Hydrogel Biosensor Array Platform Indexed by Shape

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A multi-analyte sensor array platform has been developed which consists of analyte specific features that are indexed by shape. The array features are batch-fabricated lithographically from poly(ethylene glycol) diacrylate hydrogel pre-polymer and can accommodate a wide variety of different sensing chemistries. Depending on the physical scale of the sensing moiety, it is either copolymerized to the hydrogel matrix (e.g., oligonucleotides, aptamers), or it is merely physically encapsulated, an important strategy for preserving the biological activity of the larger and more complex sensing elements (e.g., antibodies, proteins, cells). This three-dimensional hydrogel sensor platform has an advantage over two-dimensional platforms in that it offers an increased signal density, and because the array is constructed of poly(ethylene glycol), it has virtually no background noise due to nonspecific adsorption of labeled analytes. To highlight the capabilities of this platform to make high signal-to-noise measurements using diverse sensing chemistries, two demonstrations are described herein that illustrate the platform's efficacy in oligonucleotide sensing and cell-based sensing.

Introduction

In the post-human-genome project era there is an increased need for genetic, proteomic, and small molecule diagnostics to help researchers combinatorially screen for etiological links between genetic variation and disease; likewise, once cause-and-effect relationships have been established, there will be a demand for low-cost, multi-analyte sensor arrays to clinically screen individuals for these markers. There are currently numerous commercially available DNA-based diagnostics[1] designed to measure either the gene expression or genetic variation of an individual. Although existing screening techniques have been marketed for use in research and some clinical settings, there still exists a need to reduce the cost of these diagnostics to enable widespread usage.

Shape-encoded hydrogel-based arrays, termed MUFFINS (mesoscale unaddressed functionalized features indexed by shape), offer a cost-efficient, versatile platform that addresses the need for high-throughput and cost-effective screening of biological materials such as oligonucleotides, proteins, and small molecules. Photolithographic methodology naturally lends itself to high-throughput mass-production, enabling thousands of identically shaped polymer features to be produced in a single lithographic exposure. Photolithographic fabrication techniques have already been optimized for use in the semiconductor industry to efficiently produce submicron microelectronics components and can be readily modified for the production of hydrogel-based sensors.[2]

Shape-based encoding offers further simplification of array fabrication. Until now, a significant portion of biosensor research and development has focused on serially fabricated platforms composed of probes arrayed on a two-dimensional surface in a Cartesian coordinate pattern, whereby each sensor feature's function is indexed by its coordinate in the array. Several examples of this array fabrication methodology include the covalent attachment of small molecular sensors,[3] proteins,[4] and DNA[5] on glass and self-assembled monolayers on gold.[6] The use of randomly ordered arrays greatly reduces the demands of device fabrication, but requires an alternative encoding/decoding strategy for determin-

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ing sensor function. Randomly ordered arrays were first demonstrated[7] using functionalized polymer beads placed randomly into a multi-well grid, containing unique fluorescent tags to identify each sensor type.[8] Although this approach parallelizes the fabrication process, the finite number of unique dye combinations limits its application. Shape-based indexing offers the advantage of a nearly infinite number of unique patterns (as demonstrated by the widespread use of bar codes) requiring only the development of reliable decoding software. Similar to the polymer bead approach, each shape-encoded sensor type can be individually mass-produced, and multi-analyte arrays can be prepared by randomly assembling a mixture of the desired sensor types.

Hydrogels and sol−gels offer several advantages over surface-bound arrays during the analyte sensing process. The three-dimensional nature of the hydrogel and sol−gel based sensors results in an approximate 100-fold increase in signal density due to the higher immobilization capacity of sensing moieties within the matrix.[9] In particular, poly(ethylene glycol) (PEG) hydrogels are a unique medium, because they prevent nonspecific binding of proteins[10] and oligonucleotides, providing greater signal-to-noise discrimination than surface arrays. Hydrogel materials can accommodate a wide array of sensing modalities that would ordinarily not fare well on two-dimensional surfaces, such as antibodies,[11] enzymes,[12] and cells.[13] Because the physical encapsulation alleviates the need for surface attachment chemistry, the sensing moieties encapsulated in gels maintain their native configuration and hence their full biological activity.[14] The compatibility of hydrogels with a wide array of sensing methodologies—living cells, proteins, DNA, and small molecules—make them an attractive approach for biological sensing. To this end, the practicality of shape encoded hydrogel sensors was investigated by designing, fabricating, and testing cell- and DNA-based sensors.

