature resolution and film uniformity; thus, a dry patterning technique is needed to overcome these limitations. Swelling and contraction due to the movement of solvent as well as the increased film thicknesses during optical lithography contribute dramatically to the reduction in feature resolution. Photolithographic patterning of dried polysaccharide films, such as chitosan, overcomes these issues by eliminating the

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solvent during the patterning step. In addition, dry patterning of polysaccharide thin films enables multi-layer devices where each layer can be uniquely formulated and functionalized.

II. EXPERIMENTS

A. Chitosan solution preparation

Stock solutions of 3.5% w/v medium molecular weight chitosan (Sigma Aldrich, St. Louis, MO) in a 1:100 mixture of acetic acid (HAc) and deionized water (DI) are prepared with a final pH ~5. The solutions are then mixed on a hot plate at 95°C for 6 to 8 hrs and triple filtered using air actuated syringes at 550 kPa (80 psig) with 25 μm, 10 μm, 5 μm, and 2.5 μm Durapore filters (Millipore, Billerica, MA). Air bubbles in the solution are then removed using centrifugation at 27,500 g for 20 min.

A 10 mg/mL stock solution of enzyme is prepared using β-D-galactosidase supplied from Sigma Aldrich. This solution is filtered once using a Whatman 0.22 μm syringe filter and mixed 1:10 into a stock chitosan solution. The solution is then thoroughly mixed and degassed using an ultrasonic bath and spin cast promptly after mixing.

Fluorescein di-β-D-galactopyranoside (FDG) from Axxora is mixed into a 0.5 mM solution and a droplet of 1-10 μL is applied to the sample using a micro-pipetter. A negative control for fluorescence is performed using a droplet of 0.5 mM FDG on a clean microscope slide and a positive control is performed with a droplet of 0.5 mM FDG mixed with a droplet of the stock enzyme solution.

B. Single Layer Fabrication Process

Single layer lithography of chitosan-protein composite films, shown in Figure 1A, starts with spin-casting the viscous solution (~600 cp) onto silicon and pyrex wafer dies at speeds between 2000-3000 RPM to achieve thicknesses between 200 nm and 1.5 μm. A 5 min bake at 70°C or 90°C dries the chitosan film, PMMA (950 PMMA C5 Resist from MicroChem, Newton, MA) is then spin-cast on the wafer at 2000 RPM for 10 sec with a 200 RPM/sec ramp rate and is then baked again for 5 min at either 70°C or 90°C. The PMMA layer protects the chitosan-protein composite films from the photoresist developer solution. Next, a 2 μm OCG-825 G-line Photoresist is spun at 2200 RPM for 30 sec, baked for 60 sec at either 70°C or 90°C, patterned using projection printing and developed using OCG-934 2:1 photoresist developer. A hard bake is performed for 10 min at either 70°C or 90°C. The dies are then etched with oxygen plasma in a PlasmaTherm parallel plate RIE system with an RF power between 50 and 150 W, an oxygen flow rate between 55 and 65 sccm and a base pressure less than 11 mTorr. Etch times are determined based on the thicknesses of the chitosan-protein composite film and the PMMA layer. Table 2 indicates the measured etch rates for a range of materials and etching conditions. The remaining photoresist and PMMA is stripped using room temperature acetone and is followed by a deionized water rinse. Nitrogen was used to dry the samples.

A minimum line width of 2 μm and less than 15% variation in thickness across the wafer are achieved with this process.

C. Multi-Layer Fabrication Process

Multi-layer chitosan patterning begins by sputtering a 25 to 100 nm silicon dioxide (SiO2) stopping layer on top of a chitosan film created with the single layer process. SiO2 is sputtered using a Randex thin film deposition system with 100 W of applied RF power and 44 sccm of 90% argon / 10% oxygen. A deposition rate of SiO2 of 4.2 Å/min is typical. Next, the single layer patterning process is repeated on top of the SiO2 stopping layer. A brief 30 to 60 sec hydrofluoric acid (HF) vapor etch is performed to remove the surface SiO2 layer (100 nm/min) [8]. An overview of the multi-layer fabrication process is given in Figure 1B.
Figure 2. Patterned chitosan structures with immobilized β-Galactosidase treated with fluorescein di-β-D-Galactosidase (FDG) viewed under a fluorescent microscope.

