Phenol biosensor based on hydrogel microarrays entrapping tyrosinase and quantum dots

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This paper describes the use of microarray-based biosensor system for the determination of phenol. Microarrays based on poly(ethylene glycol)(PEG) hydrogel were prepared by photopatterning of a solution containing PEG diacrylate (PEG-DA), photoinitiator, tyrosinase, and CdSe/ZnS quantum dots (QDs). During photo-induced crosslinking, tyrosinase and QDs were entrapped within the hydrogel microarrays, making the hydrogel microarray fluorescent and responsive to phenol. The entrapped tyrosinase could carry out enzyme-catalyzed oxidation of phenol to produce quinones, which subsequently quenched the fluorescence of QDs within hydrogel microarray. The fluorescence intensity of the hydrogel microarrays decreased linearly according to phenol concentration and the detection limit of this system was found to be 1.0 μM. The microarray system presented in this study could be combined with a microfluidic device as an initial step to create a phenol-detecting "micro-total-analysis-system (μ-TAS)".

Introduction

Phenol and its derivatives are present in the environment as by-product contaminants from various industrial sectors and from wastewater treatment plants. Most of them are easily absorbed and have been shown to have detrimental effects on animal health.[1,2] In view of their high toxicity and persistence in the environment, the determination of phenolic compounds becomes an important subject. Commonly-used analytical methods to monitor the existence of phenolic compounds in the wastewater, such as chromatography,[3,4] are typically expensive and time-consuming, because most analysis must be performed in an off-site laboratory with associated high equipment and labor costs. Therefore, much effort has been devoted to the development of simple, sensitive, accurate and portable devices to determine the environmental presence and concentration of phenolic compounds. Among these, biosensors based on tyrosinase are simple and convenient tools for phenol assays due to their high sensitivity, effectiveness, and simplicity.[5,6] Tyrosinase is a copper-containing monooxygenase enzyme that catalyzes the conversion of phenolic substrates to catechol, which is then oxidized to quinone.[7,8] Because quinone can be electrochemically reduced to allow convenient low potential detection of phenolic compounds, amperometric detection has been widely used in most tyrosinase-based phenol biosensors.[9–12] However, optical biosensors based on absorbance and fluorescence detection have emerged as an alternative choice for detection of phenol due to several advantages over amperometric detection.[13,14] For example, the response mechanism of optical detection is very simple and reliable, and optical transducers do not disturb the reactions taking place in sample solutions. Moreover, optical sensors do not suffer from electrochemical interference from sample constituents and can be readily adapted for fiber-optic remote sensing. Abdullah *et al.* developed an optical biosensor based on the appearance of a maroon color product associated with the reaction of *o*-quinone and 3-methyl-2-benzothiazolinone hydrazone (MBTH),[15,16] and Wu *et al.* detected phenol optically using an oxygen-sensitive luminescent dye.[13] More recently, Yuan *et al.* utilized a quantum dot (QD)-based peroxidase hybrid system using the principle that quinone can quench the fluorescence of QDs.[17]

Despite recent efforts to develop optical tyrosinase-based biosensors, few studies have investigated miniaturized optical biosensors such as microarray- or microfluidic-based systems for the detection of phenolic compounds. The miniaturization of biosensors to the micrometre scale has various advantages over conventional analytic devices, including a smaller dead volume and sample consumption, lower cost, greater sensitivity, better reliability, and the ability to perform multiple reactions simultaneously. Miniaturized devices also provide a safe environment in terms of operator handling of toxic and reactive compounds, because nano/micro-volumes of sample are sufficient for analysis.

In this study, we developed a microarray-based optical biosensor to detect phenol using micropatterned poly(ethylene glycol)(PEG) hydrogels entrapping QDs and tyrosinase. On the one hand, highly crosslinked PEG hydrogel networks are capable of encapsulating proteins, and hydrogel-entrapped proteins can remain structurally intact and maintain their biological function due to the relatively inert aqueous environment within the hydrogel matrix.[18] The transparent nature of PEG hydrogels also makes them suitable for various optical detection schemes when they are used in biosensing applications.[19–21] On the other hand, QDs exhibit unique photophysical properties reflected by size-controlled fluorescence, high fluorescence quantum yields, stability against photobleaching, and sensitivity to surface ligands.[22,23] These properties make QDs ideal candidates for optical sensing applications and they have been utilized to detect

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various molecules such as glucose, *Escherichia coli*, copper ions, hydrogen sulfide, and phenol derivatives.[24,25] To develop miniaturized phenol biosensors exploiting the advantages of both hydrogels and QDs, we fabricated PEG hydrogel-based microarrays using photolithography, and quantitatively detected phenol by quinone-induced fluorescence quenching of QDs. Entrapped tyrosinase maintained its catalytic activity and could convert phenol into quinone within the hydrogel micropatterns. We also demonstrated that our phenol-sensitive hydrogel microarrays could be incorporated into a microfluidic device for potential use in a micro-total-analysis-system (μ-TAS) to detect phenol in the wastewater discharge.

