Cellulose Fibril Patterning for Real Time Studies of Cellulase Kinetics

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Abstract:

Cellulases are key enzymes in the breaking down of cellulose into fermentable sugars used for bioethanol production. Lignocellulose-based biofuels have shown to be a viable alternative to fossil fuels. However effective production of cellulosic bioethanol is dependent on understanding kinetic interactions between cellulases and cellulose. A technique for immobilizing cellulose fibrils on solid substrates using a polymer lift-off method that allows for real time imaging of cellulase-cellulose interactions has been developed.

Photolithographically patterned features on a thin polymer coating allow exposure of selected areas of the solid substrate to the cellulose fibrils. Incubation of the cellulose followed by drying, and subsequent polymer lift-off results in the immobilization of cellulose aggregates and yields specific morphologies depending on the pattern size used for the immobilization. The specific pattern attributes the various patterned features allow for imaging of immobilized cellulose mats, particles, or fibrils. Cellulose and *Thermobifida fusca* cellulases Cel6B and Cel9A can be fluorescently tagged and in combination with cellulose immobilization, can be imaged via fluorescence microscopy. This fluorescence-based system can be used to elucidate interactions between cellulose fibrils and the cellulases involved in depolymerization.

As part of this project, novel patterns containing registration and alignment marks were developed. These patterns can be used to identify specific cellulose features and image them in real time. Furthermore, the registration marks included in the novel patterns allow the tracking of specific cellulose features over prolonged time-lapsed experiments. Immobilization and identification through photolithographic patterning in combination with quantitative fluorescence microscopy techniques will result in a better understanding of cellulose-cellulase interactions. This can provide a more thorough understanding of the steps involved in the depolymerization of cellulose into fermentable sugars, which will aid the formulation of enzyme cocktails that improve the efficiency and reduce the cost of bioethanol.

Introduction:

Cellulases are instrumental in the depolymerization of cellulose fibrils into fermentable sugars for bioethanol production. Development of the most effective cocktail of enzymes for the depolymerization of cellulose fibrils into fermentable sugars is dependent upon understanding the intrinsic molecular mechanisms of cellulases. In turn, the design and creation of effective cellulase cocktails will dramatically reduce the cost of bioethanol production from lignocellulosic biomass.

Techniques that would allow for the evaluation of enzyme performance on insoluble cellulose materials ranging from single fibrils to complex cellulose mats are important for observing on-off rates and processive behaviors of cellulases. Effective imaging of these behaviors mandates high spatial and temporal imaging resolution, and in order to achieve these parameters, cellulose fibrils must be immobilized on a solid substrate.

This project developed a polymer lift-off technique that allowed for cellulose immobilization on a solid substrate by photolithographically patterning an evaporated polymer coating on a glass substrate. The immobilization of cellulose fibrils was achieved by selectively exposing the underlying glass surface and then removing the unpatterned cellulose by lifting off the polymer coating.

Experimental Procedure:

Polymer lift-off surfaces with specific pattern designs for fibril immobilzation were fabricated on 170 μm thick fused-silica wafers ranging in diameter from 15 mm to 54 mm. The wafers were initially cleaned with three successive 10 minute baths in acetone, isopropanol and deionized water, followed by drying under a nitrogen stream. Further removal of organic contaminants was performed by 10 min of oxygen plasma cleaning (60 mTorr, 50 SCCM 0_2, 150W, Oxford PlasmaLab 80+ RIE System). The back sides of the wafers were attached to adhesive tape to protect them from polymer deposition. A 1 μm thick conformal coating of di-para-xylylene (Specialty Coating Systems) was evaporated onto the wafer surfaces, after which the adhesive tape was removed.

Shipley 1827 photoresist was spun over the polymer coating on the wafers to a thickness of 3.5 μm and baked at 90°C for 1 minute. Photolithography was performed using an HTG System IIIHR Contact Aligner. The exposed photoresist was

developed in AZ300-MIF developer (Clariant Corp., AZ Electronic Materials) for 1 min, followed by a 1 min rinse in deionized water and dried under a nitrogen stream.

Exposed regions of the polymer layer were etched in a reactive oxygen ion plasma chamber (60 mTorr, 50 SCCM 0_2, 150W, Oxford PlasmaLab 80+ RIE System). Residual photoresist was removed by successive 10 minute baths in acetone and isopropanol.

Results and Conclusions:

The polymer lift-off technique was successful in immobilizing cellulose fibrils on solid substrates, and allowed for high resolution fluorescence imaging of cellulose-cellulase interactions. Different patterned features yielded varying cellulose aggregate sizes. 10 μm features in width were most effective in immobilizing single cellulose fibrils and small fibril aggregates. 10 μm diameter dots were successful in isolating single cellulose fibrils important for imaging. Features smaller than 10 μm were not consistent in immobilizing cellulose fibrils, as most material was removed in the lift-off process and only small fibrils remained in patterned features. Patterns larger than 10 μm in width yielded larger cellulose aggregates and cellulose mats. Pattern alignment schemes allowed for identification of specific pattern features and cellulose fibrils.

