Piezoelectric Inkjet Printing of Biomimetic Surfaces for Enzyme Encapsulation

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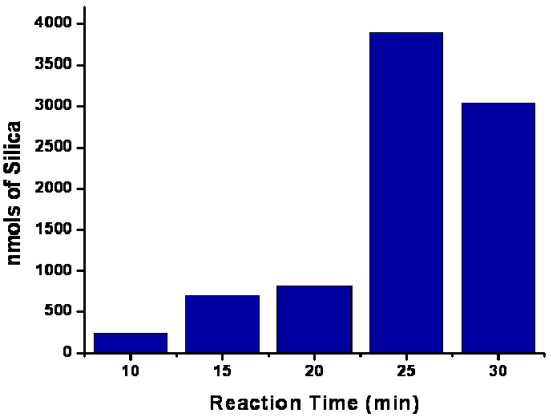
Abstract

The intricacies involved in the formation of nanostructured cell walls of marine diatoms have been a source of inspiration for a generation of developmental biologists, chemists, and material scientists. It is understood that the biomineralization of the cell wall is initiated on the surface of an internal valve known as the silica deposition vesicle (SDV), which provides a localized environment where cell wall biogenesis is completed as silica condensation is catalyzed by long-chain polyamine moieties or cationic polypeptides. A number of biomimetic analogs to the silica precipitating peptides have been developed and characterized in vitro. Although they are recognized as excellent examples of bio-inspired templates for metal oxide synthesis, these mimics have yet to successfully mimic the signature spatial and supramolecular control that is seen within the diverse structures of the diatom cell wall. For this reason, we have employed piezoelectric inkjet printing with the Dimatix Materials Printer (DMP) as an alternative, rapid prototyping method of deposition for the two-dimensional patterning of templated micro-structured silica. The flexibility associated with DMP deposition provided reproducible spot sizes and enabled tunable surface control that could serve as the basis for future functionalized surfaces.

Recent materials deposition techniques including solenoid jet printing, lithography and liftoff patterning, and direct ink write (DIW), have been used to immobilize a variety of silica precipitating precursors.[1-3] All methods provided a unique approach towards the advancement of controllable templating for patterned metal oxides; however, each was beset by their own limitations. These included large, non-uniform spots (solenoid jet printing), high temperature reaction conditions (lithography and liftoff), or inherently slow (40 µm s^{-1}) patterning conditions (DIW), rendering them unfavorable for the rapid production of functional material patterns under ambient conditions.[1, 2] For these reasons, we have employed piezoelectric inkjet printing with the Dimatix Materials Printer (DMP) as an alternative, rapid prototyping (8 m s^{-1}) method of deposition for the two-dimensional (2-D) patterning of templated micro-structured silica. The flexibility associated with DMP deposition provided reproducible spot sizes and enabled tunable surface control, specific for each reaction environment.

A composite ink was developed with a viscosity of 18.5 mPa and was printed onto gold, or sapphire substrates. The components of this ink included 8.6 wt % generation 4 (G4) PAMAM dendrimer in polyethylene glycol (PEG) (25% w/v), and phosphate buffer (100mM, pH 7.5). The PAMAM dendrimer was used as the defined silica condensing template as its primary amine moieties are effective mimics of the propyleneamine units found in silica precipitating peptides.[4] These dendrimer templates have been shown to rapidly precipitate aggregated nanospheres of silica *in vitro* at neutral pH.[5-7] To investigate the interfacial properties of the composite ink on a gold substrate, the contact angle of a 2 µL sample was determined to be 33.10°. This low contact angle (< 90°) indicates that the localized stability and confined rheological properties of the immobilized ink are loosely packed on the high energy, hydrophobic substrate.[8]

Figure 1. *Silica quantitation over10-30 minute reaction time with monosilicic acid.*



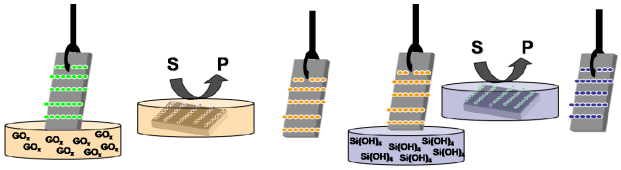
The area of printed dendrimer remained constant at 48 mm^2.