Experimental

Materials

Poly(ethylene glycol) diacrylate (PEG-DA)(MW 575), photoinitiator 2-hydroxy-2-methylpropiophenone (HOMPP), phenol, sulfuric acid, n-heptane, carbon tetrachloride, 3-(trichlorosilyl)propyl methacrylate (TPM), Bradford reagent, and tyrosinase (from mushroom, 1000 units/mg) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). 8 μM solution of Core/shell CdSe/ZnS quantum dots (QD) (Qdot® ITK™, emission wavelength 585 nm) and phosphate buffered saline (PBS, 0.1 M, pH 7.4) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). QDs are coated with a polymer layer that has carboxyl group, which allows facile dispersion of the quantum dots in aqueous solutions with retention of their optical properties. Poly(dimethylsiloxane) (PDMS) elastomer was purchased as Dow Corning Sylgard 184 (Midland, MI, USA). The chrome sodalime photomask for photolithographic patterning of hydrogels was purchased from Advanced Reproductions (Andover, MA, USA).

Equipment

Photopolymerization was performed using a 365 nm, 300mW cm^{-2} light source (EXFO Omnicure UV spot lamp, Mississauga, Ontario, Canada). A Zeiss Axiovert 200 microscope equipped with an integrated color CCD camera (Carl Zeiss Inc., Thornwood, NY, USA) was used to obtain fluorescence images. Image analyses were performed using commercially available image analysis software (KS 300, Carl Zeiss Inc.). The fluorescence intensity of QDs in solution was investigated using a QM-1 fluorescence spectrometer (Photon Technologies International, Monmouth, NJ, USA). The presence of QDs within hydrogel micropatterns was confirmed using transmission electron microscopy (TEM). For TEM analysis, a hydrogel micropattern was fabricated on a copper grid, which was then inserted into a Tecnai F12 transmission electron microscope (Philips Electron Optics, Netherlands) operating at an acceleration voltage of 15 kV.

Fabrication of hydrogel microarray entrapping QDs and tyrosinase

Hydrogel microarrays entrapping QDs and tyrosinase were fabricated by photolithography. Purified PEG-DA was dissolved in PBS to form a 50% w/v solution. 10 μL of 2-hydroxy-2-methylpropiophenone was added to 1 ml of PEG-DA solution to initiate photopolymerization. The final precursor solution was prepared by adding 100 μL of QD solution and 1 mg of tyrosinase to 1 mL of PEG-DA and the photoinitiator solution. The 50 μL of precursor solution was then dropped onto glass substrates and covered with a photomask containing microarray patterns. Upon exposure to UV light for 1 s, only exposed regions underwent free-radical-induced gelation and became insoluble. The desired microstructures were obtained by washing away unreacted precursor solution with water so that only the hydrogel microarrays remained on the substrate surface. The glass surface was modified with TPM to improve the adhesion of hydrogel microarrays on the surface by creating surface-tethered methacrylate groups capable of covalent bonding with hydrogel during photopolymerization.[20] When hydrogel microarrays were prepared inside microchannels, the microchannels were filled with precursor solutions and then exposed to UV light for 1 s through a photomask that was aligned on the top of the glass slide. By flushing the channels with PBS, the desired hydrogel structures were obtained inside the microchannels. Fig. 1 shows a schematic diagram of the fabrication of the hydrogel microarray. The possibility of enzyme leaching from the hydrogel was investigated by determining the amount of released enzyme to the buffer solution using the Bradford assay. Until they were

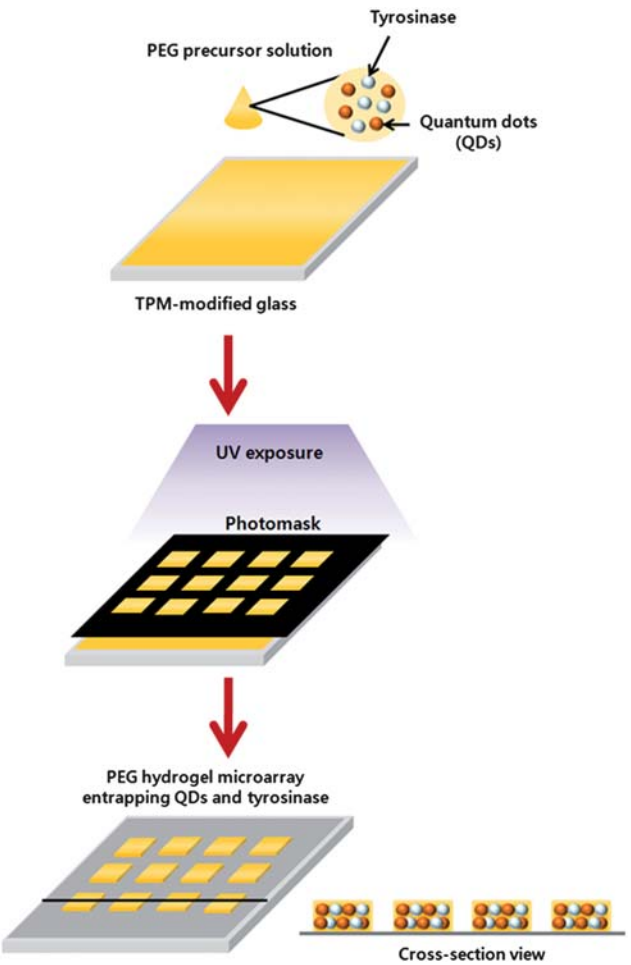

Fig. 1 Schematic diagram of preparing hydrogel microarrays entrapping QDs and tyrosinase.