Acknowledgments:

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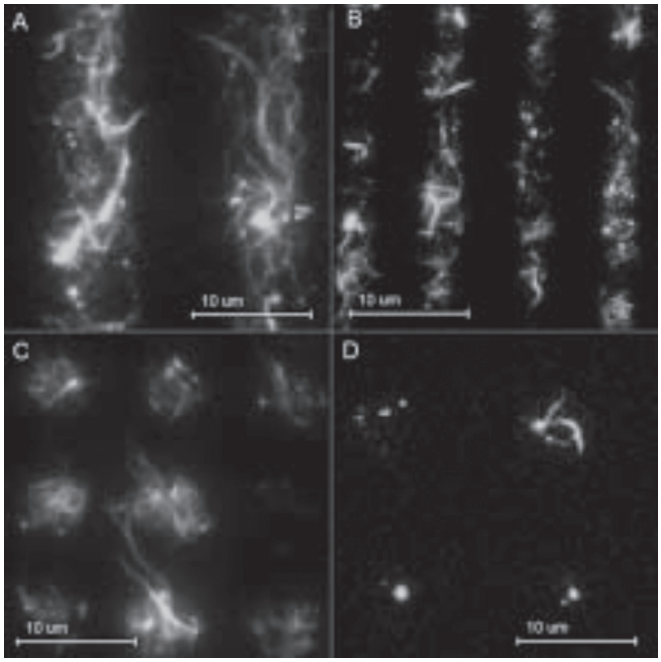

Figure 1: Imaging of immobilized cellulose particles on solid substrate. A) 10 µm wide lines immobilized cellulose agregates and celulose bundles with some single fibrils. B) 5 µm wide lines immobilized single cellulose fibrils and smaller cellulose bundles. C) 10 µm dots immobilized single cellulose fibrils and cellulose bundles. D) 5 µm diameter dots immobilized single fibrils and very few larger bundles.

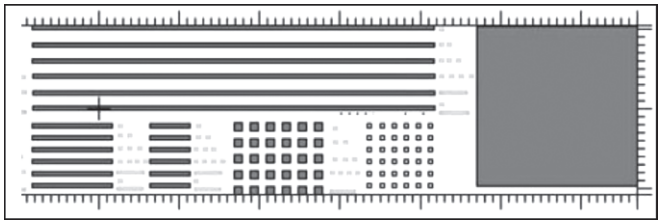

Figure 2, upper middle, right: Solid substrate pattern design with alignment scheme. Different feature sizes (ranging from 5 µm to 500 µm in width) were designed to immobilize various cellulose bundle sizes for imaging.

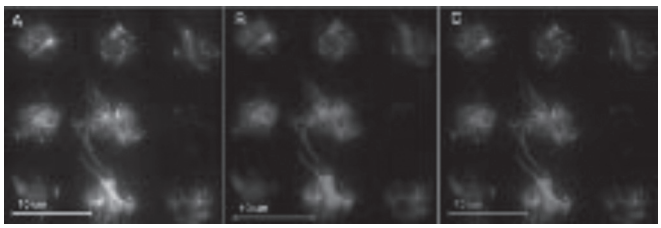

Figure 3, lower middle, right: 10 µm diameter dots incubated with Thermobifida fusca cellulases allowed for imaging of cellulose-cellulase interactions. A) Cellulose incubated with AF488-Cel6B enzyme. B) Cellulose incubated with AF 647-Cel9A enzyme. C) Combined fluorescence of both Cel6B and Cel9A.

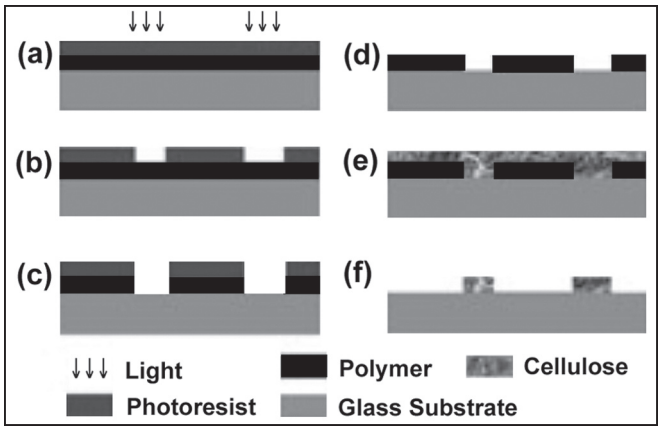

Figure 4, bottom: Surface patterning and cellulose immobilization through polymer lift-off. (a) Glass surface is coated with a thin polymer layer and photoresist is spun on and exposed though a mask. (b) Wafer is developed and exposed photoresist areas are washed away. (c) Exposed polymer is reactive ion etched with oxygen ion plasma, exposing the underlying glass surface. (d) Left over resist is removed. (e) Cellulose in aqueous solution is dried on the patterned surface. (f) Polymer is lifted-off leaving behind cellulose fibrils and fibril bundles in patterned areas.