Ultraviolet Photolithographic Development of Polyphosphazene Hydrogel Microstructures for Potential Use in Microarray Biosensors

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Polyphosphazenes that bear both methoxyethoxyethoxy and cinnamyl side groups were synthesized and evaluated for use as hydrogels incorporated into micrometer-scale biosensor arrays. Polymers with the general formula $[NPR_xR'_y]_n$ where R = $OCH_2CH_2OCH_2CH_2OCH_3$ and R′ = OCH═CHCH_2Ph ($x = y = 1$; $x = 2$, $y = 0$) were synthesized. The polymers were cross-linked to form hydrogels by exposure to ultraviolet radiation ($\lambda = 320-480$ nm) in the presence of a photoinitiator. Hydrogel microstructures in the size range 50−500 μm were fabricated using standard photolithographic techniques. The resolution and dimensions of these microstructures were examined by optical microscopy, scanning electron microscopy, and profilometry. The resultant three-dimensional hydrogel microstructures were used to encapsulate enzymes for biosensor applications. The enzymatic activity of encapsulated horseradish peroxidase (HRP) was examined as a model system. The HRP catalyzed reaction between H_2O_2 and Amplex Red to produce a fluorescent product, resorufin, was confirmed by fluorescence microscopy.

Introduction

Microfabrication techniques are used increasingly in different applications to give structures with capabilities that exceed those of conventional macroscopic systems. Patterned polymeric films have been used in various miniaturized systems such as organic electronic devices, biomedical engineering, sensors, etch resists, optical communications, thin film optical elements, flat panel multicolor displays, and microfluidics.[1] In addition, polymer-based *hydrogels* (water-soluble polymers that have been cross-linked) also play an important role in miniaturized systems. Typically, hydrogels are created in miniaturized systems based on multiple step photolithography including exposure and development.[2] The main reason for integrating hydrogels into miniaturized systems is that their aqueous microenvironment and three-dimensional architecture is appropriate for encapsulation of various biomolecules such as proteins, nucleic acids, and even whole cells.[2−7] Because of these unique properties, hydrogel microstructures have been used for numerous applications such as drug delivery, biosensors, DNA hybridization, and cell encapsulation.[8−13] The three-dimensional structure of a hydrogel can also be exploited to control flow inside microchannels.[14] In this research, we have initiated the use of a new class of hydrogels for sensor applications. These are polyphosphazene hydrogels fabricated into microstructures within the size range 50−500 μm. Polyphosphazenes are a highly tailorable class of polymers that possess a phosphorus−nitrogen backbone as shown in Figure 1.[15] Several water-soluble polyphosphazenes have been prepared that can be covalently or ionically cross-linked to form hydrogels.[16−18] The utility of polyphosphazene hydrogels as platforms for enzyme and cell immobilization on a macroscale has also been demonstrated.[19,20] In addition, polyphosphazenes that bear photoreactive side chains based on cinnamates, chalcones, or allyl groups have been

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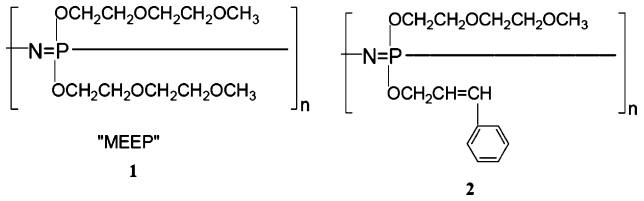

Figure 1. Polyphosphazenes with alkyl ether and cinnamyl side groups for hydrogel formation via UV photolithography.

developed.[21−23] Here, we bring together these different aspects to optimize the system for applications such as microarray biosensors or as hydrogel elements in microfluidic devices.

The use of polyphosphazenes for this application has some attractive aspects and advantages over typically used carbon-based polymers. Polyphosphazenes possess several features that make them useful for the design of hydrogel microstructures via photolithography. Compared to many commonly used polymers for UV cross-linking, for example, difunctionalized poly(ethylene glycol)[9,10] (PEG), polyphosphazenes have several useful advantages. First, in principle, each repeating unit can bear two functional side groups; thus, a high loading of the photoreactive moiety may be attained. PEG typically has only two photoreactive sights per polymer chain. Second, the photosensitivity of the polymer can be varied over a broad range based on rational design of the side group chromophores. Third, the phosphorus−nitrogen backbone is transparent to mid- and long-wavelength UV radiation, unlike many carbon-based polymers that tend to absorb UV light in this range. Thus, the probability of side reactions by chain cleavage is reduced. Fourth, the phosphazene system imparts greater synthetic flexibility and thus more control over the chemical and physical properties of the material.[15] This allows the design of many variations of the polymer for specific applications. For example, hydrophobic side groups can be attached to the phosphazene backbone in varied ratios to control or minimize water absorption while still maintaining a larger number of photoreactive side groups than functionalized PEG. In the current study the following proof-of-concept experiments were addressed. (1) Determination of the resolution obtained by photolithography (220−400 nm UV radiation) of polymer patterns in the size range 50−500 μm diameter. (2) Hydrogel formation by this method was investigated and the dimensions of the gel were measured. (3) The activity of horseradish peroxidase (HRP) immobilized within these microstructures was examined.