Table 1. Sequence Identity of Each of the Probes Incorporated into the Hydrogel Sensors for the DNA Detection Assay and Their Corresponding Shape Encoding

probe name	probe sequence	feature shape
probe A	5′-/Acry/ATACCAGCTTATTCAATT-3′	square
probe B	5′-/Acry/ATACCACCTCATTCAATT-3′	circle
probe C	5′-/Acry/ATACCACCTTATTGAATT-3′	triangle
control	none	plus

Experimental Section

Materials. Poly(ethylene glycol) diacrylates (PEG-da) with weight-average molecular weights of 575 and 700 were purchased from Aldrich. All prepolymer formulations are described in weight percent. Darocur 1173, a liquid, photoinitiated free-radical generator, was obtained from Ciba-Geigy. Functionalized single-stranded DNA sequences for the single-nucleotide-polymorphism (SNP) detection experiment were purchased from Integrated DNA Technologies. Propidium iodide was obtained from Molecular Probes. The synthesis of the BODIPY−digoxigenin conjugate, as well as genetic engineering of the cells, has been described elsewhere.[15]

DNA Covalent Attachment Verification Study. The sensors for the DNA encapsulation study were prepared from a prepolymer solution consisting of 48% 12 μM DNA 18mer in distilled water, 50% PEG diacrylate MW 575, and 2% Darocur 1173 as photoinitiator. A 3′-rhodamine tagged oligonucleotide [AATTCAATAAGGTGGTAT(Rh)] was physically encapsulated in the cross-shaped sensors. A 3′-rhodamine tagged oligonucleotide with a polymerizable 5′ methacrylamide modification [(Acry)ATACCAGCTTATTCAATT(Rh)] was incorporated into the prepolymer mixture of the pentagon-shaped sensor. The triangle control contained the same methacrylamide modified DNA sequence, but without the rhodamine tag, while the square control contained no oligonucleotides. After polymerization, the hydrogels were soaked three times for 3 h in a fresh 1-mL aliquot of hybridization buffer, at which time the hydrogels were imaged.

Pre-Polymer Formulations. Sensors for the DNA detection assay were made from a pre-polymer solution consisting of 25% PEG-diacrylate MW 700, 2% Darocur 1173 photoinitiator, and 73% probe DNA diluted to 90 μM in DI water (see Table 1 for the shape assignment for each probe). The high percentage of photoinitiator was required for some sensing applications developed involving live cells, as extended exposure to UV radiation significantly reduced cell viability. Although this same concentration was not required for the other sensing studies, for the sake of consistency, all hydrogel sensors were prepared with the same photoinitiator concentration. Sensors for the digoxigenin sensing experiment were made from a pre-polymer solution consisting of 3.23 mL of DI water, 1.25 mL of PEG-da MW 575, 170 μL of 1 M NaOH (to elevate the pH of the pre-polymer to 7.4 before cells were added), 50 μL of Darocur 1173, and 300 μL of *Escherichia coli* in PBS (the optical density of the cell mixture was approximately 50 at $\lambda = 600$ nm).

