PRINTING BIOLOGICAL LIQUID ON HYDROPHOBIC 3D ELECTRODES

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ABSTRACT

This paper presents for the first time a programmable and scalable 3D electro-bioprinting (3D-EBP) process for patterning bionanoreceptors, cysteine-modified *Tobacco mosaic virus* (TMV1cys), on high-density micropillar array electrodes. The structural hydrophobicity in high aspect ratio geometries of micro/nano devices poses a critical challenge for assembling 3D biomaterial-device interfaces. Here, we have successfully integrated electrowetting principles with a modified state-of-the-art bioprinter for automated, high-throughput, and large-scale patterning of TMV1cys particles on hydrophobic 3D electrodes. The 3D-EBP processed bionanoreceptors maintained both structural and chemical functions as characterized via SEM and fluorescence microscopy. Overall, the innovative 3D biomanufacturing process creates excellent opportunities for advancing on-demand bio-integrated devices including multiplexed biosensors and bioenergy harvesting devices.

INTRODUCTION

The convergence between biochemistry and micro/nano manufacturing technologies has brought revolutionary advancements in the development of miniaturized devices and biomaterial integrated systems [1], [2]. Technology for bio-device integration has rapidly evolved over the past two decades based on biochemistry principles, processing methodologies learned from the micro/nano fabrication industry, and instrumental science/engineering. The diversity of biofabrication technologies have led to greater understanding of biomolecular activities through on-chip characterizations, and is enabling rapid advancement of high-density, multiplexed bioarrays necessary for biosensing and diagnostic applications.

Building upon the variety of methods, many researchers have demonstrated integration of biological materials with three-dimensional transducers in an effort to achieve enhanced bioelectronics performance at a miniaturized system scale [3], [4]. Three-dimensional structures at micro- and nano- scales offer a number of beneficial properties compared to their two-dimensional counterparts, allowing for an extensive range of applications including micro energy storage/harvesting device [5], micro/nano actuators [6], micro thermal management devices [7], water-repellent surfaces [8], optical modulators [9], 3D VLSIs [10], etc. The primary attractive feature of these approaches is the high density of physical interfaces between system components and materials within the restricted surface area budget, which can directly translate into large enhancements in performance. Moreover, precise control over the arrangement of the structures through microfabrication or emerging 3D printing technologies further highlights the utility of three-dimensional components with increased surface-to-volume ratio, functional uniformity and tunability.

One of the interesting characteristics present in small scale 3D structures is their limited wetting property known as structural hydrophobicity [11]–[13]. When micro- or nano- scale structural components are densely arranged, the wetting of 3D cavities with liquids is limited by surface tension at the solid-liquid, liquid-air,

and solid-air interfaces. Considering most biological materials are stored in buffered aqueous solutions due to the narrow biological stability windows, the structural hydrophobicity can be a significant limiting factor when attempting to introduce biological materials into the 3D cavities for device functionalization. In our recent report, we have provided experimental evidence to highlight the potential limitation using cysteine-modified *Tobacco mosaic virus* (TMV1cys) and Au-coated Si micropillar array (μ PA) electrodes. In addition, the electrowetting principle - which controls surface wettability with applied electric potential - has been introduced as an enabling technique for uniform and localized immobilization of the bionanoreceptors on densely-arranged μ PA electrodes [14], [15].

Here, we have successfully integrated the electrowetting-assisted biofabrication technique with a customized bioprinter for automated, high-throughput, and large-scale printing of the bionanoreceptors on 3D electrodes. The simple system components, optimized printing process parameters including extrusion pressure/time and printing distance, combined with the electrowetting process determined from our previous work, allows a novel 3D bioprinting technique with excellent repeatability and consistency, ultimately offering a unique function to existing bioprinting systems to bring an on-demand programmable, scalable, and readily adaptable 3D biofabrication technology.

MATERIALS AND METHODS

Hydrophobic 3D Electrodes and Biological Ink

Figure 1: Au-coated μ PA electrodes and their wettability. (a) Fabrication process and dimensions for the μ PA electrodes. A comparison of TMV1cys solution droplet (10 μ L) contact angles on (b) planar Au and (c) Au-coated μ PA (SAR=10). (d) A plot of TMV1cys solution droplet contact angles on Au-coated μ PA at different SARs. The relationship between the contact angle/hydrophobicity and SARs follows the trend anticipated by Cassie-Baxter equation.