Experimental Section

General Materials. Hexachlorocyclotriphosphazene (Otsuka) was recrystallized from hot heptane and sublimed under vacuum before use. Poly(dichlorophosphazene) was obtained by the thermal ring opening polymerization of hexachlorocyclotriphosphazene at 250 °C. Sodium hydride (95% dispersion in mineral oil) (Aldrich) and cinnamyl alcohol (Aldrich) were used as received. Methoxyethoxyethanol (Aldrich) was dried over calcium hydride, vacuum-distilled, and stored over molecular sieves. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl under an argon atmosphere. Nuclear magnetic resonance (NMR) data were obtained using a Bruker 360 MHz instrument. ^{1}H NMR spectra were referenced to tetramethylsilane. ^{31}P NMR spectra were obtained using phosphoric acid as an external reference.

Polymer Synthesis. *Synthesis of Polymer **1***. The synthesis of poly[bis(methoxyethoxyethoxy)phosphazene] (MEEP) was carried out according to a method reported previously.[16] The resultant solution was concentrated and the polymer was dialyzed against deionized water (2 days) followed by methanol (2 days) and then dried under vacuum. ^{31}P NMR: −7 ppm. ^{1}H NMR: δ = 3.4 ppm (m, etheric).

*Synthesis of Polymer **2**.* Cinnamyl alcohol (5.76 g, 0.043 mol) was added slowly to a suspension of sodium hydride (1.032 g, 0.043 mol) in THF (50 mL). The reaction was allowed to proceed until the sodium hydride was consumed. The resultant sodium alkoxide was added dropwise to a fresh solution of poly(dichlorophosphazene) (5.0 g, 0.086 mol) in THF (700 mL) and the reaction mixture was stirred overnight at room temperature. An aliquot of the reaction mixture was removed and analyzed by ^{31}P NMR spectroscopy. A solution of sodium methoxyethoxyethoxide ether (0.8 equiv) in THF was then added dropwise to the partially substituted polymer and the mixture was allowed to react overnight. The resultant solution was concentrated by rotoevaporation and dialyzed (12−14000 MW cutoff) against deionized water (2 days), followed by methanol (2 days). Polymer **2** was isolated by evaporation of the contents of the dialysis tube. The final product was dried under vacuum at 42 °C. Characterization data ^{31}P NMR: −7 ppm. ^{1}H NMR: δ = 3.3 ppm (m, etheric), 4.1 ppm (m, alkene), 6.4 ppm, 7.1 ppm (br, aromatic).

Preparation of Hydrogel Microstructures on the Glass Substrates. Hydrogel microstructures from polymer **1** and polymer **2** were prepared via photolithography. Solutions of each polymer in tetrahydrofuran (1 g/5 mL) were prepared and 2.5 mg of benzophenone (Aldrich) was added as a photoinitiator to each polymer solution. The resultant solutions were spin-coated at 2000 rpm for 10 s onto clean glass slides, to produce uniform films approximately 10 μm in thickness. A photomask with micrometer-sized patterns (50−500 μm) was placed directly above the polymer film which was illuminated for 3−5 min. These films behave as negative photoresists. Areas exposed to UV light formed a cross-linked network and were insoluble in the developer solvent. Unexposed regions of polymer were rinsed away with ethanol or deionized water. An ultraviolet light source (EFOS Ultracure 100ss Plus, UV spot lamp, Mississauga, Ontario) or an EXFO Lite System, Model E3000 equipped with a liquid light guide, 750 mm × 5 mm 50 W metal halide lamp, with a band-pass filter (320−480 nm) (INPRO Technologies, Inc., Frederick, MD) was used in all experiments.