Cell Culture. The *E. coli* used in the cell-based sensor assay were cultured using the following protocol. A 2-mL starter-culture was prepared from frozen-stock. Cells were incubated in Terrific Broth (TB) medium supplemented with 2% w/v glucose and 200 μg/mL ampicillin on a shaker table at 25 °C overnight. Next, 2 mL of fresh medium (TB w/2% glc and 200 μg/mL amp) were inoculated with 20 mL of the starter culture and incubated on a shaker table at 37 °C. When this culture

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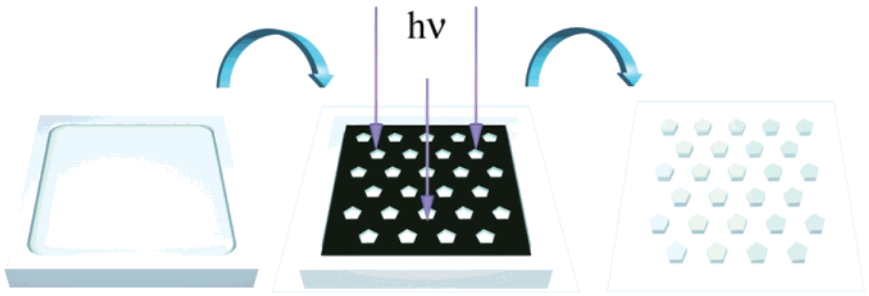

Figure 1. Shape-encoded sensor fabrication process: (1) liquid pre-polymer with the desired sensing moiety is poured into a Teflon substrate, (2) the prepolymer is exposed through a mask placed on top of a glass slide, and (3) the slide is rinsed to remove unpolymerized monomer and yield the cured shape-encoded sensors.

Table 2. Sequence and Attached Fluorophore of Each of the Target DNA Used to Test the Hydrogel-Bound Sensors in the DNA Detection Assay

target name	target sequence	fluorophore	excitation/emission λmax (nm)
target A	5′-/TATGGTCGAATAAGTTAA/6FAM/−3′	6-Fam	494/520
target B	5′-/TATGGTGGAGTAAGTTAA/TAMRA/−3′	TAMRA	559/583
target C	5′-/TATGGTGGAATAACTTAA/Cy5/−3′	Cy 5	648/668

reached growth phase (after approximately 2 h), signified by the optical density of the culture approaching a value of 0.5 (optical density measurements were taken at 600 nm), the cells were induced to express and display antibody fragments on their surface by adding 2 μL of 100 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). IPTG is a chemical analogue of galactose which cannot be hydrolyzed by the enzyme b-galactosidase. Therefore, it is an inducer for activity of the *E.coli* lac operon by binding and inhibiting the lac repressor without being degraded. The cells were cultured in this induction medium for 3 h, after which they were centrifuged into a pellet. The supernatant was aspirated away and discarded, and the pellets were resuspended in phosphate-buffered saline (PBS) to an optical density of 50.

Exposure Tool and Hydrogel Fabrication. Broadband ultraviolet radiation from a 200-W high-pressure mercury arc lamp (Oriel) was used for curing. The bulb was housed in an Oriel shutter enclosure that collimated the radiation to approximately a 15-cm diameter area and filtered out wavelengths below 365 nm. The nominal intensity of the collimated light was 20 mW/cm^2, as measured by a Molectron PowerMax 5200 intensity meter. An Oriel 68810 arc lamp power supply, coupled with an Oriel 68705 igniter, was used to power the bulb. The shutter was controlled by an Oriel 8160 timer.

Sensors were prepared by placing the prepolymer solution between two standard 1 in. × 3 in. glass slides with 250-μm thick cover slips acting as spacers. The curing time for hydrogel sensors was reduced by increasing the photoiniator content, to minimize the exposure of photosensitive sensors to UV light. The curing time was determined empirically to be 25 s based on the physical properties of the hydrogels and their shape under a microscope. Sensors were rinsed with DI water after exposure to remove any unreacted pre-polymer and stored until use in the hydrated state.

Hybridization Media (DNA detection assay). The DNA detection assay was conducted in a hybridization mixture composed of 300 μL of the following constituents in DI water: 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 M urea, 5 M formamide, and 8 μM of each of the three fluorescently labeled target DNA sequences (see Table 2). The sensors for the DNA assay were rinsed in a buffer solution containing the same ingredients as the hybridization mixture less the target DNA.

