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A humidity-sensitive hydrogel-*Bacillus* spore composite for micropatterning of biomolecular gradients

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A composite material consisting of *Bacillus subtilis* spores suspended in a humidity sensitive hydrogel can be used to pattern biomolecules in different concentrations directly onto glass surfaces using a mechanical micromanipulator. By altering the relative humidity surrounding the composite gel during deposition, surface concentration of patterned biomolecules can be controlled and varied to create user-defined, biomolecular surface concentrations. © *2013 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4817971]

Biological gradients are an integral facet of life, influencing cellular phenomena such as chemotaxis,[1] directional cell growth,[2] and differentiation.[3,4] Patterning of artificial gradients has been used in diverse applications ranging from biosensing[5] to cell culture systems.[6] Several methods have been employed to create such gradients including diffusion-based microfluidics,[7] laser desorption,[8] and photochemistry.[6,9] However, such methodologies often require complex equipment and harsh treatments which prevent the widespread use of gradient patterning.[10] Here we present a patterning strategy based upon a composite material formed by suspending *Bacillus subtilis* spores within a humidity sensitive hydrogel (Figure 1(a)). Previous studies have demonstrated the ability of *B. subtilis* to swell and absorb water from humid environments in addition to acting as microscale inkwells when loaded with fluorescent dyes.[11–13] By saturating spores with a fluorescent dye and subjecting the composite to various humidities, we are able to control the concentration of dye released from the hydrogel surface. We show that the spores can be used to engineer the dye concentration within the hydrogel, and hence can be exploited as an additional parameter to control the dye release process (Figure 1(b)).

The surface coat of a spore is a porous material with a slightly negative charge.[14,15] This negative charge is believed to facilitate the absorption and retention of positively charged dyes such as avidin-fluorescein isothiocyanate (avidin-FITC) through electrostatic interactions.[16] This interaction allows the spores to retain higher concentrations of dye relative to the surrounding environment and also has been shown to protect the dye from photobleaching.[11] However, the direct use of spores to pattern on surfaces proves difficult due to the brittle nature of spore aggregates and the small size of spores (approximately 1 μm).[17] In order to circumvent this problem, we suspended spores in a stimuli-responsive hydrogel[18] to improve the structural integrity of the spores while maintaining access to environmental humidity. In addition, the large size of the hydrogel construct (50–100 μm) facilitated manual manipulation using microtweezers,[19] see Figure S1 in the supplementary material,[20] thus allowing easy, user-controlled patterning using the hydrogel composite.

Hydrogel spore composites were prepared by drying either 0.1 ml or 0.2 ml of *B. subtilis* spores, 10^7 CFU (Mesa Labs) and suspending the resultant spore powder into 0.9 ml of 25.9 mg acrylamide (Sigma-Aldrich), 75.2 μl methacrylic acid (Sigma-Aldrich, distilled to remove inhibitor), 12.2 mg *N,N′*-methylenebisacrylamide (Polysciences Inc.), and 75 μl *N,N,N′,N′*-tetramethylethylenediamine (Sigma-Aldrich) dissolved in deionized (DI) water. Gelation was induced by adding the solution into a 0.1 ml aqueous solution of 0.35 M ammonium persulfate and subsequently vortexing to ensure uniformity in spore distribution.[18] A negative charge control was made by pipetting 0.5 μl poly(lactic-co-glycolic acid) (PLGA) microspheres (1% aqueous suspension of 1 μm microspheres, Phosphorex Inc.) into the solution prior to gelation in order to mimic the charge and size of spores within the gel.

We verified the binding and retention of avidin-FITC by plating the hydrogel composites on a glass slide, saturating the hydrogel with avidin-FITC (Sigma-Aldrich), and triple rinsing with running deionized water for 5 min. Figure 2 shows that the hydrogel-spore composite retains dye near the gel surface while a hydrogel-only control shows no significant retention of dye. When the hydrogel composite was examined under bright field microscopy, spores were seen at very high concentrations near the surface of the gel. The concentration of spores decayed quickly from the surface to the hydrogel center (see Figure S2 in the supplementary material).[20] Since the hydrogel in this control experiment was synthesized directly on the surface of the glass slide, it is believed that the spores were pushed towards the hydrogel surface by convective forces and were subsequently set in place when gelation occurred. The high concentration of spores on the outer edge of the hydrogel composite explains why fluorescence was seen only in the vicinity of the surface after rinsing. The water removed all dye from the hydrogel, except the dye protected by the spore coat. Another control was prepared by saturating