Once the customized ink was developed, it was loaded into the print cartridge, where a specific voltage is applied to each nozzle of the printhead, releasing a droplet on the order of 10 pL.[9] Because the DMP is a piezoelectric instrument, a user-controlled, pulsed voltage induces an internal stress in the form of a pressure wave inside the nozzle of the printhead (16 nozzles total, spaced 256 µm apart).[9, 10] In these studies, the maximum voltage pulse was 24 V, and the frequency of the pulses was maintained at 1.0 kHz, ensuring that the same amount of material was deposited during each print cycle. The printed spot sizes were constant and independent of the substrate at 36 ± 2 µm. Additional properties of the patterned dendrimer were characterized using UV-vis spectrophotometry and scanning electron microscopy (SEM). Varying the spot spacing, the number of print cycles, and the reaction time with monosilicic acid resulted in silicified structures reminiscent of patterned silica on the cell wall of diatoms.

Initial experiments were aimed at characterizing the silica precipitating reactivity of the dendrimer patterns (Figure 1). In these studies, samples were printed eight times with a 46 µm spot spacing on R-plane sapphire substrates. Each pattern was then reacted with a solution monosilicic acid (113 mM in phosphate buffer, pH 7.5) at various time points. The multiple print cycles over larger areas were necessary to produce highly reactive multilayer films, increasing the amount of accessible surface amines. As the reaction time increased, the corresponding amount of precipitated silica increased until 25 min of reaction time (Figure 1). Silica was quantitated using a modified *β*-silicomolybdate assay against a previously determined silicate standard curve.[5, 11, 12] With the aread of printed dendrimer fixed at 48 mm^2, the amount of detectable silica ranged from 242 nmole silica under 10 min of reaction time to 3897 nmole silica under 25 min of reaction time (Figure 1). After 25 min of reaction time, the amount of silica decreased to 3038 nmole silica, inferring that the maximum reaction potential has been reached.

To investigate this reaction behavior, the nanoscale reactivity of a printed spot of dendrimer (printed four times, 96 µm spacing) was investigated using electron microscopy as a function of the temporal development of silica. Unlike its *in vitro* counterpart that occurs within seconds,[5] the *in vivo* reaction between the patterned dendrimer and monosilicic acid did not form spontaneous nanospheres, suggesting that the 2D activity of the printed dendrimer was retarded by its heterogeneous composition. Similar to its *in vitro* counterpart, however, the *in vivo* dendrimer mediated silica condensation reaction suggested three distinct phases over defined time-points: nucleation, which initiated a phase change on the printed spot (1-3 min); nanospherical precipitation, which was seen only after the onset of contact pin line effect (5-20 min); and aggregation of the nanoparticles around the exterior of the spot (25-30 min). It is believed that this final phase change begins the pattern inhibition; as evidenced by the decrease in observed silica. Furthermore, examinations of time points beyond 30 min were not possible due to non-specific gelation of the aqueous monosilicic acid under these reaction conditions.

Figure 2. *A schematic representation for glucose oxidase encapsulation within the printed dendrimer spots. After the enzyme is encapsulated, the composite patterns are reacted with monosilicic acid for 20 min. Subsequent patterns were then washed extensively before enzyme activity was tested.*



The physical interpretation of the evolving dendrimer patterns has offered a unique perspective for the development of biologically relevant, reactive mesoporous materials. In previous studies using standard primary amine concentrations (20 mM), the PAMAM dendrimer has served as a controllable template for the encapsulation of glucose oxidase under ambient reaction conditions.[13] Once encapsulated within the dendrimer-silica matrix, the enzyme was shown to remain stable over time, suggesting an electrostatic stabilizing effect of the dendrimer template. The immobilization of free enzyme has potential applications in the next generation of biosensing devices;[14] for this reason, a series of preliminary reactions were conducted using the dendrimer patterns. Here, the number of print cycles and the amount of glucose oxidase encapsulated were optimized, as their subsequent reactivity was tested.

