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Photolithographic Fabrication of Poly(Ethylene Glycol) Microstructures for Hydrogel-based Microreactors and Spatially Addressed Microarrays

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Abstract We describe the fabrication of poly(ethylene glycol) diacrylate (PEG-DA) hydrogel microstructures with a high aspect ratio and the use of hydrogel microstructures containing the enzyme β-galactosidase (β-Gal) or glucose oxidase (GOx)/horseradish peroxidase (HRP) as biosensing components for the simultaneous detection of multiple analytes. The diameters of the hydrogel microstructures were almost the same at the top and at the bottom, indicating that no differential curing occurred through the thickness of the hydrogel microstructure. Using the hydrogel microstructures as microreactors, β-Gal or GOx/HRP was trapped in the hydrogel array, and the time-dependent fluorescence intensities of the hydrogel array were investigated to determine the dynamic uptake of substrates into the PEG-DA hydrogel. The time required to reach steady-state fluorescence by glucose diffusing into the hydrogel and its enzymatic reactions with GOx and HRP was half the time required for resorufin β-D-galactopyranoside (RGB) when used as the substrate for β-Gal. Spatially addressed hydrogel microarrays containing different enzymes were micropatterned for the simultaneous detection of multiple analytes, and glucose and RGB solutions were incubated as substrates. These results indicate that there was no cross-talk between the β-Gal-immobilizing hydrogel micropatches and the GOx/HRP-immobilizing micropatches.

Keywords: Micropatterning, hydrogel, photolithography, poly(ethylene glycol) diacrylate (PEG-DA), microreactor, microarray

Micropatterning techniques are emerging as important tools in a wide variety of fields including biochips, bioelectronics, and fundamental studies of cell biology [2, 3, 7, 13, 24]. As interest in micropatterning has increased, many different techniques have been developed such as photolithographic procedures, soft lithography, and dip-pen lithography [6, 15, 22, 23]. Among these techniques, the micropatterning of photopolymerizable hydrogel using photolithographic techniques has considerable merit. For example, photolithographic techniques have allowed the creation of complex biomolecule patterning *in situ* in closed environments such as microfluidic channels and photopolymerizable hydrogels, which can be used to develop highly cross-linked polymer networks capable of biomolecule entrapment for numerous sensing techniques [9, 25].

Poly(ethylene glycol) (PEG) hydrogel is a biocompatible, nontoxic, nonimmunogenic, and hydrophilic polymer with excellent properties, and has been used widely in biomaterials, biotechnology, and medicine [5, 11, 12, 18, 19]. Furthermore, PEG hydrogel functionalized with diacrylate groups can participate in photopolymerization reactions. Therefore, the advantages of photolithographic techniques can be applied to the micropatterning of hydrogels. Revzin *et al.* [16, 17] fabricated PEG hydrogel microstructures to investigate their potential use in optical sensors and in designing cellular microenvironments. Zhan *et al.* [26] integrated PEG hydrogel micropatches into the channels of microfluidic devices, and PEG hydrogel micropatches have been used as microsensors for quantitative pH sensing. Koh *et al.* [14] reported a simple method for controlling the spatial positioning of mammalian cells and bacteria on substrates using patterned PEG hydrogel microstructures. In a previous study, we reported a novel technique for manufacturing photopolymerizable hydrogel microparticles containing biocatalysts by using the immiscibility of hydrogel and oil solutions in microfluidic systems combined with photopolymerization [10].

In this study, the hydrogel was micropatterned by the photopolymerization of poly(ethylene glycol) diacrylate (PEG-DA) solution with 365 nm UV light. This was extended to the fabrication of hydrogel microstructures with high aspect ratios. To demonstrate the potential use of hydrogel-based microstructures as microreactors, β-galactosidase (β-Gal) or glucose oxidase/horseradish peroxidase (GOx/HRP) was trapped in arrays of hydrogel microstructures

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and the diffusion behaviors of substrates into the hydrogel microstructures were investigated. Spatially addressed hydrogel microarrays containing different enzymes were also created using a multistep photolithographic method, and the fabricated hydrogel micropatches were used as biosensing components for the simultaneous detection of multiple analytes.