The Si micropillar array (μ PA) electrodes have been fabricated through a standard microfabrication process as described in Figure 1a. A Si wafer is etched down via DRIE using a photolithographically patterned negative photoresist (NR9-1500PY, Futurrex) as the etch mask. The resulting Si μ PAs are passivated by 500 nm PECVD SiO₂ followed by sputtering of Cr (30 nm)/Au (200 nm). The pillar aspect ratio was held constant at 10 (height: 70 μ m, diameter: 7 μ m) while the spacing aspect ratio (SAR) varied from 3.3 to 10 to investigate the effect of the structural hydrophobicity level on the final pattern size/resolution.

The biomaterial used in this work is genetically-modified TMV expressing cysteine residues on its outer surface (TMV1cys, the details of genetic modification and purification protocols can be found in previous report [16]). A TMV1cys solution of 0.2 mg/ml concentration in 0.1 M, pH 7 phosphate buffer (PB) solution is used as the bio-ink for all experiments. The enhanced metal binding properties of TMV1cys, such as self-assembly onto Au surfaces and surface metallization with Ni, provide effective means for characterization of functionalization morphology after the electrowetting assisted bioprinting process.

As an initial step, the structurally hydrophobic properties of the μ PA electrodes were characterized via contact angle measurements of 10 μ L sessile drops of the TMV1cys solution on Au-coated planar (Figure 1b) and μ PA (Figure 1c) electrodes. While the planar Au surface exhibits a hydrophilic nature with a 65° contact angle, the μ PA surface yields more hydrophobic characteristics as indicated by the 132° contact angle (SAR=10). This is close to the theoretically expected value (136°), calculated based on the *Cassie-Baxter* equation (Eq. 1), where θ^* is the apparent contact angle on the μ PAs, ϕ_s is the fraction of the solid in contact with the TMV1cys solution, and θ_0 is the contact angle on the planar Au substrate [12].

$$\cos \theta^* = -1 + \phi_s(1 + \cos \theta_0) \quad (1)$$

Figure 1d plots both theoretically expected (derived from *Cassie-Baxter* equation (Eq. 1) with $\theta_0=65^\circ$, and ϕ_s calculated from the pillar geometry) and experimentally acquired contact angles (θ^*) indicating that the hydrophobicity of the μ PAs decreases with an increase in SAR. The disparity between the two can be attributed to the gentle pressure applied when loading the droplet onto the μ PAs via syringe tips and hydrophilic surface (Au) inducing liquid pinning onto the pillar tips [14]. Such wetting state is only valid when there is no external disruption force due to the hydrophilicity of the Au surface. Once the *Cassie-Baxter* state is disrupted with an external force (e.g. mass loading), a faster transition of the wetting state - indicated by the droplet spreading into the cavities (transitioning to the *Wenzel* state) - is observed with the lower SAR μ PAs (data not shown). It becomes a significant challenge to introduce the solution into narrower cavities (SAR ≥ 7) that do not easily transition to a *Wenzel* state, which can be attributed to less pressure loading per spacing segment. In other words, the wider spacing reduces ϕ_s which increases the fraction of the liquid droplet that is supported by the micropillars [11]. The TMV1cys droplets on such high SAR μ PAs are readily movable with no traceable liquids remaining on the electrode surface.

Integration of 3D-EBP with a Bioprinter

The 3D electro-bioprinting (3D-EBP) technique has been integrated with a state-of-the-art commercial 3D bioprinter (3D Bioplotter, Envision TEC) by simply connecting a function generator (Agilent 33220A) to the printing nozzle and the substrate as described in Figure 2a. The built-in graphical user interface allowed a highly automated and programmable 3D-EBP with precise control over nozzle pressure, printing distance, array

size/density, and solution temperature. As the 3D Bioplotter is mainly designed for printing materials carried in hydrogels (e.g. gelatin, alginate, corn starch, etc.), the controllable pressure and time ranges for ink extrusion at the nozzle are optimal for higher viscosity fluids compared to the buffer solution used in this work. Through multiple iterations at the lowest extrusion time and pressure ranges, the optimal process parameters have been determined as 0.3 second extrusion at 0.1 bar with a distance from the nozzle to the substrate set at 100 μ m. It should be noted here that the optimization of the process parameters strongly depends on the nozzle size and the viscosity of the ink solution.