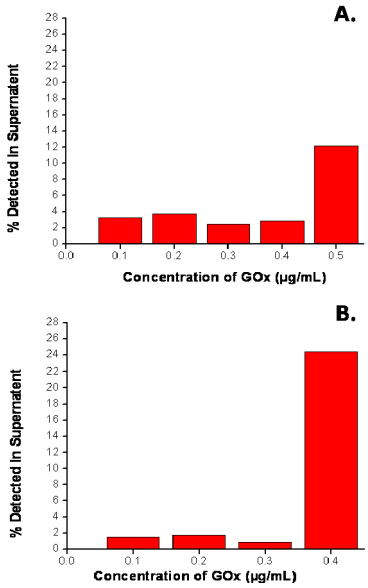


Figure 3. *Percent of GO_X lost as quantified using the Bradford Assay against a previously determined standard curve. The enzyme encapsulated patterns were reacted with monosilicic acid (20 min) and washed repeated with buffer prior to quantification. In these studies, the number of print cycles varied from **A.** 8x printing to **B.** 2x printing into a 96 well plate. Controls of wells that had no printed dendrimer exhibited zero enzyme loss.*

The controlled deposition of functional materials such as proteins, cells, polymers, and antibodies required for cell-based biosensors,[15-19] bone tissue engineering supports,[20] or antimicrobial devices[21] has recently gained attention as the demand for new hybrid biological devices has increased.[9, 22, 23] Previously, instruments, such as chemical vapor deposition (CVD) and stereolithography apparatus (STL), have successfully reproduced the nanometer scale deposition of semiconducting materials required for the rapid prototyping in industrial device manufacturing; however, their high temperature reaction environments and time consuming protocols are unsuitable for functional material depostion.[24] For this reason, we have encorporated piezoelectric inkjet printed of the biomimetic, dendrimer ink to control the encapsulation of varying concentrations of glucose oxidase.

First, the composite dendrimer ink was printed into individual wells of a standard 96 well plate (printed 2-8x, in the 36 mm^2 area of the reaction well). Then, a 10 mg/mL stock solution was prepared by dissolving glucose oxidase in 0.25 mM phosphate buffer at pH 7. After appropriate dilutions, a 200 µL aliquot of glucose oxidase was added to the reactive wells and incubated for 10 minutes to allow interactions between the enzyme and dendrimer. Next, 20 µL of HCl hydrolyzed 1 M tetramethylorthosilicate (TMOS) was added to the reaction mixture and incubated for 15 minutes to allow for complete formation of the plated silica. The precipitated particles were centrifuged at 3000 RPM and washed three-fold with 0.1M potassium phosphate buffer at pH 6 (Figure 2).

The amount of glucose oxidase not encapsulated was quantitated by the Bradford assay from a standard curve of known concentrations of glucose oxidase. Briefly, 160 µL of enzyme test solution was added to 40 µL of BioRad Protein Assay solution concentrate, mixed for 5 minutes, and observed by UV-Vis at 595 nm. The working enzyme concentration range for this study was 0.8-80 µg/mL.

Activity of the encapsulated enzyme was determined by the glucose oxidase activity assay (Worthington Biochemical Corporation) with slight modifications. First, an *O*-dianisidine dye reagent was prepared by adding 100uL of 1% *O*-dianisidine solution to 12 mL of 0.1M potassium buffer at pH 6. From this dye reagent, 192 µL was added to 25 µL of 18% β-D-glucose (concentrations of glucose reflect consideration of mutarotation in solution) and 8.35 µL of a 0.2 mg/mL solution of horseradish peroxidase (HRP). Next, the resulting mixture was added to the GOX-containing wells and the change in color was observed for 5 min at 460 nm.