MATERIALS AND METHODS

Materials

PEG-DA, with an average molecular weight of 575, and 2-hydroxy-2-methylpropiophenone (photoinitiator) were obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). 3-(Trichlorosilyl)propyl methacrylate (TPM), for surface modifications, was purchased from the Fluka Chemical Co. (Milwaukee, WI, U.S.A.). Glucose oxidase (GOx), horseradish peroxidase (HRP), and β-galactosidase (β-Gal) were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Resorufin β-D-galactopyranoside (RGB) and Amplex Red (*N*-acetyl-3,7-dihydroxyphenoxazine) were purchased from Molecular Probes (Eugene, OR, U.S.A.). Phosphate-buffered saline (PBS, pH 7.4) consisted of 150 mM NaCl, 4 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 in deionized water. All chemicals were of reagent grade or better.

Fabrication of PEG-DA Hydrogel Microstructures

To fabricate the PEG-DA microstructures, glass slides were cleaned for 20 min in a piranha solution consisting of a 3:1 ratio of 50% (w/v) aqueous solution of H_2SO_4 and 30% (w/v) H_2O_2 (*Caution: this mixture reacts violently with organic materials and must be handled with extreme care*), and then rinsed with deionized water and ethanol, and dried with nitrogen gas. The cleaned substrates were functionalized with TPM to bind the hydrogel microstructures covalently to the glass substrate *via* the TPM monolayer. This was accomplished by dipping the glass substrates into a 5 mM TPM solution of a 4:1 ratio of heptane-carbon tetrachloride for 1 min [17, 21]. At the next step, cover-glass anchors of 110 μm thickness were placed onto the TPM-functionalized substrate, and the hydrogel precursor solution, consisting of PEG-DA and 1% (v/v) photoinitiator, was dropped onto the area between the two cover-glass anchors, as shown in Fig. 1. The hydrogel precursor solution was spread well over the whole area between the cover-glass slides by capillary action after a dark-field, hole-type chrome photomask with 100 mm diameter had been aligned with the area to be micropatterned. The PEG-DA solution was photopolymerized by exposure to 365 nm UV light (EFOS Ultracure, UV spot lamp, Mississauga, ON, Canada) for 5–15 s. Following polymerization, the glass substrates were washed with PBS buffer to remove the unpolymerized

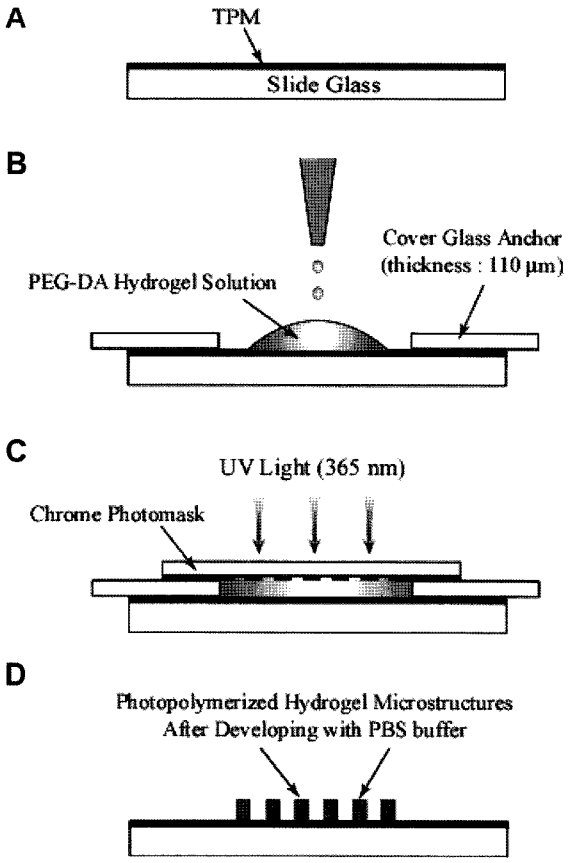

Fig. 1. Schematic illustration of the process used to fabricate PEG-DA hydrogel microstructures.
A. Glass substrates are functionalized with TPM. **B**. Hydrogel precursor solution is dropped onto the area between two cover-glass anchors that are placed onto the TPM-functionalized substrate. **C**. The chrome photomask is aligned and the micropatterning PEG-DA hydrogel undergoes UV photopolymerization. **D**. The unpolymerized hydrogel solution is removed with PBS buffer.

hydrogel solution. To characterize hydrogel microstructures, scanning electron microscopy (SEM) images were obtained on a JSM-6700F field-emission scanning electron microscope, using an accelerating voltage of 10.0 kV.