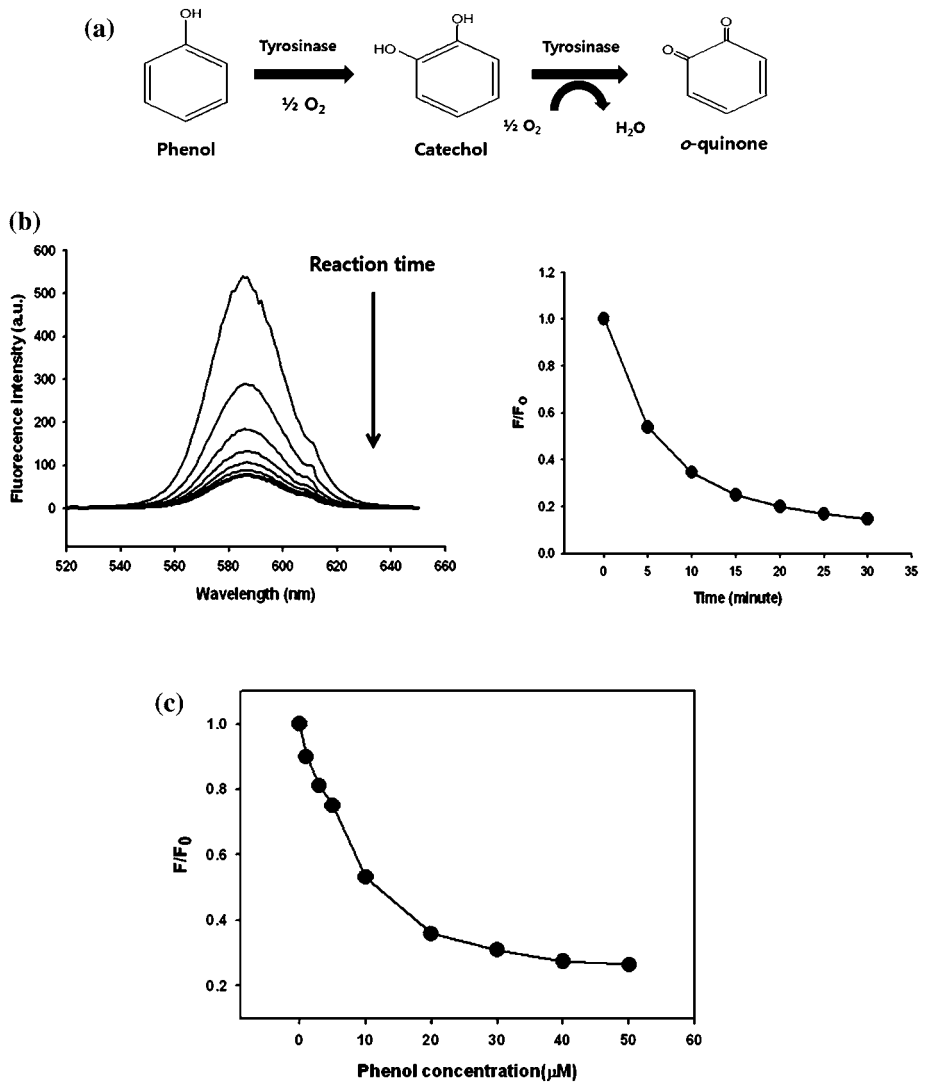

Fig. 2 Phenol assays using QDs and tyrosinase in free solution. (a) Reaction mechanism of tyrosinase-catalyzed oxidation of phenol to produce *o*-quinone, (b) Change of fluorescence spectra (left) and relationship between F/F_o and reaction time (right) by tyrosinase-catalyzed reaction of phenol (phenol concentration: 10 μM), (c) Relationship between F/F_o and phenol concentration (reaction time: 5 min).

used, all the hydrogel microarrays were immersed in PBS solution to prevent drying and stored in the dark at 4 °C.