Hybridization Media (Cell-Based Assay). All cell assays were conducted in a hybridization mixture composed of a standard 1× phosphate-buffered saline (1× PBS final concentrations are 137 mM sodium chloride, 10 mM phosphate, 2.7 mM potassium chloride, and pH is 7.4) with target analyte and propidium iodide at a final concentration of 100 nM and 15 μM, respectively. The labeled target was synthesized by chemically conjugating the fluorophore BODIPY FL to digoxigenin, the analyte to which the surface displayed antibody chains bind. After hybridization, sensors were rinsed in a rinse buffer composed of 1× PBS containing 50 μM of a mild commercially available nonionic detergent, NP-40.

Imaging. An Olympus IX-71 inverted microscope was used for fluorescent imaging. The light source for the microscope was a 100-W high-pressure mercury arc lamp driven by an Olympus BHL-RFL-T3 power supply. A 4× objective coupled with a 10× eyepiece (total 40× magnification) was used for all images. An Olympus MagnaFire SP digital camera was used to record the fluorescent micrographs. Different optical filter sets were used depending on the fluorophore: TAMRA was imaged with a U-MWG2 filter set, Cy5 was imaged with a custom filter set ordered from Leeds Instruments (catalog 41008), and both 6-FAM and BODIPY FL were imaged with a U-MWB2 filter set.

Hydrogel Swelling. To characterize the swelling behavior of the hydrogels, the degree of swelling was measured at 25 °C for various concentrations of PEG-da MW 575 and 700 prepolymer in water. After the initial hydrogel mass was measured, the hydrogel was allowed to swell in DI water for 24 h, after which the post-swell mass of the hydrogel was measured. The percent swelling of each hydrogel formulation was characterized by the equation $[(m - m_0)/m_0 \times 100\%]$, where m_0 denotes the preswelling mass and m denotes the post-swelling mass of each hydrogel.

Results and Discussion

Sensor Fabrication. Photolithography was selected as the fabrication method because of the ease, precision, and reproducibility with which complex shaped materials can be prepared. In the modified contact lithographic process (Figure 1), the PEG-da pre-polymer solution containing the desired sensing moiety was dispensed into a milled Teflon basin and a photomask was placed on top, in direct contact with the pre-polymer. The appropriate UV exposure dosages were determined empirically for each pre-polymer formulation, but averaged approximately 200 mJ/cm^2. UV irradiation cross-linked the material beneath the transparent portions of the mask and caused the resultant patterned hydrogel features to adhere to the photomask (Figure 1). The unpolymerized pre-polymer was rinsed away with water, and the hydrogel sensor features were physically removed from the mask. The resulting highly cross-linked structures immobilized the desired sensing moiety in a three-dimensional permeable aqueous network.