Enzymatic Reactions in PEG-DA Hydrogel Microstructures

A mixture of 450 μl of PEG-DA hydrogel solution, 50 μl of β-Gal (1 mg/ml) in PBS buffer (pH 7.4), and 1% (v/v) initiator was dropped between cover-glass anchors to a thickness of 110 μm, followed by UV-induced photopolymerization for 5 s through the photomask. After washing the unpolymerized hydrogel solution, 50 μM RGB in PBS buffer (pH 7.4) was incubated as the substrate. To investigate multienzyme reactions of glucose molecules, a mixture consisting of 450 μl of PEG-DA hydrogel solution, 25 μl of GOx (2 mg/ml), 25 μl of HRP (1 mg/ml), 10 mM Amplex Red, and 1% (v/v) initiator was placed between the cover-glass anchors for the fabrication of enzyme-trapping

hydrogel micropatches. After UV-induced photopolymerization for 5 s, 20 mM glucose in PBS buffer (pH 7.4) was applied as the substrate. The time-dependent fluorescence intensity of the hydrogel micropatches was analyzed by collecting fluorescence micrographs every 30 s.

RESULTS AND DISCUSSION

Characterization of Micropatterned Hydrogel Microstructures

Parts A and B of Fig. 2 show scanning electron microscopy (SEM) images and surface topography of the generated hydrogel micropattern, respectively. The surface topography was determined with a white-light confocal microscope (Nanofocus μSurf, Duisburg, Germany). The average height of the microstructures was 110 μm, which was the same as the thickness of the cover-glass anchor. The diameters of the hydrogel microstructures were typically 102 μm at the top and 104 μm at the bottom, indicating that no differential curing occurred through the thickness of the hydrogel microstructure. The radicals formed at the top and bottom of the PEG-DA solution during UV exposure cured the hydrogel at about the same rate because PEG-DA is highly UV transparent. Therefore, the gradient conversion through the thickness was minimized. This approach makes it possible to fabricate high-aspect-ratio microstructures. High-aspect-ratio microstructures have several advantages, such as a higher ratio of active surface area to substrate surface area, potentially higher packing density of the microstructural elements, and higher throughput in continuous-flow systems, compared with those of low-aspect-ratio microstructures [1]. These features result in good sensitivity and performance in biochemical reactions and are important in studying the effects of three-dimensional