Analysis of Polymer Micropatterns. Pattern development was confirmed with the use of an optical microscope (Carl Zeiss Inc., Thornwood, NY). Micropatterns were analyzed by scanning electron microscopy (SEM) at the electron microscope facility at The Pennsylvania State University. SEM examination of polymer micropatterns was carried out using a JEOL JSM 5400 model electron microscope. Polymer micropatterns were coated with a layer of gold/palladium (10 nm) with a BAL-TEC SCD050 sputter-coater before analysis. The micropattern images were obtained by adjusting the SEM stage to a 40° tilt angle. The average height of the hydrogel microstructures was measured with a profilometer (Vecco Instruments, Plainview, NY).

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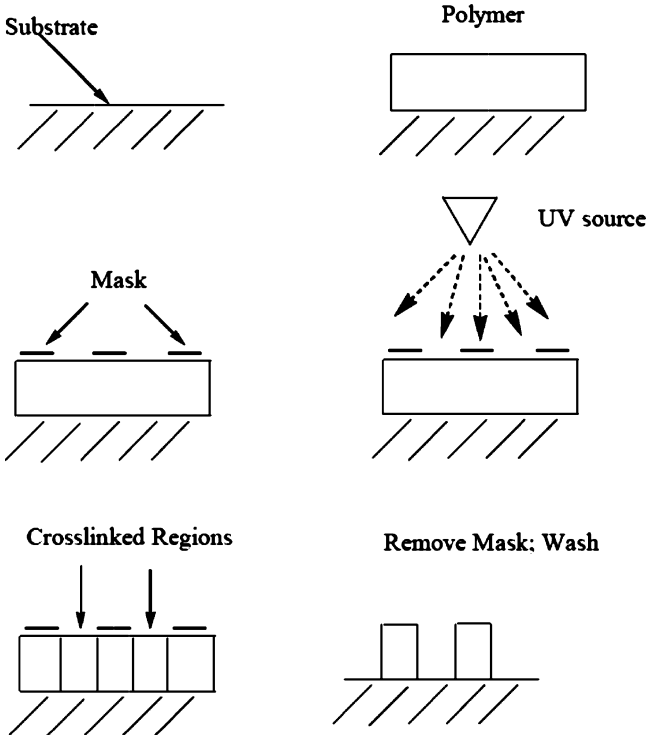
Enzyme Immobilization within Hydrogel Microstructures. Horseradish peroxidase (HRP, EC 1.11.1.7, Type VI, 290 units/mg) was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Horseradish peroxidase (HRP) phosphate buffered solution (2 mg/mL) was added to a solution of polymer **2** and was encapsulated in UV cured hydrogel microstructures. An enzyme assay was performed to determine HRP activity after exposure to UV illumination in the following manner. HRP-immobilized hydrogel microstructures were exposed to 5 μL of H_2O_2 (1 μM) with 5 μL of *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex red, 10 μM). The HRP-catalyzed reaction between hydrogen peroxide and Amplex red was monitored by fluorescence microscopy. Images were acquired with a Zeiss Axiovert 200 microscope equipped with an integrated color CCD camera (Carl Zeiss Inc., Thornwood, NY).

Enzyme Assays of Immobilized HRP. *(A) Activity of Immobilized Enzymes as a Function of Enzyme Concentration*. An assay was performed with immobilized HRP in which the enzyme concentration was varied while the substrate concentration remained constant. Briefly, a solution of polymer **2** was prepared in deionized water to obtain a final polymer concentration of 12 mg/mL. 2,2-Dimethoxy-2-phenyl acetophenone (DMPA) (100 mg) was dissolved in 0.4 mL of *N*-vinylpyrrolidinone (NVP) as a photoinitiator/co-initiator system and added to the polymer solution. HRP in phosphate buffered solution was added to a series of polymer **2** solutions to obtain final enzyme concentrations of (2.0, 1.0, 0.5, 0.2, and 0.02) mg/mL, respectively. Polymer concentrations were adjusted to maintain 12 mg/mL in each enzyme solution. Two films of each polymer **2**/enzyme conjugate solution were spin cast (1000 rpm, 30 s) onto glass slides (2.5 cm^2). The resultant films were used as negative photoresists for UV photolithographic pattern development. Three separate regions of each film were patterned with 500 μm diameter dots of cross-linked polymer. The polymer micropatterns were rinsed with deionized water to remove any un-cross-linked polymer. The activity of HRP entrapped in micropatterned hydrogels of polymer **2** was monitored by fluorescent microscopy. Each region of patterned polymer dots was examined individually so that all assays were performed in triplicate. Hydrogen peroxide/water solution (H_2O_2, 3%) was added (5 μL) to each region of patterned polymer dots. The hydrogels were left in contact with the H_2O_2 for 1 min to allow complete absorption of the peroxide solution. Then the hydrogels were wetted with 5 μL of substrate solution (Amplex red, 5 mg/mL) and incubated for 2 min. The relative fluorescent intensities of the hydrogel-immobilized enzyme-catalyzed reactions were monitored over a 5-min period by fluorescence wide field microscopy (Olympus BX-60 equipped with a Texas Red fluorescent cube). As a control experiment, solutions of HRP (0.02−2.0) mg/mL in phosphate buffered solution were exposed to ultraviolet radiation under the same conditions that were used to prepare hydrogel micropatterns. These solutions were assayed with Amplex red and compared to hydrogel-immobilized HRP with respect to enzyme activity.

