An Organophosphate Sensor Based on Photo-crosslinked Hydrogel-entrapped *E. coli*

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This paper describes a whole cell sensor using *E. coli* entrapped within photocrosslinked hydrogel beads. Hydrogel beads containing organophosphorus hydrolase (OPH)-expressed *E. coli* were prepared by adding a hydrogel precursor solution containing the *E. coli* to an oil phase using a precision syringe pump, forming droplets, and photopolymerizing them. The beads showed good monodispersity with an average size of 1.2 mm. We detected organophosphates (OPs) using the beads. The detection relied on a pH-sensitive fluorescence dye that responds to protons produced from the intracellular OPH reaction with the OPs. This sensor could detect up to 80 μM of paraoxon with a detection limit of 3 μM . The enzyme activity of *E. coli* entrapped within the hydrogel beads showed stable enzyme activity for at least two weeks. This whole cell sensor will be implemented in a microfluidic system by directly photopolymerizing the hydrogel precursor solution within microfluidic channels.

Keywords Hydrogel, organophosphate, whole cell sensor

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Introduction

Organophosphates (OPs) have long been used as pesticides, insecticides and warfare reagents.^{1,2} Many of them have been known to be neurotoxins by inhibiting the acetylcholinesterase (AChE) enzyme in nerve cells.³ OPs can be detrimental to human health even at a low exposure level because of their severe toxicity. Organophosphate poisoning has been a serious problem in developing countries that heavily rely on pesticides and insecticides for increasing crop or food production.⁴ Therefore, scientists have been interested in developing an effective method for both detecting and detoxifying OPs.

It has been known that organophosphate hydrolase (OPH) enzyme efficiently hydrolyzes OPs.³ Because of its specificity, the OPH enzyme has been used for sensing⁵⁻⁷ and detoxifying OPs.^{8,9} However, a major problem of this naked OPH enzyme is that it shows a significant decrease in enzyme activity and stability after immobilization of the enzyme within or on a matrix.¹⁰ This problem arises from the sensitivity of the naked enzymes to environmental conditions.

Alternatively, several groups have reported OP sensors based on cellular OPH probes.¹¹⁻¹⁵ In these reports, cells were usually trapped behind a membrane¹³ or encapsulated within a monolithic agarose¹¹ or poly (vinyl alcohol) cryogel.¹⁵ Then, the product of the substrate reaction with OPH was detected using absorption spectrometry,¹¹ potentiometry,^{13,15} and amperometry.¹⁴ The entrapment of whole cells within polymer matrices provides a convenient preparation of sensors while avoiding the need for enzyme extraction and purification.^{16,17} In

addition, the activity of the intracellular enzyme can be stable for a longer time than that of the purified enzyme. The microbial density within the matrices can be conveniently controlled by changing the appropriate ratio of the polymer and microbials. Furthermore, the collection of microbial-containing hydrogel beads is facile after use.

Although the approaches mentioned above are effective in detecting certain OPs, they have limitations. First, the absorption and amperometric methods cannot be universally used, because not all of the OPs produce chromophores or electroactive species after hydrolysis. Thus, the observation of a change in the proton concentration resulting from OP hydrolysis is more applicable than the previous two detection methods in the sensing of various OPs. But, since the potentiometry is generally less sensitive than other detection methods, it is necessary to trace the change in the proton concentration using an alternative method to potentiometry. Secondly, the polymer matrices mentioned above for immobilizing cells are inconvenient to use for a portable microchip-sized sensor. For example, under the necessity of furnishing a high-throughput OP assay it is difficult to imagine using them within microfluidic channels having sizes ranging from tens to hundreds micrometers, because of difficulty to control the size and shape of those polymers.

In this report we describe a hydrogel-entrapped *E. coli* sensor for detecting OPs. The *E. coli*, which were genetically engineered to express OPH,¹⁸ were immobilized within hydrogel beads by photopolymerization. Detection of the protons produced from the intracellular OPH reaction with an OP relied on 5,6-carboxysemaphthofluorescein (SNAFL), a pH-sensitive fluorescence dye.¹⁹ Using these hydrogel bead sensors, we demonstrated that paraoxon, an OP neurotoxin, can be quantitatively detected with a comparable detection limit to other whole cell OP sensors. Although we tested these hydrogel

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beads for an OP sensor as a proof-of-concept experiment, the ease of size and shape control resulting from photopolymerizable property of hydrogels will enable them to be applied to a microchip size biosensor²⁰⁻²² and remediation.

Experimental

Chemicals

Poly(ethylene glycol) diacrylate (PEG-DA, $M_w = 575$), 2-hydroxy-2-methylpropiophenone, inhibitor remover resins, paraoxon, heavy mineral oil, and light mineral oil were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI). The stabilizer contained in the PEG-DA was removed using the inhibitor remover resins in a glass column. The PEG-DA was then stored in a 4°C refrigerator prior to use. Paraoxon was purified as described in a literature procedure.¹⁸ The purified paraoxon stock solution (1 mM) was dissolved in deionized water and stored in a freezer at -20°C. Tryptone and yeast extract were obtained from Becton Dickinson (Sparks, MD). 2-(*N*-cyclohexylamino)-ethanesulfonic acid was obtained from EM Science (Gibbstown, NJ). SNAFL was obtained from Life Technologies Co. (Eugene, OR). All aqueous solutions were prepared using 18 M Ω -cm water (Thermo-Barnstead, Waltham, MA).