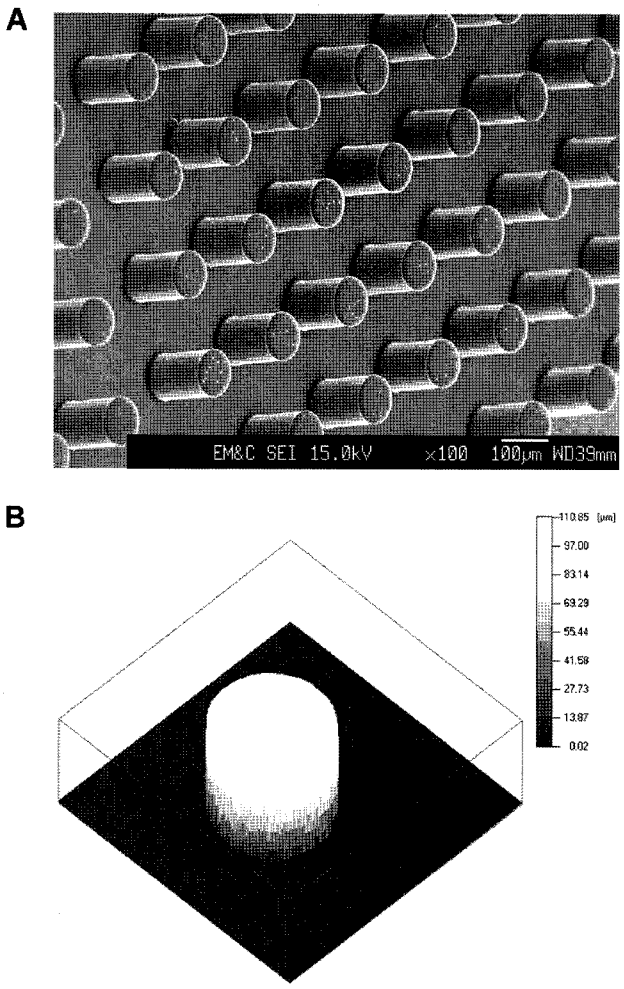

Fig. 2. Hydrogel microstructures were fabricated with cover-glass anchors of 110 μm thickness and a photomask with a 100 μm diameter (UV irradiation time, 5 s).
A. Scanning electron micrograph of the fabricated hydrogel microstructures. **B.** Confocal micrograph showing differential curing through the thickness of the hydrogel microstructure.

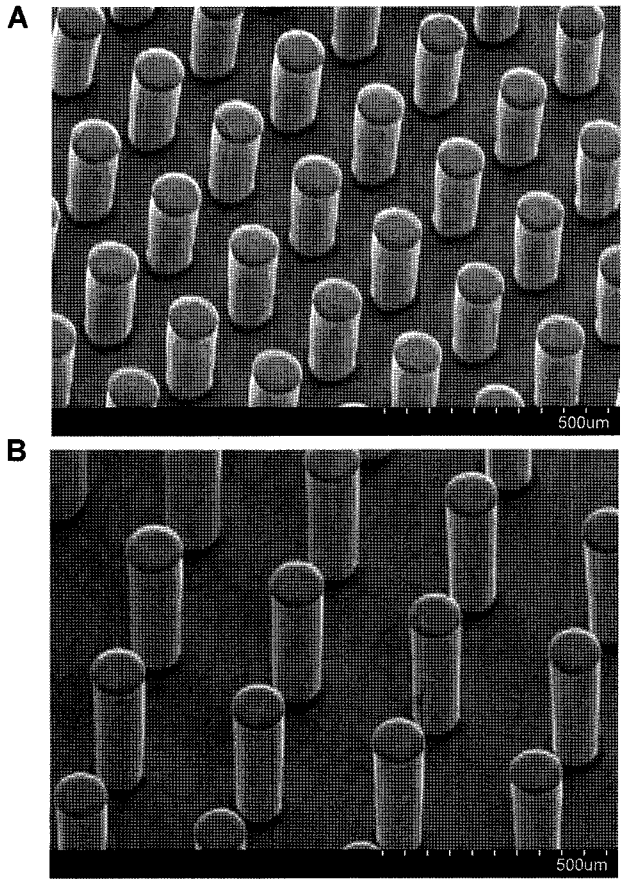

Fig. 3. Scanning electron micrographs of (**A**) hydrogel microstructures with an aspect ratio of 2 (diameter of the photomask, 100 μm; thickness of cover-glass anchor, 220 μm; UV irradiation time, 10 s) and (**B**) those with an aspect ratio of 3 (diameter of the photomask, 100 μm; thickness of cover-glass anchor, 330 μm; UV irradiation time, 15 s).

topological influences on the functions of cells and proteins. Recently, microstructure arrays with high aspect ratios have been required for better color resolution in multicolor display panels. Hydrogel microstructures with aspect ratios of 2 and 3 have been fabricated simply by overlapping two and three sheets of cover-glass anchors of 110 μm thickness, respectively (Fig. 3). The diameters of the hydrogel microstructures with an aspect ratio of 2 were 105 μm at the top and 103 μm at the bottom, and those with an aspect ratio of 3 were 107 μm at the top and 102 μm at the bottom. Neither type showed a significant gradient conversion through the thickness of the microstructure. This method has advantages compared with other high-aspect-ratio patterning techniques. For example, embossing techniques, which are one of the representative techniques for high-aspect-ratio microfabrications, need a tapered mold with antistick coating to facilitate demolding. The effects of the process and material parameters should be considered for good replication [4]. However, our approach is relatively simple and does not require a tapered mold or antistick coating, although the period of UV irradiation should be considered carefully to prevent undercuring or overcuring.