Fabrication of a microfluidic device

Microchannels in PDMS were obtained by curing a 10 : 1 mixture of PDMS prepolymer and curing agent against a Si master that had a negative pattern of the desired microchannel defined with SU-8 50 negative photoresist (Microlithography Chemical Corp, Newton, MA).[26] After curing for 3 h at 60 °C, the PDMS replica was removed from the master and oxidized in an oxygen plasma (Harrick Scientific Co., Ossining, NY) with a glass slide for 1 min. Bringing the oxidized PDMS and glass slide into conformal contact resulted in an irreversible seal and thus formed an enclosed microchannel. These microchannels were treated with dilute TPM solution in perfluorooctane for 10 min immediately after sealing to enhance the adhesion of the hydrogel microarray to the microchannels. To make inlet and outlet ports in the microfluidic device, several holes were punched through the PDMS replica using a 16-gauge needle. Polyethylene tubes were inserted into these holes and then connected to a syringe pump (Harvard Apparatus, Holliston, MA) to complete the microfluidic device. This microfluidic device was mounted on the stage of a microscope for real-time fluorescence detection and imaging.

Phenol detection

Phenol dissolved in PBS (pH 7.4 and 30 °C) was reacted with tyrosinase that was immobilized within the hydrogel microarray or free tyrosinase. The change in fluorescence intensity of QDs due to the reaction between phenol and tyrosinase was monitored and quantified using fluorescence microscopy and fluorescence spectrometer. All the fluorescence quenching date was obtained based on the control samples that did not contain phenol. For phenol detection using hydrogel microarray, a drop of phenol solution was placed onto microarray and fluorescence intensity was measured after given intervals.

Results and discussion

Tyrosinase can catalyze the oxidation of phenolic compounds to catechols *via* hydroxylation with molecular oxygen; these catechols are subsequently dehydrogenated to *o*-quinone. The principle that we used to develop a QD-based biosensor for phenolic compounds in this study is quenching of QD fluorescence intensity by quinone intermediates (electron acceptors) produced from the tyrosinase-catalyzed oxidation of phenolic compounds as shown in Fig. 2a.[17,27] To validate this detection mechanism, 10 μM of phenol was reacted with tyrosinase in the presence of QDs and the resulting change in fluorescence emission intensity was monitored. Fig. 2b illustrates the fluorescence spectra and change in F/F_0 value (F and F_0 represent the fluorescence emission intensity at 585 nm in the presence and absence of phenol, respectively). As shown in these figures, the fluorescence intensity decreased with reaction time due to the formation of more quinones, which are capable of quenching the emission of QDs by adsorbing on the surfaces of QDs. Same trends were obtained from other QDs (Qdot® amino (PEG) quantum dots, Invitrogen Corp.) which had different surface chemistry (amine-terminated) and emission wavelength (525 nm), demonstrating that fluorescence quenching by quinone intermediates was general phenomena. Degree of fluorescence quenching reached maximum value after 30 min for all the range of phenol concentration. When the F/F_0 value was plotted against the phenol concentration (Fig. 2c), it decreased almost linearly up to a certain concentration of phenol (~10 μM), and reached a plateau at higher phenol concentrations, as expected for Michaelis–Menten kinetics. A plot of the Lineweaver–Burk equation yielded a maximum rate (V_{max}) of 0.012 s^{-1} and a Michaelis constant (K_m) of 0.13 mM. The detection limit of phenol was about 10 nM under these conditions.

After confirming that phenol can be successfully detected by quenching of QD fluorescence intensity by tyrosinase-catalyzed oxidation, hydrogel microarrays entrapping QDs and tyrosinase were fabricated by photolithography using the ability of PEG-DA to gel upon exposure to UV light. The formation of PEG hydrogels is based on the UV-initiated free-radical polymerization of the acrylate end group on PEG derivatives. That is, when exposed to UV light in the presence of a photoinitiator, acrylate groups form reactive free radical sites which react with each other, thus resulting in the formation of polyacrylate networks highly cross-linked with PEG. As shown in Fig. 3a, a clearly defined array of hydrogel microstructures was successfully fabricated with no residual polymer on the substrate. Based on the fluorescence images (Fig. 3b), which shows that only the hydrogel microarray region emitted fluorescence, it is clear that QDs were successfully entrapped within hydrogel microstructures and that the QD-containing precursor solution covered with photomask was completely removed by the developing process. Furthermore, the fluorescence intensity profile shown in Fig. 3b indicates that the fluorescence intensity of the hydrogel microarray was nearly identical at each spot, and fluorescence was almost homogeneous within the single hydrogel microstructure. These results imply that the amount of QDs within each of the hydrogel microstructures was similar and that QDs were evenly distributed in the hydrogel microstructure. Fig. 3c shows TEM images of a PEG hydrogel micropattern entrapping QDs that was fabricated on the TEM grid. Hydrogel