Preparation of hydrogel beads containing *E. coli*

The *E. coli* strains (plasmid pJK33) have OPH enzyme expressed in cytoplasm.¹⁸ The procedures for the cell culture and the preparation of a hydrogel precursor solution are described in detail in the Supporting Information. Hydrogel beads containing bacteria cells were prepared as follows. First, 50 mL of a mixed mineral oil consisting of a heavy (50 v/v %) and a light mineral oil (50 v/v %) was poured into a 100-mL graduated cylinder. Then, 0.5 mL of a cell-suspended hydrogel precursor solution prepared as described in the Supporting Information was taken with a 1-mL plastic syringe equipped with a 32 G needle (0.11 mm inner diameter, BD, Franklin Lakes, NJ). Using a syringe pump (Harvard Apparatus, Boston, MA), the gel precursor solution was added to the mineral oil in the graduated cylinder at a rate of 10–70 μ L/min. A fiber-optic-equipped UV light source (365 nm, OMNICURE S-2000, EXFO Life Science, Ontario, Canada) was illuminated to polymerize the hydrogel precursor droplets formed in the mineral oil. Figure 1A shows a schematic diagram of the experimental set up for producing hydrogel beads. The UV exposure time was 5 s. After the polymerized beads were collected with a suction filter, a mineral oil on the bead surface was lightly wiped off with a paper towel, and then rinsed with a copious amount of 150 mM NaCl. The *E. coli*-containing hydrogel beads in a 150-mM NaCl solution were kept in a 4°C refrigerator prior to use.

Test of enzyme activity of hydrogel-entrapped *E. coli*

The enzyme activity of OPH was measured using paraoxon as the enzyme substrate. The absorbance of *p*-nitrophenol, the hydrolysis product of paraoxon, was observed at 400 nm ($\epsilon = 17000$) in a pH 9.0 buffer (see Fig. 2 for the hydrolysis reaction). Five different batches of hydrogel beads were prepared and their activities were examined using 100 μ M of paraoxon in 100 mM of a pH 9.0 CHES buffer at 40°C. For the measurement, eight hydrogel beads were added to a plastic cuvette. Immediately after the paraoxon solution was added to the cuvette, the absorption of the solution was measured at 400 nm every 30 s for 5 min using a UV-VIS spectrophotometer

Fig. 1 (A) Schematic diagram showing the experimental set up for generating hydrogel beads. The syringe pump was placed for the syringe needle to direct at a vertical angle of the mineral oil layer in a graduated cylinder. (B) Photo of hydrogel beads containing *E. coli* collected after photopolymerization. The average size of the bead was 1.2 ± 0.1 mm.

(8452A, Agilent, Santa Clara, CA), while the solution in the cuvette was stirred with a magnetic stirrer. The tests were repeated four more times. The intracellular enzyme activity was calculated by obtaining the initial slope of the time-dependent absorption curve. The average of five measurements was compared among the different batches. The effect of the number of beads on the reaction rate was also probed by observing the rate of the absorption change vs. the number of beads in the solution.

pH calibration of SNAFL dye

SNAFL dye has been known as a pH-sensitive, ratiometric fluorescence dye.¹⁹ The fluorescence intensity (FI) ratio of the two emission peaks at 540 nm (acid peak) and 635 nm (isosbestic point) depends on the solution pH. To construct a calibration curve of the FI ratio of SNAFL vs. solution pH, we relied on the OPH enzyme reaction to continuously change the solution pH. First, 100 μ M paraoxon was used to initiate the enzyme reaction in the presence of *E. coli*-containing beads and 1.3 μ M of SNAFL dye. The FI ratio of the solution was

measured using a spectrofluorometer (LS 50B, Perkin Elmer, Waltham, MA) equipped with a biokinetic accessory (Perkin Elmer) having a thermostat and stirrer. The thermostat temperature was set at 40°C. To measure the solution pH, a needle tip micro pH electrode (Model 9863BN, Thermo Scientific, Waltham, MA) was placed in the cuvette so as not to block the beam path in the spectrofluorometer. The excitation wavelength was set to 514 nm. While the FI ratio of the two emission peaks was collected at every 4 s interval for 2400 s, the pH data were acquired every 4 s using the computer-interfaced pH sensor. Using these measured data, the curve of the FI ratio of SNAFL vs. the pH was constructed.