Hydrogel Microstructures as Microreactors for Enzymatic Reactions

In the next set of experiments, to demonstrate the potential use of hydrogel-based microstructures as microreactors, we fabricated an array of hydrogel micropatches containing enzymes. Glass substrates were treated with TPM to covalently bond the hydrogel micropatches to the glass substrates. The formation of hydrogel microstructures from PEG-DA is based on the free-radical polymerization of the acrylate end groups appended to the PEG derivatives. The photoinitiator dissociates upon exposure to UV radiation, creating highly reactive methyl radicals that attack the unsaturated carbon-carbon double bonds of the acrylate functionality, thus initiating free-radical polymerization [21]. Similarly, the reaction between the acrylate groups in the PEG-DA and the acrylate moieties of the TPM self-assembled on the glass surface results in the covalent bonding of the hydrogel micropatches to the glass substrates. Therefore, the PEG hydrogel micropatches do not detach from the surface during washing. Fig. 4A shows fluorescence micrographs of hydrogel micropatches obtained after 50 μM RGB in PBS buffer (pH 7.4) had been incubated as the substrate. Nonfluorescent RBG is hydrolyzed to D-galactose and fluorescent resorufin by β-Gal catalysis. Fluorescence micrographs were collected every 30 s for 6 min and the time-dependent fluorescence intensities of the hydrogel patches were plotted to trace the dynamic uptake of RGB substrate into the PEG-DA hydrogel micropatch. Fluorescence intensity showed no significant change for the first minute because the RGB

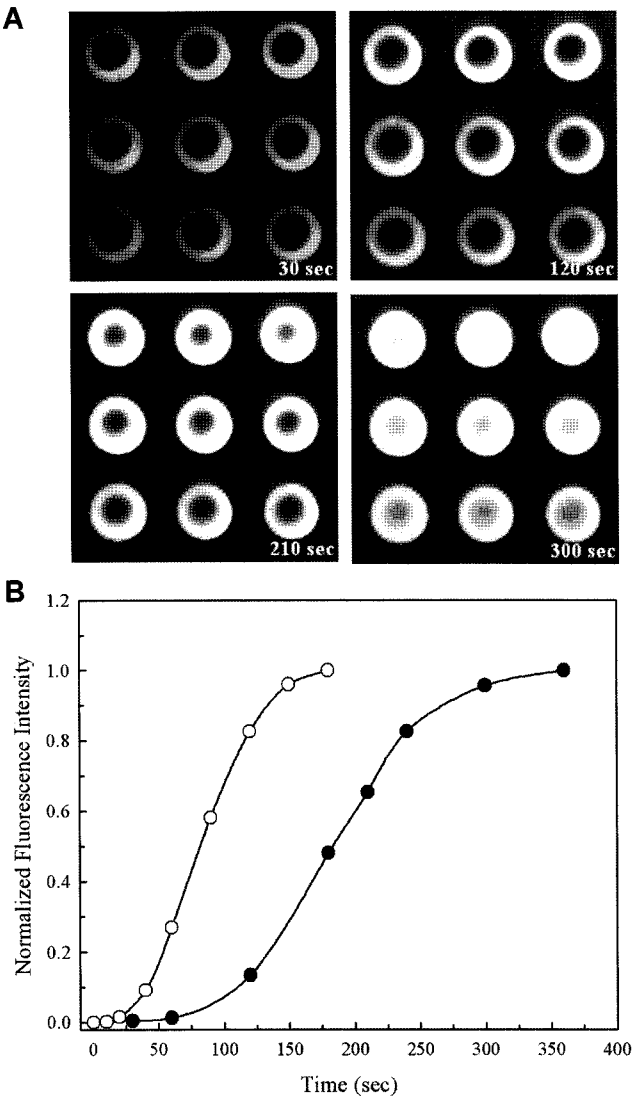

Fig. 4. A. Time-dependent fluorescence micrographs of β-Gal-trapping hydrogel microstructures after a solution containing 50 μM RGB was incubated as substrate. **B.** Normalized fluorescence intensity profiles as a function of time for β-Gal-containing microstructures (●) and GOx/HRP-immobilizing hydrogel microstructures (○).