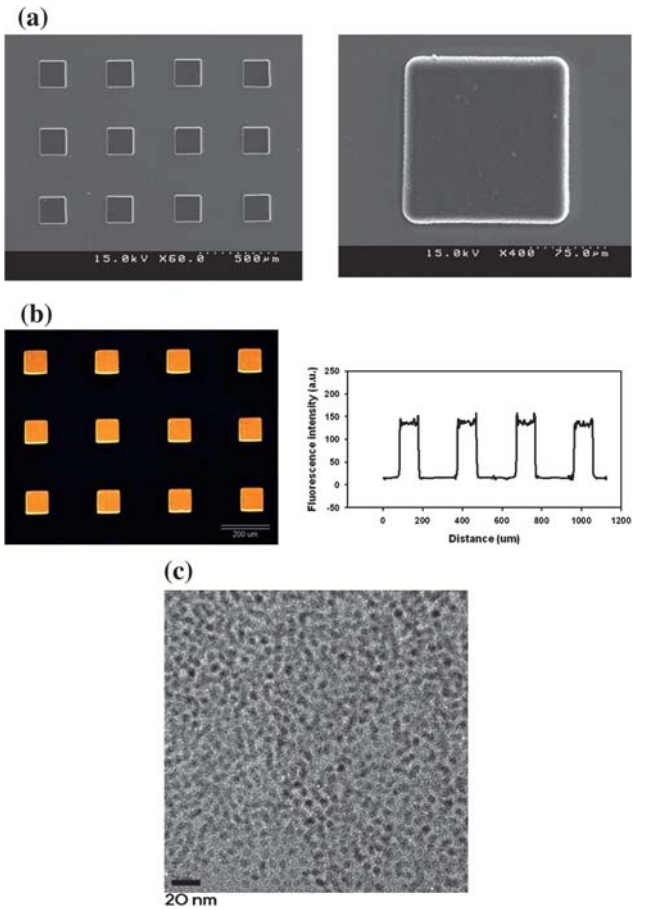

Fig. 3 PEG hydrogel microarray entrapping QDs and tyrosinase. (a) SEM images, (b) Fluorescence image (left) and fluorescence intensity profile across a hydrogel micropattern (right), (c) TEM image of QDs entrapped within hydrogel microarray.

microstructures remained on the TPM-modified substrates when exposed to an aqueous environment for a week due to covalent bonding between the hydrogel microstructures and the surface-tethered methacrylate groups on the substrate.

To demonstrate that the hydrogel microarray entrapping QDs and tyrosinase had the ability to detect phenol based on the same mechanism that was demonstrated in the solution state, phenol solution was reacted with the hydrogel microarray. It was found that there was a difference in fluorescence emission intensity from the hydrogel microarray before and after the enzyme-catalyzed reaction. That is, hydrogel microarrays exposed to phenol-containing solution had weaker fluorescence intensity than those prior to phenol introduction due to the quenching of QDs by the quinones produced as a result of the reaction between hydrogel-entrapped tyrosinase and phenol within the hydrogel microstructures (Fig. 4a). This result demonstrates that tyrosinase maintained its catalytic activity within the hydrogel microarray after the photopatterning process. Fig. 4b shows the time-dependent decrease of the F/F_0 value in the hydrogel microarray due to the reaction between the hydrogel-entrapped tyrosinase and 10 μM phenol solution. Similar to the results observed in the solution state, the fluorescent intensity decreased with reaction time. However, in terms of the initial slopes of the