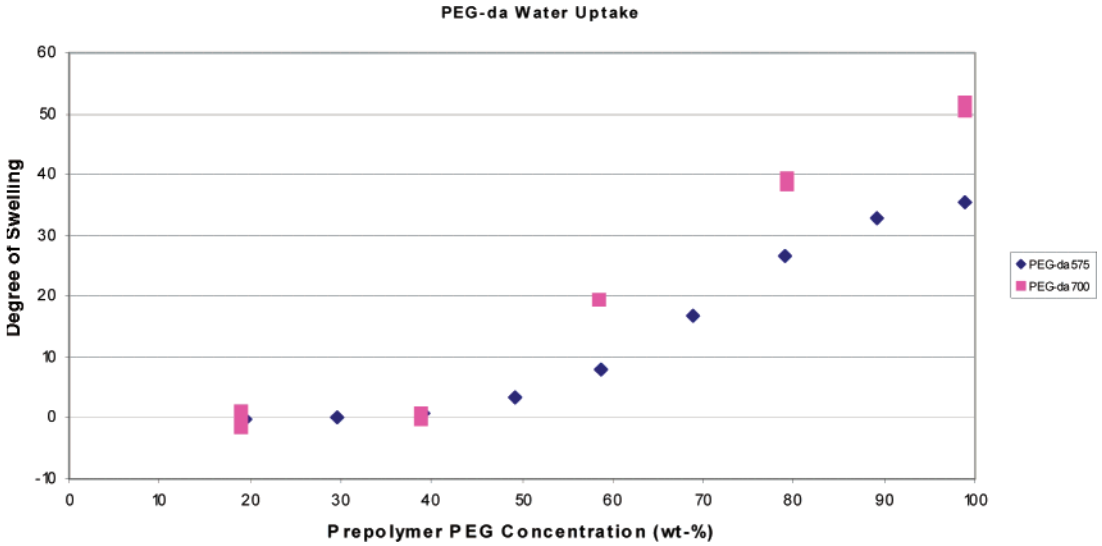

Figure 2. Degree of swelling for hydrogels as a variable of PEG-da pre-polymer concentration in water for PEG-da MW 575 and 700.

The swelling, analyte transport, and mechanical properties of the hydrogel are a function of the cross-linker/water ratio in the initial pre-polymer mixture. A variety of different hydrogel samples were prepared with varying concentrations of PEG-da MW 700. Analysis of the swelling properties of these samples demonstrated that hydrogel samples fabricated from pre-polymer composed of less than 40 wt % PEG-da had negligible swelling (Figure 2). Hydrogels that exhibited swelling were avoided, as excessive swelling distorts the shape of the sensors and causes damaging forces in subsequent hydrogel-based sensing devices due to significant volume changes. Similar empirical studies determined that hydrogels with a PEG-da concentration greater than 20 wt % had sufficient physical integrity to enable sensor handling during the study. Hence, all sensor demonstrations described herein utilized a pre-polymer PEG-da concentration of 25 wt %.

The DNA oligomers used in this study (18 bp length) exhibit sufficient transport in the MW 700 PEG-da hydrogel to enable their diffusion in and out of the hydrogels in a matter of hours. Much larger oligomers (e.g., 41 bp length) diffuse very slowly in PEG-da MW 700 formulation. Increasing the molecular weight of the poly(ethylene glycol) diacrylate cross-linker provided a noted improvement in transport of larger DNA oligomers at the expense of increased swelling. Methods that enable diffusion of even larger oligonucleotides and small proteins are presently being investigated by incorporating a porogen during the polymerization step.

Analysis of randomly ordered arrays requires a sophisticated encoding system that can reliably identify the function of each sensing unit. To demonstrate the potential of shape-based encoding, dot-array patterns have been designed that will enable millions of different hydrogel features to have unique tagging and be rapidly decoded with an automated recognition system.[16] However, to demonstrate the viability of the hydrogel medium for sensing applications, fewer unique shapes were required so simple geometric shapes were selected as the initial lithographic goal.

The mask fabrication technique chosen for photolithographic printing depends primarily on the size of the sensor features to be patterned. Well-resolved hydrogel features with sizes as small as 10 μm have been prepared using more costly and time-consuming chromium-on-glass mask writing techniques.[13] However, since the goal of this study was merely to demonstrate sensing in the hydrogel platform, more convenient, lower-resolution mask preparation techniques were used. The hydrogel sensors in this study were fabricated using laser-printed transparency film for masks. The printed transparency provided sufficient resolution to prepare 1−2-mm sensor features and could be fixed to a transparent glass slide to provide the functional photomask.

Once separate batches of sensor features are produced, they must be assembled into a single array, or "chip", to provide a functional device. Arraying techniques involving physical confinement and self-assembly have been developed,[17] however, they are not required for simple demonstration of the hydrogels sensing capabilities and, therefore, will not be discussed in detail here.

Sensing Demonstrations. The efficacy of the hydrogel medium as a sensor platform was demonstrated using two different sensing chemistries: single-stranded DNA (ssDNA) and genetically engineered *Escherichia coli*. The *E. coli* was designed to express single chain antibody fragments on their surface.