(B) Activity of Immobilized Enzymes as a Function of Substrate Concentration. To determine the sensitivity of the hydrogel-immobilized HRP to a range of substrate concentrations, an enzyme assay was carried out as described below. Polymer **2**/enzyme conjugate solutions were prepared in a 5:1 ratio as described above to give final HRP concentrations of 0.625 mg/mL. The polymer **2**/enzyme solutions were spin cast (1000 rpm, 30 s) onto eight clean glass slides (2.5 cm^2). Each polymer-coated slide was UV spot-cured to produce a patterned region with 500 μm diameter dots. The un-cross-linked polymer film was rinsed away with deionized water. Four solutions of the substrate (Amplex red) in deionized water were prepared with the following concentrations: (5.0, 2.5, 1.25, and 0.625) mg/mL. The micropatterned regions were treated with 3% hydrogen peroxide solution followed by a solution of Amplex red in the same manner described above. Fluorescent intensities of the hydrogel-immobilized enzymes were monitored as a function of substrate concentration with fluorescent microscopy.

Scheme 1. Fabrication of Hydrogel Micropatterns Using UV Photolithography



Results and Discussion

Preparation of Hydrogel Microstructures on the Glass Substrates. Poly[bis(methoxyethoxyethoxy) phosphazene] (**1**) (Figure 1) readily forms cross-linked hydrogels by exposure to gamma rays or ultraviolet to radiation in the presence of a photoinitator.[16,17,24] Thus, polymer **1** was chosen for the initial photolithography experiments. However, pattern development of polymer **1** met with only limited success. This was attributed to the relatively low sensitivity of the alkyl ether side groups to UV radiation. It was concluded that incorporation of a photoreactive side group in an equal ratio to the alkyl ether groups should improve pattern development and still permit hydrogel formation. Polymer **2** (Figure 1) was chosen because the photoreactivity of other polymers that contain cinnamyl groups is well-known.[25]

Micropatterns of polymer **2** were prepared in the manner described below. Polymer films were subjected to UV photolithography for 300−360 s as depicted in Scheme 1.

The regions of the polymer film exposed to ultraviolet radiation formed a cross-linked polymer network. Thus, polymer **2** became insoluble in common solvents such as tetrahydrofuran, ethanol, and water. The irradiated film was

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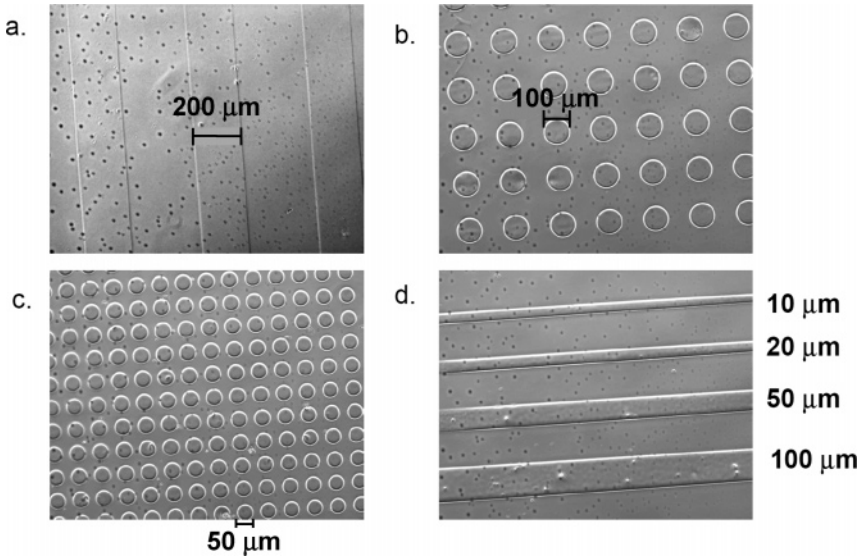

Figure 2. (a) Micropatterned lines of polymer **2** 100 μm width, 200 μm line spacing. (b) Micropatterned dot array of polymer **2** 100 μm diameter. (c) Micropatterned dot array of polymer **2** 50 μm diameter. (d) Micropatterned lines of polymer **2** in the range of 10−100 μm line width.