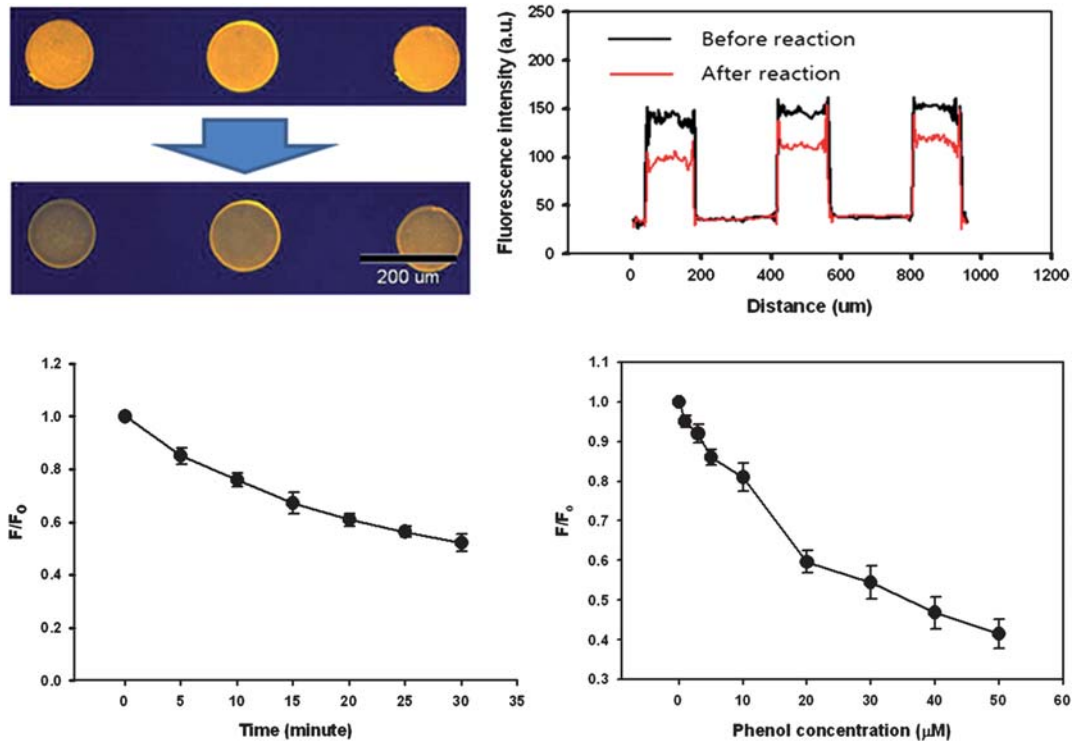

Fig. 4 Phenol assays using QDs and tyrosinase within PEG hydrogel microarrays. (a) Change of fluorescence intensity by reaction between phenol and tyrosinase, (b) Change of F/F_0 according to reaction time (phenol concentration: 10 μM), (c) Change of F/F_0 according to phenol concentration (reaction time: 5 min).

curves, which determine the reaction rate, the value obtained using the microarray system was about five-fold lower than that obtained in the free solution system, because in the microarray system, enzymes are entrapped within the crosslinked hydrogel, which acts as a mass transport barrier. Fig. 4c indicates that there was a linear correspondence between the F/F_0 values and the concentration of phenol in the two different ranges (0–20 μM and 20–50 μM) with a detection limit of 1.0 μM, which is the US Environmental Protection Agency (EPA)-estimated wastewater discharge limit of phenol. Values for V_{max} and the apparent K_m of entrapped tyrosinase were 0.0062 s^{-1} and 0.465 mM, respectively. The change in K_m value is related to the affinity of the enzyme for the substrate; a high apparent K_m value suggests that the enzyme has a lower affinity for the substrate. Therefore, the hydrogel-entrapped tyrosinase had a higher K_m value than the free enzyme. However, our previous study demonstrated that by using higher molecular weight of PEG, the accessibility of the substrate to the active site of the hydrogel-entrapped enzyme could be facilitated, resulting in the enhanced reaction rate.[28]

To investigate the reliability of the hydrogel microarray system for phenol sensing, three different microarray devices that were prepared independently were exposed to the same concentration of phenol and F/F_0 values were plotted as a function of reaction time (Fig. 5). The results obtained using different microarrays were consistent, indicating no major device-to-device error. One important advantage of using immobilized enzymes for biosensing over free enzymes is reusability. To demonstrate this, one hydrogel microarray was exposed to the same concentrations of phenol repeatedly for a one week period and the relative sensitivity obtained each day was compared with that of the first day. Between every reaction, the hydrogel microarray was incubated with PBS buffer solution to remove unreacted phenol and produced quinone. It was found that fluorescence intensity of QDs within hydrogel microarray completely recovered after washing process. Fig. 6a demonstrates that the hydrogel microarray maintained about 84% of its initial sensitivity after a week of period. During this experiment, we also confirmed that neither the enzymes nor the QDs leached out of the hydrogel microarray,

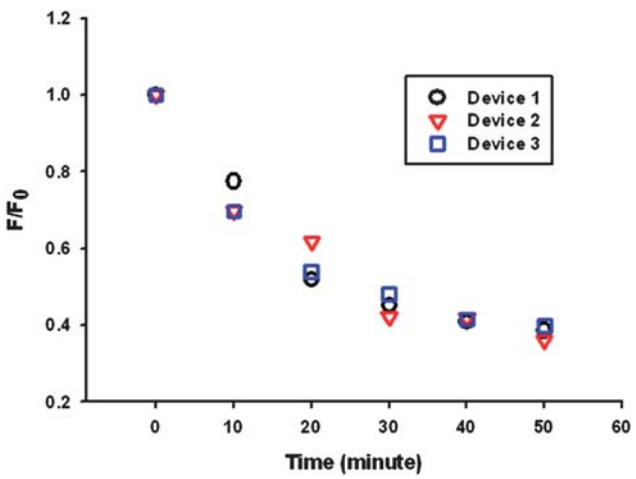

Fig. 5 Plot of F/F_0 as a function of reaction time for three independently-prepared microarray systems.