Fluorescence intensity was calculated by subtracting the intensity of the background from the fluorescence intensities measured for the enzyme-immobilizing hydrogel microstructures. The excitation and maximum emission wavelengths were 563 and 587 nm, respectively.

takes time to diffuse into the hydrogel and react with β-Gal. However, the fluorescence intensity increased sharply between 1 and 5 min with the formation of fluorescent resorufin. This accumulated within the hydrogel patches, reaching a steady state after 300 s [Fig. 4B (●)]. We then used these hydrogel micropatches to investigate multienzyme reactions of glucose molecules. Other experimental conditions, including different percentages of hydrogel and UV irradiation times, were the same as those described in the

previous section, because the diffusion of molecules in polymeric matrices may be related to various chemical factors (hydrogen bonds, electrostatic interactions, and hydrophobic interactions between the molecules and matrix) and physical factors (hydrodynamic radii, conformations of the molecules, and the presence of binding sites) [8]. Fig. 4B (○) shows the time-dependent fluorescence intensity changes of the hydrogel micropatches after incubation with 20 mM glucose in PBS buffer (pH 7.4). The glucose molecules incubated on the hydrogel micropatches diffused into the gel and reacted with GOx. Gluconic acid and H_2O_2 were generated by the GOx-catalyzed reaction, and then fluorescent resorufin was formed *via* the HRP-catalyzed reaction between H_2O_2 and Amplex Red [20]. The fluorescence intensity of the fluorescent resorufin accumulated within the hydrogel patches, reaching a steady state after 150 s. This value is half the time required for the RGB/β-Gal reaction to reach steady-state fluorescence. This result can be explained as follows: (1) The diffusion of glucose molecules into the hydrogel micropatches was faster than that of the RGB molecules; and/or (2) the time required for glucose to react with the enzymes was shorter than the reaction time of RGB with β-Gal.

Spatially Addressed Hydrogel Microarrays as Biosensing Components for the Detection of Multiple Analytes

Array systems of spatially addressed molecules on planar supports are one of the most powerful and versatile structures for rapid screening assays and sensor devices. Here, our micropatterning technique using photopolymerizable hydrogels and a photolithographic method was extended to create spatially addressed hydrogel microarrays containing different enzymes. The fabricated hydrogel micropatches were used as biosensing components for the simultaneous detection of multiple analytes. The spatially addressed hydrogel microarrays were prepared by the following procedures. A PEG-DA hydrogel solution containing GOx, HRP, and Amplex Red was photopolymerized by UV irradiation through the photomask on glass slides treated with TPM. After the remaining unpolymerized PEG-DA hydrogel