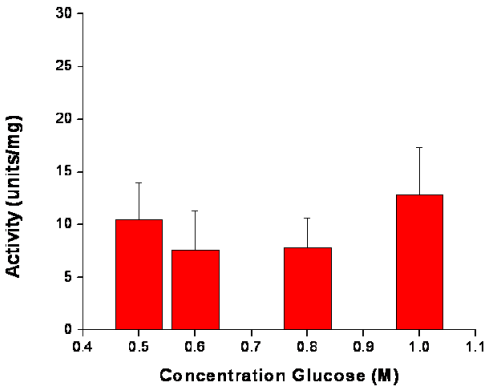


Figure 4. *The quantifiable activity of GO_X at varying substrate concentrations. Controls of wells without printed dendrimer exhibited zero activity.*

In these optimization studies, the maximum concentration of GO_X that would still result in near 100% encapsulation was tested using wells that contained patterned dendrimer (printed 8x at 46µm spot spacing). It was determined that 0.4 mg/mL was the optimal concentration for complete encapsulation (Figure 3). At 0.5 mg/mL, ~12% of the enzyme was observed in the supernatant and subsequent washes after encapsulation, suggesting that ~80-100 µg is the maximum loading concentration. To confirm this, a solution of 2 mg/mL GO_X was encapsulated and showed a 73.4% loss of enzyme, which resulted in 106 µg being encapsulated. It should be noted, however, that at this high printing concentration (8x printed), it was difficult to observe the color change of the oxidized *0*-dianisidine (Figure 3a). As a result, a less concentrated printing concentration was used (2x printed, Figure 3b). At this concentration, it was determined that the maximum loading capacity was 0.3 mg/mL, which was still higher than expected, since the 8x printing had an optimal loading concentration of 0.4 mg/mL. This is most likely the result of electrostatic repulsions between the enzyme molecules that reduces their electrostatic attraction to the dendrimer.

The activity of the printed, encapsulated enzyme was studied against free enzyme in solution. It was determined that the free enzyme showed optimal activity at 6 µg/mL, varying glucose concentrations from ~5-30 mM (Figure 4). Consequently, GO_X was encapsulated at 6 µg/mL and activity was first tested using a much higher glucose concentration (0.5-1.0 M) with the assumption that the encapsulation and plating would significantly decrease the activity of the enzyme. At these concentrations, it was determined that the activity of the enzyme is 15 units/mg (Figure 4). Compared to the free enzyme, the printed dendrimer exhibited roughly 10-fold slower activity. Future work will focus on lowering the substrate concentration until Michaelis-Menten and Lineweaver-Burk kinetics can be obtained and compared to the free enzyme. However, these preliminary results have indicated promising leads in developing controllable surfaces.

The number of reported biological mimics is ever growing; however, controlling the reproducibility of functional mimics and ensuring that their activity is unchanged over time under ambient conditions has proven to be one of the most challenging tasks for successful, functional mimics. Utilizing piezoelectric inkjet printing as a non-contact rapid prototyping method for templating the microscale patterning of dendrimer templates, has offered a unique way of utilizing nature as inspiration in developing highly reactive patterns . By controlling the length of reaction time with silicic acid, the spot spacing, and the number of print cycles, this highly reactive 2D could serve as the basis for future functionalized surfaces.

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Leila Deravi received her BS in chemistry from the University of Alabama (2005) and is beginning her forth year as a graduate student in the Department of Chemistry PhD program at Vanderbilt University. She is working under Dr. David Wright, and her research focus is applied biomaterials chemistry. Here, she develops functional biomaterial inks for deposition using the Fujifilm Dimatix materials inkjet printer. Once printed on a surface, the subsequent properties of the patterns are characterized for materials applications.