Figure 2: (a) Overview of the programmable 3D-bioprinting system integrating 3D-EBP with a commercial bioprinter. (b) Illustration of TMV solution impinging onto the μ PA from teflon-modified needles. The solution impinging onto the μ PA surface without electrowetting (left) keeps a spherical shape without further access/spreading into the microcavities (*Cassie-Baxter* state), while the droplet impinging with electrowetting (right) selectively wets the underlying cavities (*Wenzel* state).

In this initial demonstration, a 32-gauge stainless steel needle (inner diameter: 100 μ m, outer diameter: 260 μ m) was utilized with a hydrophobic modification at the tip via dip-coating with Teflon; the tip was dipped into Teflon solution (TeflonTM AF 400S1) and cured at 180°C for 3mins. A syringe was connected during the curing step to continuously flow air through the tip to prevent blockage during the curing process. The hydrophobic coating at the tip was critical to isolate the droplet forming towards the substrate during extrusion (Figure 2b); otherwise the extruded droplets would roll-up to the outer metallic surface causing inconsistency in droplet size and printing failures.

The electrowetting voltage was continuously applied during the printing process by seamlessly integrating the function generator to the metallic -nozzle and -printing bed without hindering the printing operation. Based on our previous work, a sinusoidal voltage signal of 1.2 V_{rms} at 10 kHz was applied to induce uniform and localized wetting of the TMV1cys ink into the μ PAs [14]. All processes were conducted at room temperature with ambient humidity. Both the print-bed and the bio-ink cartridges were kept at 10°C for extended stability of TMV1cys particles throughout the multiple printing processes. The bio-printed electrodes were incubated overnight in a humid chamber for self-assembly of TMV1cys particles onto the electrode surface through thiol-Au binding [15].

RESULTS AND DISCUSSIONS

Bio-ink Printing on 3D μ PA Electrodes

Figure 3 compares the printing processes on μ PA electrodes (SAR: 10) under two different conditions emphasizing electrowetting as an enabling method. When the printing process is conducted without applying the electrowetting voltage (Figure 3a), the extruded droplets at the printing nozzle do not settle onto the μ PA surface, and collectively accumulate at the nozzle until falling onto the surface due to gravity (the droplets loaded onto the μ PA surface does not penetrate into the cavity). However, with the application of the electrowetting technique (Figure 3b), the 3D-EBP allowed consistent dispensing of ~ 1 μ L of the bio-ink on the μ PA electrodes throughout the programmed array patterns by successfully introducing the bio-ink into the designated micro-cavities. The spacing between the droplets has been set to 1 mm to prevent adjacent droplet from merging. Particularly for creating discrete patterns, the liquid merging between the neighboring droplets becomes problematic for lower SAR μ PA electrodes as they are more susceptible to lateral spreading of liquid during the electrowetting process (further discussed in the next section). However, the merging event can be utilized for creating continuous patterns on the 3D substrate.

Figure 3: Optical images comparing the TMV1cys solution droplet printed μ PA electrodes (SAR=10). (a) Hydrophobic nature of the μ PA electrodes repels the droplets being loaded onto the surface resulting in failure in printing/patterning the droplets. (b) Continuous application of electrowetting voltage during the printing process allows successfully patterning of uniform droplet volumes on the μ PA electrodes.

Functionalization Morphology and Pattern Sizes

In order to characterize the TMV1cys printed on the μ PA electrodes using SEM, an electroless Ni coating was performed after the overnight incubation step. As shown in Figure 4a, all μ PAs featuring different SARs/densities were successfully coated with the TMV1cys at the printed spots. The top-down SEM images taken at the droplet boundaries confirm the localized patterning of the TMV1cys within the 3D substrate with more surface functionalized

particles identified on the printed side (left from the light-blue line) compared to the non-printed area (right from the light-blue line). However, it should be noted that the TMV1cys density on the μ PA electrodes with higher SAR is noticeably less compared to that observed from the lower SAR electrodes. This can be attributed to limited TMV1cys diffusion from top to bottom of the electrodes for dense microstructures. Comparing with the μ PAs without electrowetting (Figure 4b), the nanoparticles assembled on the lower bottom of the pillar surfaces in Figure 4a (SAR: 10) confirm that the ink solution has successfully reached the bottom surface with electrowetting, indicating that the use of higher concentration ink can help increase the biofunctionalization density on high SAR μ PA electrodes.