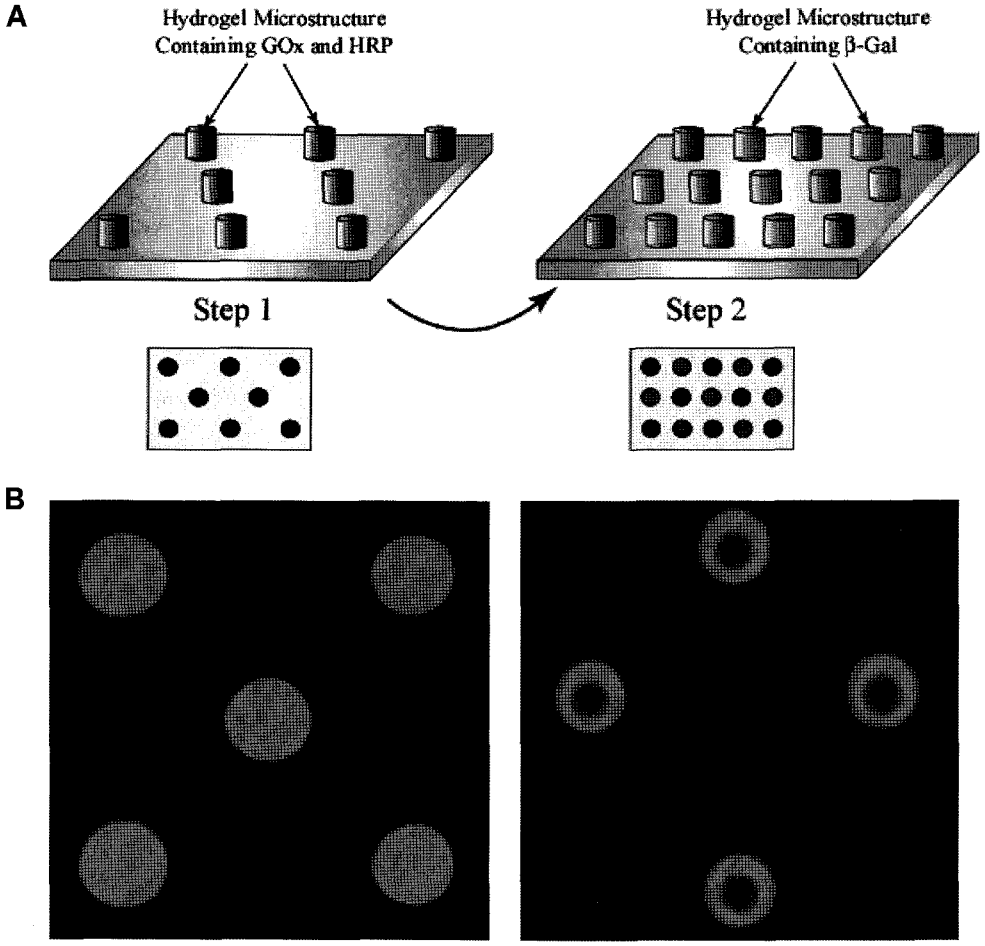

Fig. 5. A. Illustration of the two-step process used to create spatially addressed hydrogel microarrays containing different enzymes for the simultaneous detection of multiple analytes. **B.** Fluorescence micrographs of hydrogel microarrays after incubation with glucose (left) and RGB (right) as analytes. Fluorescence intensities were measured after 150 s.

precursor solution had been washed off, hydrogel solution containing β-Gal was photopolymerized by aligning the photomask with the areas between the hydrogel arrays containing GOx/HRP/Amplex Red (Fig. 5A). Fig. 5B (left) shows the fluorescence micrograph after the 20 mM glucose solution had been incubated on the spatially addressed hydrogel arrays. A fluorescence micrograph was taken at steady state, reached after 150 s. Fluorescence was observed in the micropatches containing GOx and HRP, but no fluorescence was detected in the hydrogel arrays containing β-Gal. These results indicate that there was no cross-talk resulting from the release of enzymes between the GOx/HRP-trapping hydrogel and the β-Gal-immobilizing hydrogel. Furthermore, when the hydrogel arrays were exposed to 50 μM RGB solution, only the β-Gal-trapping hydrogel micropatch arrays showed fluorescence (Fig. 5B, right). These results demonstrate that the spatially addressed hydrogel arrays could be used as biosensing components for the detection of multiple analytes. With this approach, it is also possible to place more than two kinds of hydrogel micropatches on a single sensing surface, each containing a different enzyme, another catalyst, or reporter.

We have demonstrated a micropatterning technique using PEG-DA hydrogel and a photolithographic technique that can be used to fabricate microstructures with a high aspect ratio. The fabricated microstructures can be used as microreactors for enzyme reaction and biosensing components for the simultaneous detection of multiple analytes. The study of the diffusion behaviors of substrate molecules in hydrogels is also important in designing applications for hydrogel materials that involve biosensor activity and drug delivery. We believe that our approach will contribute to the characterization of hydrogel-matrix-based systems such as controlled drug delivery systems and biosensing systems with acceptable sensor response times. This technique can be extended to the measurement of multiple analytes by placing two or more hydrogel micropatches, each containing a different enzyme, on a single sensing surface.

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