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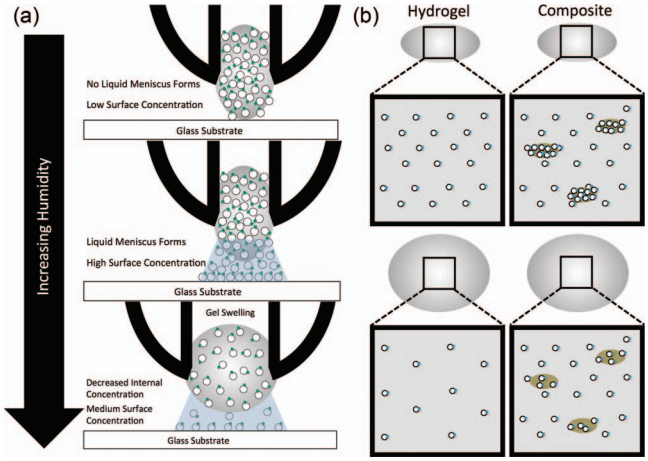

FIG. 1. (a) Schematic illustrating the patterning procedure used to create user-defined surface concentrations. At low humidity levels, a meniscus does not form at the gel-substrate interface leading to low levels of dye transfer. At mid-ranged humidities, a liquid meniscus forms allowing transfer of the dye to the surface at high concentrations. As the humidity increases further, the gel absorbs the water from the humidified air, diluting the internal concentration of dye, and thus decreasing the patterned surface concentration. (b) Schematic illustrating the ability of negatively charged spores to bind positively charged dye, thus increasing the internal concentration of dye compared to the hydrogel control.

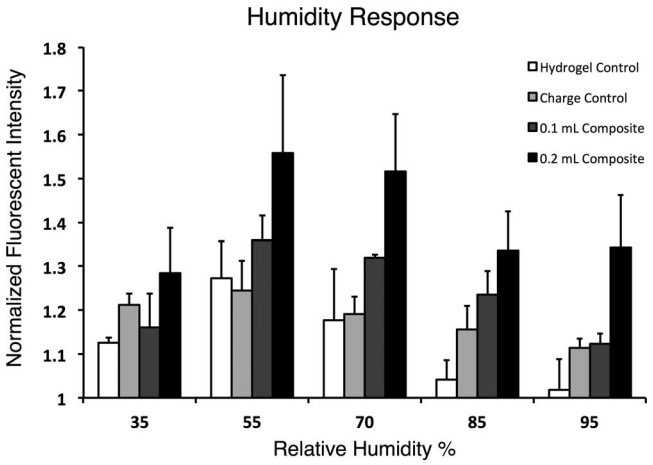

FIG. 3. Summary of the humidity response experiment. Error bars represent standard deviation of 3 experiments. Note the large increase in intensity as the hydrogel is hydrated by increased relative humidity followed by a gradual decline as the internalized dye is diluted.

the hydrogel composite with a 1 M magnesium chloride solution in order to mask the negative surface charge of the spores. After the rinse, the magnesium control showed essentially no retention of dye, suggesting that the spore surface charge aids in the retention of dye.

We next characterized the humidity response of hydrogels with different concentrations of spores. We also performed control experiments with hydrogels devoid of spores (hydrogel control) and those that had negatively charged PLGA microspheres instead of spores (charge control). A humidifier and a hygrometer were used to produce and measure the humidity in a chamber. Briefly, samples were saturated with avidin-FITC and allowed to dry. Small aliquots of each gel (approximately 50 μm in diameter) were grasped using a mechanical micromanipulator. The gel was placed in the humidity chamber for 2 min prior to patterning to acclimatize to the environment. However, the glass slide remained outside the chamber until immediately prior to patterning in order to avoid condensation on the surface. The gel was lowered until a meniscus formed on the glass slide, patterning a small circular spot on the surface. The gel sample was re-soaked fully for each humidity point to eliminate the effects of dye depletion from this experiment. The slide fluorescence profiles were immediately imaged to minimize photobleaching and were imported into a MATLAB algorithm for analysis. The intensity of each image was normalized to that of the background intensity of the glass slide and was averaged over the patterned area. This procedure was repeated for five different relative humidities (Figure 3) and was run in triplicate for each humidity point.