DNA Sensing. In a manner analogous to the traditional DNA microarray platforms, a ssDNA probe sequence was immobilized into the hydrogel matrix of each feature. The ssDNA probe served as a molecular recognition element capable of specifically binding its complement through base-pair interactions (In general, the hybridization conditions of the assay are maintained such that the probe sequences only anneal to the target analytes that are 100% complementary). The probe

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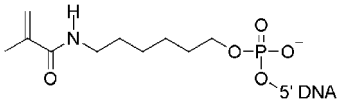

Figure 3. ssDNA probes used in the DNA detection assay possessed the 5′ modification illustrated above to enable covalent attachment during the photolithographic fabrication of the sensor features.

sequences were covalently attached to the PEG-da hydrogel matrix via a methacrylamide modification made to the 5′ end of the oligomer (see Figure 3); this functional group copolymerized with the acrylate moieties of the hydrogel matrix during the photopolymerization process, thereby permanently anchoring the probe in the sensor feature.

To verify the covalent attachment of the DNA oligomers to the hydrogel, a control experiment was devised to compare the diffusion of DNA oligomers with and without a polymerizable methacrylamide group. Pentagon-shaped hydrogels were prepared using a prepolymer with a 12 μM concentration of a 3′ rhodamine tagged 18-mer oligonucleotide and a 5′ methacrylamide group which was expected to be covalently attached to the hydrogel during the polymerization step. A cross-shaped hydrogel was prepared with a different DNA 18-mer containing the 3′ rhodamine modification without a polymerizable 5′ endgroup. Two controls were added: one a triangle with unlabeled DNA with a methacrylamide linkage, and the other a square with no added oligonucleotides. After polymerization the hydrogels were rinsed 3× for 3 h with buffer solution. Neither control exhibited fluorescence. The sensor with the polymerizable DNA oligomer exhibited a bright fluorescence signal that was unaffected by the multiple rinses, whereas the DNA oligomer that was merely physically entrapped exhibited an obvious attenuation of the initially bright signal after repeated rinsing (Figure 4). As expected, the hydrogel with physically entrapped DNA exhibited some fluorescence near the core, where diffusion from the hydrogel would be slowest.

In addition, the extent to which DNA was covalently attached to the hydrogels during photopolymerization was quantified by UV absorption measurements. The amplitude of the characteristic DNA absorption band around 256 nm was quantified in a hydrogel prepolymer with added DNA, and in a prepolymer blank. After polymerization, the resulting hydrogels were soaked in deionized water for 24 h and the UV absorption spectra of the washes were quantified, revealing that greater than 95% of the acrylamide-modified DNA remained immobilized in the hydrogel.

One of the most difficult tasks required of a DNA microarray is distinguishing between two target analytes whose sequences differ by only a single nucleotide. To ensure that this hydrogel platform meets that demand a demonstration was designed such that three sensors were fabricated with 18-mer probes differing by a single base pair from a reference sequence. Likewise, to evaluate whether the target analytes would bind to the hydrogel matrix nonspecifically, a fourth sensor feature, serving as a control, was fabricated with no probe DNA (see Table 1 for the probe identity and shape key).

A sensing assay was performed by placing the loose sensor features in a hybridization mixture containing all three fluorescently labeled 18-mer DNA sequences, referred to as "target sequences". Each of these target sequences was 100% complementary to one of the different probes. To distinguish between the three target sequences, each target was labeled with a unique fluorophore (see Table 2 for target sequence identity and labeling scheme).

After the hybridization the features were rinsed in a buffer solution containing no DNA and imaged on a fluorescence microscope. One bright-field and three fluorescence micrographs were captured; each fluorescence micrograph used an optical filter set specific for one of the three different target fluorophores (Figure 5). These micrographs illustrate the ability of the sensor's features to accurately identify each target sequence correctly with negligible cross-hybridizational noise. It should be noted that the fluorescence signal observed from these features is much stronger at the periphery of the shapes, owing to the slow diffusion of oligonucleotides into these hydrogels. However, the signal response is sufficiently strong to easily identify the hydrogel shape, and therefore the identity of the probe. A detailed study of the mass transport in hydrogels has provided a solution to this problem, and will be the topic of a subsequent paper.