Figure 4: Process evaluation using SEM. (a, cross-section view) TMV1cys particle coating on all sidewalls of the μ PAs has been achieved (observed as surface textures). (a, top-down view) Also, clear functionalization boundary has been observed at the wetting edges (blue-dotted line) of each of the μ PAs highlighting the pattern fidelity enabled by structural hydrophobicity. (b) The clean surface of the μ PAs processed without electrowetting further emphasizes the electrowetting as an enabling technique for bio-printing. (c) The pattern sizes ranged from 0.89mm² to 0.37mm² (N=5) following the hydrophobicity levels of the μ PAs. (scale bars: 10 μ m).

The dependence of the pattern/spot sizes on the SARs/pillar densities have been characterized by estimating semi-circular pattern boundaries observed from top-down SEM images. Figure 4c compares the pattern sizes measured from the μ PA electrodes with different SARs at the respective contact angles - representative of their hydrophobicity levels. The lower SAR μ PA electrodes resulted in a larger pattern size due to the facile spreading of the bio-ink into the microcavities with the electrowetting (under the identical process settings, which is in a good agreement with our previous investigation). The statistical analysis using five different locations of a printed array show a high consistency in pattern size per SARs, emphasizing the excellent uniformity and repeatability of the 3D-EBP process.

Scalable Biofunctionalization Density

Biochemical activity of the printed TMV-1cys particles is evaluated using a sulfhydryl (-SH on cysteine) specific fluorescent labeling reagent, Fluorescein-5-Maleimide (Thermo Fisher Scientific). As our previous report confirmed that the electrowetted TMV1cys on μ PAs enhances bionanoreceptor density by surface area enhancement (SAE) factors anticipated from the 3D electrode geometries [14], this work has focused on achieving scalable nanoreceptor density per different SARs of the μ PAs. Figure 5a

compares top-down fluorescent microscopy images taken from the printed spots on different electrode geometries. Under an identical exposure time, TMV1cys printed on μ PA electrodes resulted in a significantly higher fluorescence compared to the planar electrodes with an excellent patterning fidelity (Figure 5a, bottom-right), due to the underlying microstructure. However, there is a noticeable discrepancy between the change in increment factor of the fluorescence intensity and the SAE factor calculated based on the μ PA geometry (Figure 5b). This is attributed to the lower density of TMV1cys on the high SAR μ PAs as supported by the SEM characterizations in Figure 4a, indicating that control over concentration of bio-ink may need to be optimized to obtain highly controllable functional scalability of the resulting nano/micro/bio-integrated components.

Figure 5: Characterization of fluorescence intensity from (a) TMV1cys functionalized on planar Au and μ PA electrodes displaying SARs ranging from 3.3 to 10 (scale bars: 100 μ m). (b) A significant increase in functionalization density is achieved with the μ PAs as reflected in the increase in fluorescence intensity ($N=5$).

CONCLUSIONS

This work offers a unique function to existing bioprinting systems bringing a simple, innovative, and technology enabling 3D biofabrication through integration of the core manufacturing technique based on the electrowetting principle. The presented system is fully automated, scalable, and readily adaptable for a wide range of applications. While the presented demonstration utilizes a particular biomaterial of interest to our work (TMV1cys), this manufacturing technology can be applicable for integrating other micro/nanomaterials (DNA, proteins, cells, carbon nanotubes, graphene, etc.), stored in aqueous media, onto conductive hydrophobic electrodes. Possible applications include areas developing energy storage/harvesting devices, biochemical sensors, optical metamaterials, heat management devices, superhydrophobic surfaces, and more. Overall, the innovative 3D biomanufacturing process generates excellent opportunities for advancing on-demand bio-integrated devices opening up unprecedented possibilities in micro/nano/bio integrated fabrication technologies and platforms.

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