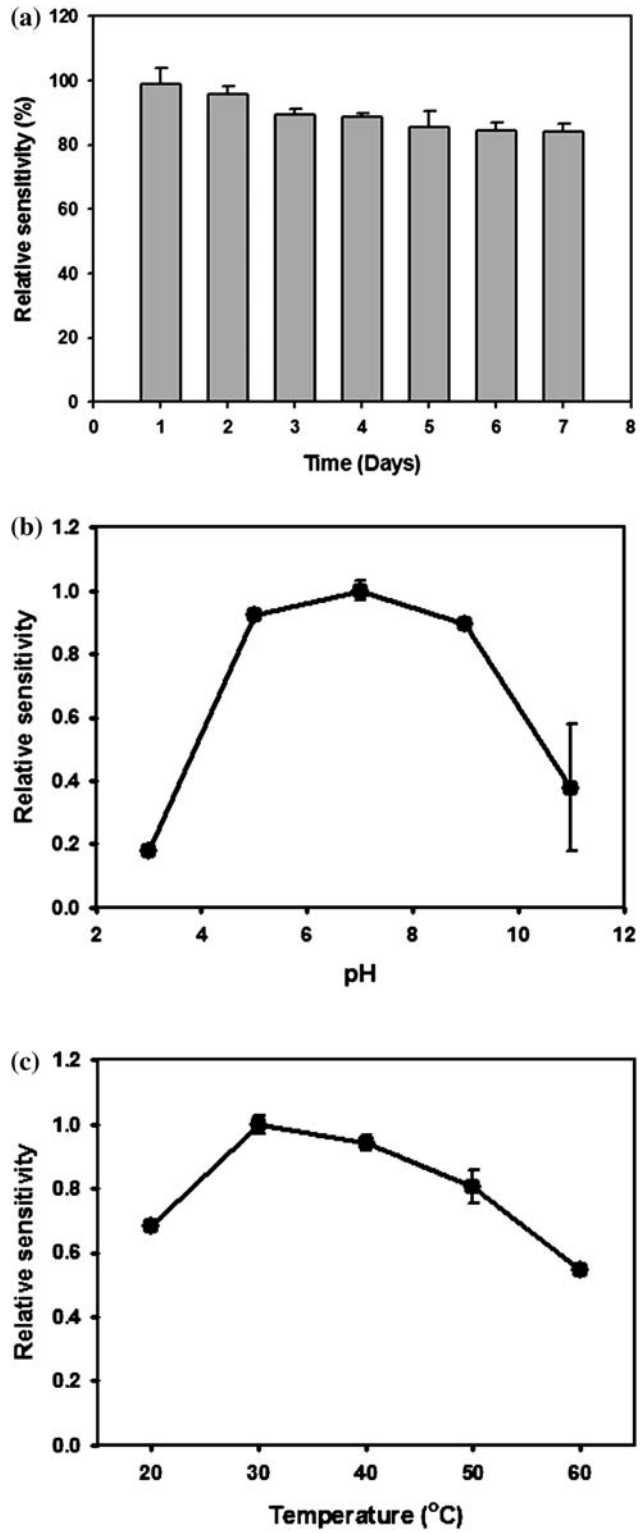

Fig. 6 Stability of microarray-based phenol biosensor. (a) Stability over a week, (b) Effect of pH on the sensitivity, (c) Effect of temperature on the sensitivity.

suggesting that the mesh size of PEG hydrogel used in this study was large enough to allow the diffusion of small molecules such as phenol, but was also small enough to prevent leaching of the encapsulated enzyme and QDs. Furthermore, effects of pH and temperature on the sensitivity of this device were investigated as other stability tests. As shown in Fig. 6b and 6c, these devices were stable in the range of pH 5–9 and 30–50 °C, maintaining more than 80% of best sensitivity.

On the basis of these results, we prepared hydrogel microstructures entrapping tyrosinase and QDs inside microchannels. Using a well-established method to create microfluidic devices, approximately 300 μm wide and 50 μm deep microchannels were created in PDMS and irreversibly sealed to a glass substrate to enable fluid to be pumped through the microchannels without leakage. To avoid the production of deformed structures, the introduced precursor solution was allowed to reach a stationary state and then exposed to UV light. Fig. 7a shows optical and fluorescent images of hydrogel microarrays entrapping tyrosinase and QDs inside a microchannel. Only UV-illuminated regions underwent photopolymerization and gelled inside the microchannel, and the fluorescence image shows that the unreacted precursor solution was completely removed by flushing the channel with PBS. After the hydrogel microarrays were fabricated inside the microchannels, all fluid was pumped into the microchannels at a flow rate of 5 μL min^{-1} using a syringe pump. At this flow rate, the hydrogel microstructures in the channels remained in place without buckling or migration. To investigate the phenol responses of hydrogel microarrays within the microfluidic device, 10 μM of phenol solution was introduced into the microchannels and fluorescence intensity was recorded at different time intervals. As shown by the fluorescence intensity profile and the resultant F/F_0 plot according to reaction time (Fig. 7b), the reaction between tyrosinase and phenol successfully quenched the hydrogel-entrapped QDs within the microfluidic device. When the F/F_0 value was plotted against the phenol concentration (Fig. 7c), it decreased linearly up to 10 μM phenol and reached a plateau at higher phenol concentrations. These results demonstrates the potential application of our system in a micro-total-analysis-system (μ-TAS) as a biosensor or microreactor.