D. Sample Imaging and Metrology

Film thicknesses and line widths are characterized using a KLA-Tencor Alpha Step IQ profilometer and a NanoSpec/AFT Model 3000 film thickness measurement system. Activity of the immobilized β-D-Galactosidase is observed qualitatively using a Zeiss AxioImager M1 fluorescence microscope, a FITC filter set and a QImaging 5MP MicroPublisher camera.

III. RESULTS AND DISCUSSION

A. Single layer

The viability of enzymes in the chitosan after lithography is determined by an example assay of immobilized β-D-galactosidase and an externally applied solution of fluorescein di-β-D-galactopyranoside (FDG). The hydrogel features swelled taking up the solution and substrate; thus, allowing enzyme activity. In the example assay, β-D-galactosidase cleaves the fluorescein from the FDG increasing its fluorescence signal at a peak of 514 nm when excited by 490 nm. Significant fluorescence of the features was observed when using a GFP filter set, as shown in Figure 2. The chitosan features remained bound to the silicon substrate and did not delaminate when hydrated. All features, including the smallest line features at 2 μm in width, retained activity.

B. Multi-layer

The primary concerns for multi-layer processing are protection of the lower layer and effective adhesion of the top layer. Patterning of the top layer of chitosan requires an over etch to attain high quality feature definition; thus, an etch stop is necessary to protect the underlying layer. The presence of enzymes and polymers requires a very low thermal budget for the etch stop with temperatures below 90ºC; thus, sputter deposited silicon dioxide is chosen as the etch stop. In addition, silicon dioxide can be removed using a HF vapor etch. Removing the silicon dioxide etch stop exposes the underlying layer allowing external chemicals like FDG to be applied to it for assays. A 10 min oxygen plasma etch performed at 70 W showed no measureable changes in silicon dioxide thickness and completely protected an underlying layer of chitosan. Thus, a thin layer of sputtered silicon dioxide is used as the etch stop due to its high oxygen plasma resistance and to minimize exposure to HF vapor during the etch stop removal step.

Two layers are patterned to test the multi-layer fabrication process. The first layer is an 800 nm chitosan film with immobilized enzyme on a silicon surface. A 65 nm sputtered silicon dioxide layer is then deposited on top of the first layer of chitosan to serve as the etch stop for second layer. The second layer is a 1.5 μm chitosan film. The same sets of features are patterned for both layers, with the second layer rotated 90º. The resulting multi-layer pattern is shown in Figure 3. The viability of the proteins in the first layer is then tested using the same protocol as used for the single layer. The strong fluorescence from the features with immobilized enzymes shown in Figure 3 (bright white features) showed that the enzymes survive the processing. The chitosan top layer shows some auto fluorescence; however, this intensity is noticeably lower than that of the functionalized, 1st layer. The wrinkling shown above the white pads of the features in the 1st layer is believed to be caused by interaction between the swelling hydrogel and residual silicon dioxide from the etch stop.

Figure 3. Multi-layer stack of patterned chitosan with immobilized enzymes in the bottom layer taken at 5x (above left) and closer up at 20x (above right). Images taken with fluorescent microscope with FITC filter set where bright white fluorescing features are in the bottom layer with immobilized enzymes and the lower intensity, autofluorescing features are in the top layer. Note: Auto-leveling applied to images for purposes of clarity.
IV. CONCLUSION

The advantages of enzymes immobilized in chitosan thin films are viability for extended periods of time and suspension in a robust, biocompatible matrix. Our high-resolution, enzyme-compatible patterning process enables the creation of multi-layer biologically-active devices. By patterning stacks with each layer distinctly functionalized, unlike surface functionalized structures with only a single functionalization, our process enables devices capable of multi-stage assays with higher densities of enzymes for analysis. Overall, given that the amount of analyte is limited, this allows greater sensitivity and versatility. Also, these devices can be fabricated from a greater variety of materials.

Further research for multi-layer chitosan hydrogel patterning will be focused on refining the etch stop layer and demonstration of a multi-layer assay. The current silicon dioxide layer, while providing adequate protection during patterning, introduces stress issues. While the wrinkling due to stress shown in Figure 3 does not affect the viability of the enzymes in the first layer, a more elastic etch stop layer would be preferable to prevent delamination. More enzyme/substrate combinations will be examined and the change in enzyme activity measured. With the multi-layer process, high density assays can increase throughput as well as enable a novel method to examine protein-protein interactions.

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