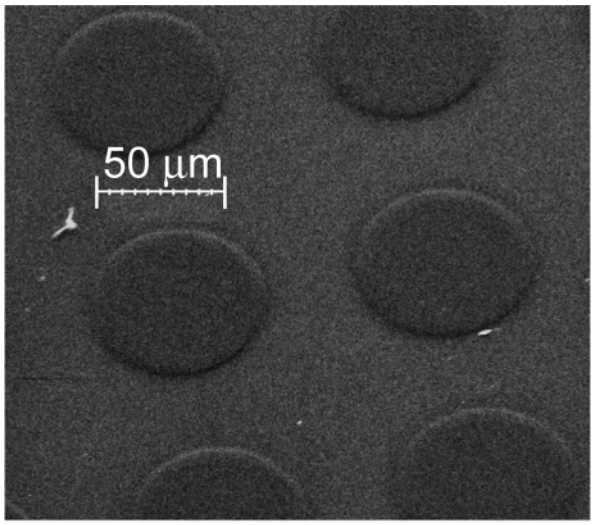

Figure 3. Scanning electron micrograph of hydrogel elements (50 μm in diameter) of polymer **2** within a micropatterned array.

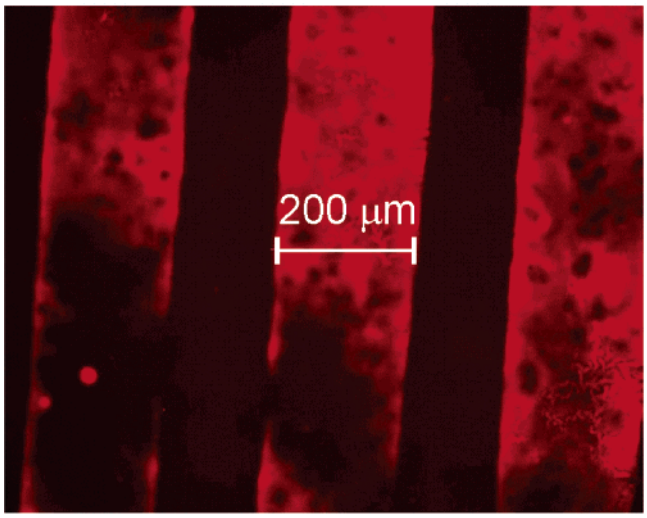

Figure 4. Fluorescence microscope image of polymer **2**/enzyme conjugate microstructure (line width = 300 μm, line spacing = 200 μm) after reaction between Amplex Red and H_2O_2. The pattern was fabricated by exposure to UV light for 200 s.

rinsed with water to remove un-cross-linked polymer so that only the projection of the transparent areas of the mask remained on the substrate surface. Polymer **2** was then examined by optical microscopy for micropattern development. The resultant patterns are shown in Figures 2a−d. Figure 2a shows that three-dimensional micropatterns, 200 μm in width, are readily formed with good resolution. Polymer **2** was also used to form micropattern arrays of different shapes from 100 μm down to 10 μm in size with good resolution (Figures 2b−d).

The morphology of these micropatterned arrays was investigated further with scanning electron microscopy. Figure 3 shows a scanning electron micrograph of a microarray in which each hydrogel element is 50 μm in diameter. Average microstructure heights were determined by profilometry measurements. The average heights of the water-swollen hydrogel micropatterns were approximately 30% more than the initial water-free un-cross-linked polymer films. The low amount of water swelling in this system indicates that hydrogels of polymer **2** possess a high cross-link density. This conclusion is supported by earlier studies from our laboratory that were focused on the water-absorption properties of MEEP hydrogels.[16] Another possible explanation for the lower swelling is that the hydrophobic cinnamyl side group limits the amount of water uptake in the cross-linked gel.