Conclusion

We have described the design and implementation of a phenol biosensor based on hydrogel microarrays entrapping an enzyme and QDs. Photolithography created well-defined hydrogel microarrays without deactivation of entrapped tyrosinase, and commercially available CdSe/ZnS QDs were successfully exploited to detect phenol within the hydrogel microarrays using the fluorescence quenching ability of quinone produced by the tyrosinase-catalyzed oxidation of phenol. Although the hydrogel microarray system possessed a slower quenching rate and consequently, a lower detection limit than the free solution system due to the mass transport limitation of the hydrogel, it was sensitive enough to detect phenol at 1.0 μM. We also demonstrated that the hydrogel microarray could be combined with a microfluidic device for potential use in a micro-total-analysis-system (μ-TAS) as a biosensor. The hydrogel microarray approach described here can be extended to other biosensors where reactions between an enzyme and target analytes can quench QDs. Furthermore, isolation of different microarrays within different microfluidic channels can enable simultaneous

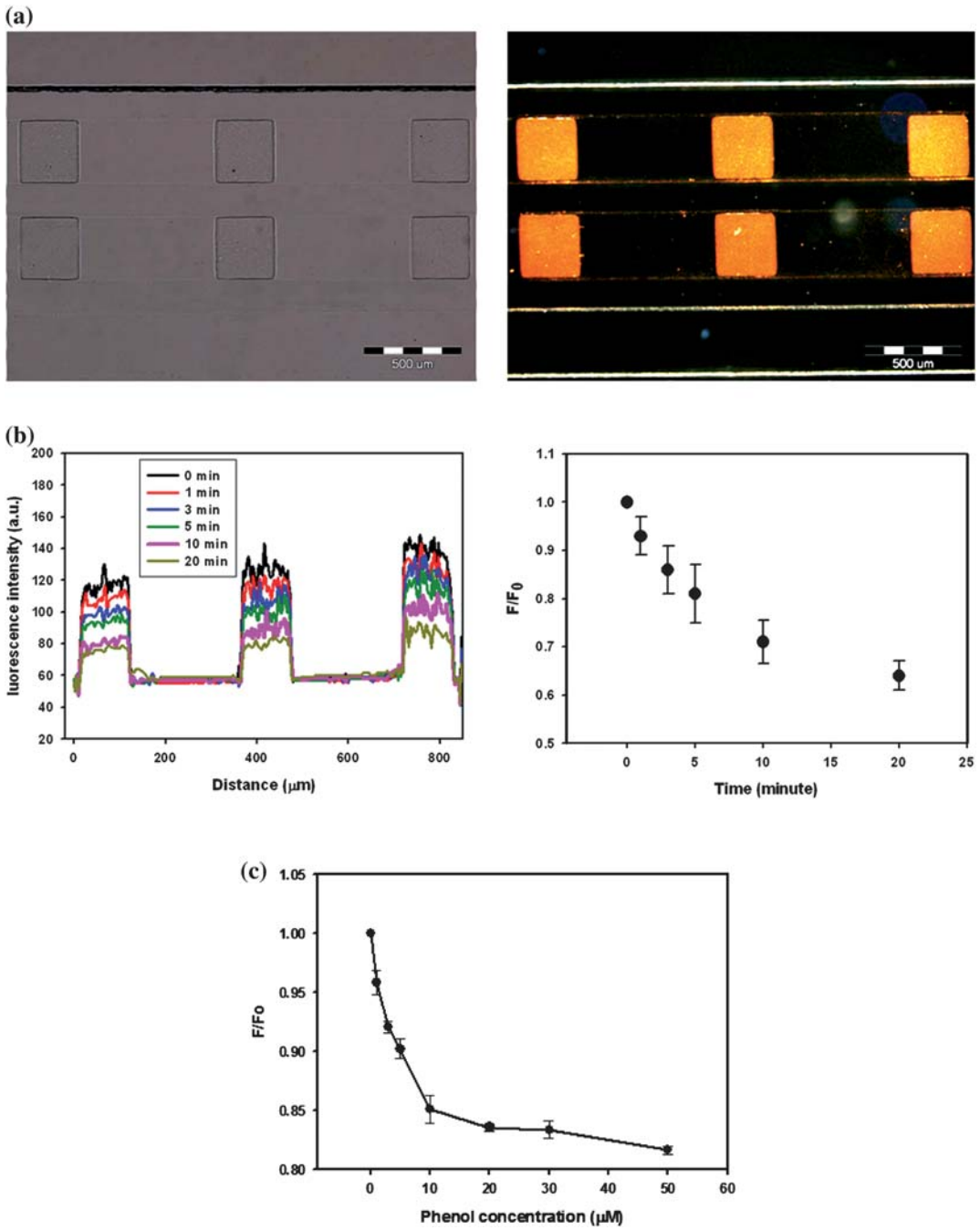

Fig. 7 Microfluidic devices incorporating phenol-sensitive hydrogel microarrays. (a) Optical and fluorescence images hydrogel microarrays entrapping tyrosinase and QDs inside a microchannel, (b) Fluorescence intensity profile (left) and the resultant F/F_0 plot (right) according to reaction time, (c) Change of F/F_0 according to phenol concentration within microchannels (reaction time: 5 min).

detection of multiple analytes and eliminate the possibility of cross-talk between enzymes.

Acknowledgements

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