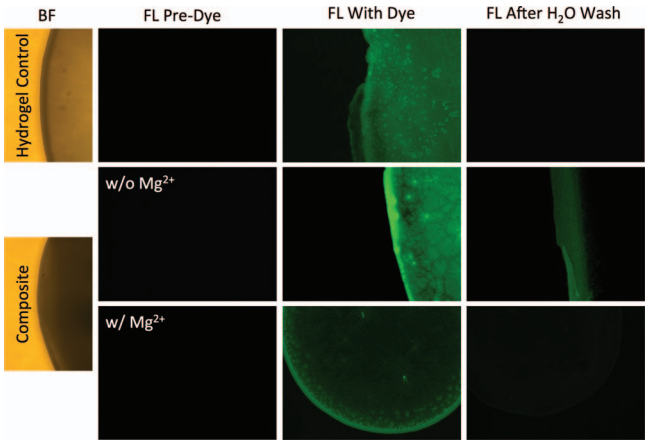

FIG. 2. Bright field and fluorescent micrographs of hydrogel (top row) and spore composites (bottom two rows) before the addition of avidin-FITC (left column), after the addition (middle column), and after 1 min wash with water (right column) demonstrating the binding of dye is at least partially charge dependent. The gel constructs can be visualized as the dark area on the right-hand side of the BF images (see also Figure S2 in the supplementary material[20]).

All experiments showed a rapid increase in fluorescent intensity followed by a gradual decline as the relative humidity was increased from 35% to 95% (Figure 3). The 0.2 ml spore composite showed the greatest increase in fluorescent intensity with 32% increase over that of the hydrogel control. Using unpaired, 2-tailed, heteroscedastic t-tests ($P < 0.1$), significant difference was seen between the composites and various controls. The 0.1 ml composite was statistically different from the charge control at 55% and 70% relative humidity and from the hydrogel control at 85% relative humidity. The 0.2 ml composite was statistically different from both controls for all relative humidity values except 35%. At 35% relative humidity, the dry gels did not have enough moisture to form a meniscus to transfer dye to the surface, leading to low initial fluorescent intensity. However, at higher humidities, the hydrogel began to absorb ambient humidity leading the formation of a meniscus and subsequent deposition of the fluorescent dye. As the humidity continued to increase, the gel significantly swelled with water, increasing in size (see Figure S3 in the supplementary material)[20] and slowly

diluting the concentration of dye within the gel, hence resulting in a decreased deposition of the fluorescent dye.

The level of fluorescent intensity deposited on the surface using the composite gels is directly attributed to the ability of the composite gel to bind higher concentrations of dye compared to the hydrogel and the charge control gel, thus creating a steeper gradient as the humidity is varied. The binding and retention experiment showed that charge plays a role in the ability of spores to bind higher levels of dye than hydrogel alone; however, the statistical difference from the charge control of the humidity response experiment suggests that the mechanism may involve more than simple electrostatic interaction. Although the spores and PLGA microspheres were of similar size, charge, and were placed into the gel in similar numbers, the porous nature of the spore coat[15] could have provided more negatively charged surface area to bind the positively charged avidin-FITC than the non-porous PLGA microspheres. As the concentration of spores within the composite is increased, the total charged surface area available for interaction increases as well. Thus, as expected, there is a positive correlation between the peak concentration of patterned dye and the concentration of spores present in the composite gel.

Additionally, the amount of negatively charged surface area within the gel affects the rate at which surface concentration decreases as humidity increases. In the hydrogel control, as the humidity increases, the internal concentration decreases due to the increased volume of water. In the composites, as the humidity increases, the internal concentration decreases as well. However, the dye bound to the charged surface has an increased diffusive flux due to the decreased concentration within the surrounding gel. This increases the diffusion from the spore to the surrounding gel, which attenuates the concentration lowering effects of increased humidity (see Figure S4 in the supplementary material[20]).

In conclusion, we have designed a *Bacillus subtilis* spore hydrogel composite that is capable of patterning positively charged biomolecules in a humidity dependent manner. By varying the surrounding humidity during patterning, user-defined gradients can be patterned. The peak intensity can be varied by changing the concentration of spores within the hydrogel composite allowing for humidity sensitivity optimization of the gel for various applications. Future studies accounting for long-term patterning hysteresis and dye depletion, along with standardization of kinetic swelling characteristics of hydrogels and spores, should allow further understanding and optimization of the hydrogel-spore composite for various applications. This patterning method shows great potential for use in *in situ*, customized biomolecular patterning without the need of complex equipment or harsh surface treatments.

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[20]See supplementary material at http://dx.doi.org/10.1063/1.4817971 for additional figures and methods.