Cell-Based Sensing. The efficacy of using cells as sensing elements within this hydrogel platform was demonstrated using *E. coli* genetically engineered to express single chain antibody fragments on their surface.[11,12] In this demonstration it was not necessary that the cells remained viable to function as sensing

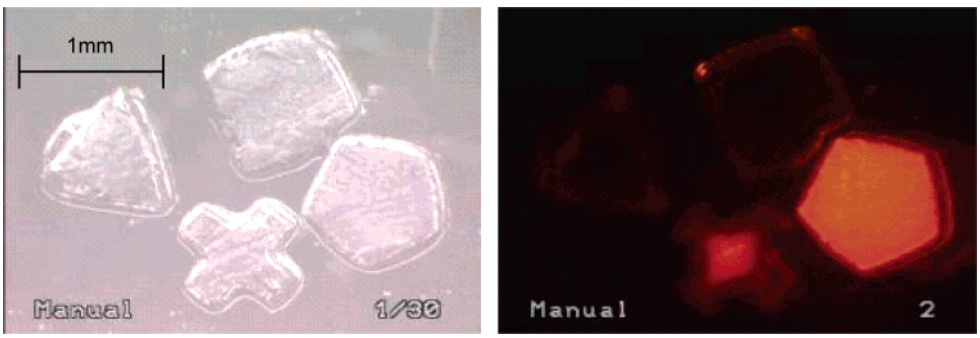

Figure 4. After multiple rinsings, the fluorescently tagged methacrylamide-modified DNA remains bound to the pentagon hydrogel while the physically encapsulated DNA without the methacrylamide group is washed out of the cross hydrogel, verifying the covalent attachment of the methacrylamide-functionalized DNA oligomer to the hydrogel matrix during polymerization.

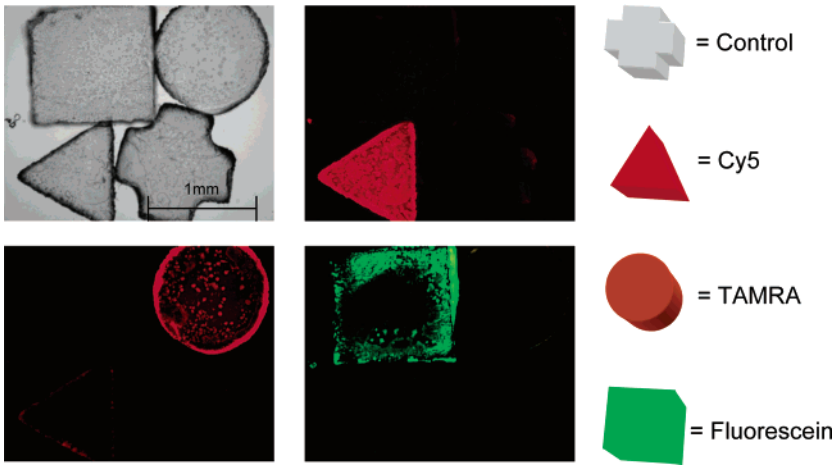

Figure 5. Micrographs of the DNA detection assay demonstrating the successful detection of each of the complementary target sequences with high signal and negligible cross-hybridizational noise.