Enzyme Immobilization and Activity within Hydrogel Microstructures. To investigate the use of polyphosphazene hydrogel microstructures for the immobilization of biomolecules, a model enzyme, horseradish peroxidase (HRP), was immobilized within the gels. As one method to demonstrate that the encapsulated enzyme maintains its activity and can react with hydrogen peroxide, an HRP-catalyzed reaction inside the hydrogel microstructures was investigated by exposing enzyme-containing hydrogel microstructures to a substrate solution that contained hydrogen peroxide and Amplex red. When HRP maintains its activity, the HRP-catalyzed reaction between hydrogen peroxide and the nonfluorescent Amplex red produces fluorescent resorufin

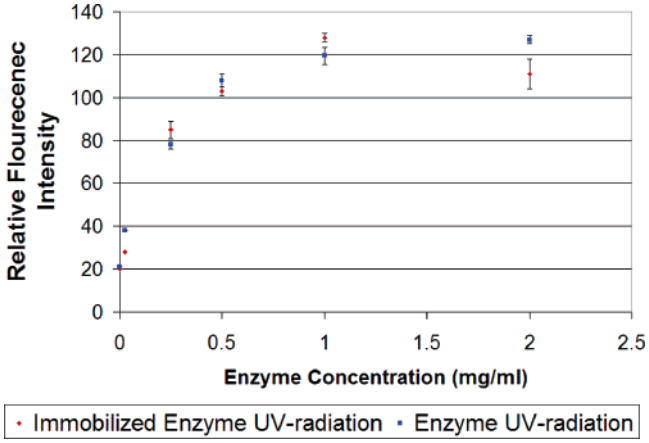

Figure 5. Dose−response curves of UV-irradiated HRP as a function of enzyme concentration, within hydrogels (open squares) and in the absence of hydrogels (closed diamonds).

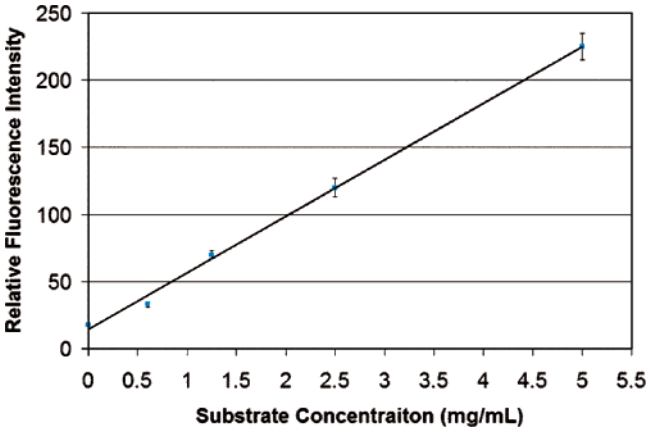

Figure 6. Activity of HRP immobilized within a hydrogel as a function of substrate concentration, measured by fluorescence intensity.

as a product. From the strong red fluorescence emitted from the enzyme-containing hydrogel microstructures, it was confirmed that encapsulated HRP molecules maintain their activity and were able to carry out the enzyme−substrate reaction. The micropattern appeared to be more fluorescent in certain regions due to the three-dimensional structure of the hydrogel (Figure 4).

The dose−response relationship between hydrogel-immobilized HRP and Amplex red was examined with enzyme assays using fluorescence microscopy. To investigate the integrity of immobilized HRP, an assay was performed in which enzyme activity was monitored as a function of enzyme concentration (0.02−2.0) mg/mL. The data shown in Figure 5 indicate that HRP immobilized within photolithographically patterned hydrogels retains enzymatic activity toward Amplex Red (5 mg/mL) at a level that is comparable to solutions of free enzyme that were exposed to the same dosage of ultraviolet radiation. To examine the sensitivity of this system, an additional assay was carried out in which the concentration of hydrogel-immobilized enzyme (0.625 mg/mL) was held constant. The activity of immobilized HRP toward Amplex red was monitored as a function of substrate concentration. The intensity of emitted fluorescence signals increased linearly with substrate concentration (0.625−5.0) mg/mL (Figure 6).

Conclusions

Polyphosphazene hydrogels that bear both methoxyethoxyethoxy and cinnamyl side groups were synthesized and evaluated for applications in UV microlithography. Hydrogel microstructures in the size range 50−500 μm were prepared using standard photolithographic techniques. Retention of enzymatic activity after the photolithography was demonstrated by the horseradish peroxidase-catalyzed reaction between hydrogen peroxide and Amplex red. Enzyme immobilization and activity within these micropatterned hydrogels was detected by fluorescence microscopy. The results of this study demonstrate the potential of polyphosphazene hydrogels for use in a range of microscale enzyme-based biosensors.

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