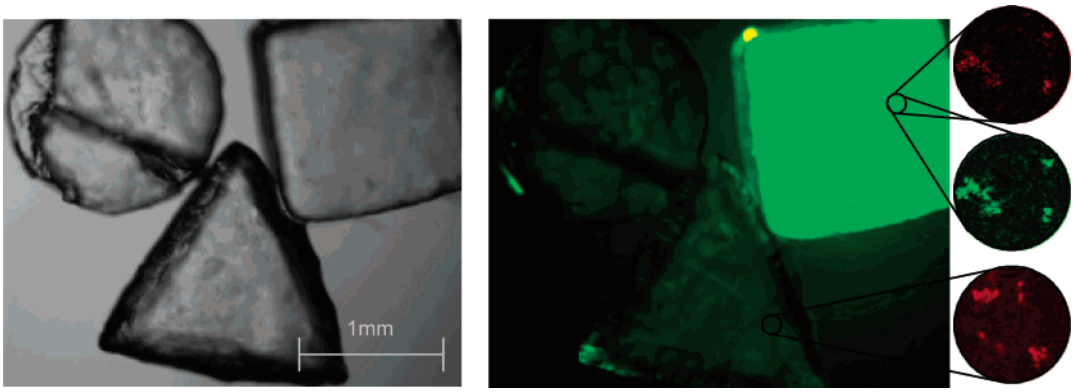

Figure 6. Brightfield (left) and fluorescence (right) micrographs of the cell-based sensing assay demonstrating successful detection of digoxigenin by the square-shaped sensor. The high-magnification, confocal micrographs verify that the signal originates from the cells within the sensor: the red signal, a propidium iodide stain, reveals the location of the cells and confirms that the green signal of the fluorescently labeled digoxigenin originates from the cells within the hydrogel.

Table 3. Specific Target Analytes of the Single Chain Variable Fragment Antibodies (scFvs) of Each Cell-Based Sensor and Their Corresponding Shape Encoding

cell's sensing functionality	feature shape
digoxigenin	square
atrazine	triangle
none	circle

elements; however, strategies have been investigated to encapsulate living cells that do keep them alive for sensing within this platform. This sensor demonstration was designed to validate the encapsulation strategy outlined earlier, as well as to illustrate that sensing moieties, when unencumbered by attachment chemistry, can yield remarkable sensing fidelity.

In this example, three sensor features were constructed and used in an assay to detect a fluorescently labeled small molecule named digoxigenin. Two of the sensors were functionalized with cells, while the third, serving as a control, was composed of hydrogel only. The cells placed in the sensors were both the same base-strain of *E. coli*; however, one had been engineered to express single-chain antibody fragments specific for digoxigenin and the other one was specific for atrazine (see Table 3). The three sensor features were incubated in a hybridization mixture containing fluorescently labeled digoxigenin as well as a live/dead stain for the cells, propidium iodide. Subsequently, the sensors were washed in a rinse-buffer and imaged on a fluorescence microscope. Two micrographs were captured, one fluorescence and the second bright-field (see Figure 6). The micrographs revealed a strong fluorescence signal coming from the sensor containing cells that express anti-digoxigenin antibodies. The sensors were also imaged on a fluorescence confocal microscope, a microscope capable of imaging discrete focal planes within the sensor. The micrographs taken with the optical filter-set specific for the propidium iodide stain (shown in red in Figure 6) reveal the location of the cells within the appropriate sensors, and the micrographs taken with the optical filter-set specific for the BODIPY FL fluorophore (shown in green in Figure 6) show the location within the sensors where digoxigenin had bound. Notice that this set of micrographs clearly illustrates that the signal is a result of specific interactions between the target analyte and the securely encapsulated cells within the appropriate sensor feature. These results highlight the utility of hydrogels to physically encapsulate biologically sensitive molecular recognition systems for their incorporation into sensory arrays.

Conclusions

The utility of the shape-encoded hydrogel sensory platform, or MUFFINS, has been demonstrated using both oligonucleotide and cell-based sensing. The PEG hydrogels exhibited negligible nonspecific binding of analytes enabling a favorable signal-to-noise ratio in the

three-dimensional sensors. In addition, both oligonucleotides and cell-bound antibodies retained their ability to hybridize specifically to the designated target, verifying that the incorporation of these materials into hydrogels does not inhibit their sensing function. These results suggest that hydrogel-based sensing offers significant promise as a medium for DNA, protein, and particularly cell-based sensing devices. Research is presently in progress to highlight the versatility of shape-based sensing, to enhance mass transport into and out of the hydrogel materials, and to develop efficient techniques for arraying these shape-encoded hydrogel sensors.

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