Analysis of paraoxon using hydrogel beads

For the paraoxon analysis hydrogel beads containing *E. coli* were added to 2 mL of 1.3 μM SNAFL in 150 mM NaCl in a cuvette, and incubated for 30 min at 40°C. Eight beads were used for the paraoxon analysis if chosen from Batch 1, 3, and 5, while six and nine beads were used, respectively, if chosen from Batch 2 and 4. After the pH of the solution containing SNAFL and the hydrogel beads was adjusted to pH 9.5, the required amount of the paraoxon stock solution was added to obtain the final concentration range of 0 to 100 μM . Immediately after adding the paraoxon, the FI ratio of SNAFL was acquired as a function of time. All measurements were done three times for each of the paraoxon concentrations.

The *E. coli*-free hydrogel beads were prepared in the same way as *E. coli*-containing hydrogel beads, except that the hydrogel precursor solution did not contain the bacteria. Using the *E. coli*-free hydrogel beads, we repeated the measurements for the whole set of the paraoxon solutions in the same manner as described above. These data were then subtracted from the data of the corresponding concentration of the paraoxon solutions obtained with hydrogel beads containing *E. coli*.

Results and Discussion

Preparation of hydrogel beads containing E. coli

Previous reports have shown that photo-crosslinked hydrogels can be easily prepared in various shapes and sizes ranging from μm to mm scale.^{6,20,21,23,24} Russell et al. showed that enzymes could be entrapped within the hydrogel bead using a drop-in-oil method combined with an air sprayer.²⁴ We devised a new method to produce hydrogel beads in a more convenient way. Instead of using an air sprayer, we used a syringe pump to form a hydrogel droplet at the tip of the syringe needle, which depended on the force of gravity to pull it down to the oil. Also, the smallest diameter of a needle (32 G) was chosen so as to minimize the size of the droplet. The size of the falling droplet from the needle was determined by the balance between the surface tension of the hydrogel at the needle tip and the gravity force. The effect of the flow rate (10–70 $\mu\text{L}/\text{min}$) on the droplet size was not observed. This is because regardless of the flow rate the droplet should grow to the point where the gravity force overcomes the surface tension of the droplet. A flow rate of 20 $\mu\text{L}/\text{min}$ was adopted for the remainder of the experiments.

While the hydrogel droplets descended to the bottom of the oil layer, they were illuminated with 365 nm UV light for photopolymerization. The measured UV irradiance was 2 W/cm². The UV irradiance and the exposure time are important factors in converting C=C of the PEG-DA and controlling the mechanical strength of the hydrogel.²⁵ A higher conversion of the PEG-DA shows a better mechanical strength. The Ft-IR data showed that almost all of the C=C bonds in

PEG-DA were converted to C-C bonds upon exposure of the PEG-DA to 2 W/cm² UV irradiance for 1 s (Fig. S1, Supporting Information). Considering the potential decrease of the UV light intensity due to scattering, we chose an exposure time longer than 1 s. The UV exposure time depends on the descending time of the droplets in the oil layer, and could be controlled by changing the ratio of heavy mineral oil (85 cps at 25°C) to light mineral oil (23 cps at 25°C). The increase in the ratio of the heavy mineral oil prolonged the descending time of the hydrogel droplets, thus increasing the UV exposure time. The descending time of the hydrogel droplets was ~5 s at 50% v/v ratio of the two mineral oil. A photo of collected beads after photopolymerization is shown in Fig. 1B. The bead size was uniform with an average size of 1.2 ± 0.1 mm (the error indicates the standard deviation of the diameters measured from 130 beads).

The number of cells contained in the hydrogel precursor solution was 5×10^7 cells/mL. Since the volume of a hydrogel bead is 5.1×10^{-4} mL, the number of *E. coli* contained in the bead amounted to $\sim 2.5 \times 10^4$ cells.

Enzyme activity test of E. coli-containing hydrogel beads

The intracellular OPH activity was tested using 100 μM paraoxon in a pH 9.0 buffer. This enzyme catalyzes the hydrolysis of paraoxon to produce *p*-nitrophenol, which shows strong absorption at 400 nm. The initial rate of the absorbance change reflects the intracellular enzyme activity. A previous study showed that most of the *E. coli* cells entrapped within photocrosslinked hydrogel (the same hydrogel that was used in this research) are viable.²⁰ It is reasonable to predict that most of the cells in the hydrogel bead will be active participating in the enzymatic reaction.

Figure 2A shows the enzyme activities of five batches of hydrogel beads prepared on different days. The enzyme activity of each batch was the average of five measurements for the batch, expressed as absorbance unit (AU) per second. The error bar indicates the standard deviation of five measurements for each batch. While the enzyme activities of Batch 1, 3, and 5 were similar to each other, those of Batch 2 and 4 were significantly different from those of the other three batches. This interbatch variability probably arose from a variation in the expression level of the OPH enzyme in *E. coli* and the number of *E. coli* cells entrapped within the bead. Note that the intrabatch variability of the enzyme activity was less than 10%.

The variation of the enzyme activities among different batches could be resolved by controlling the number of hydrogel beads. The intracellular enzyme activities were examined by changing the number of beads, while all other experimental conditions were fixed. Figure 2B shows a plot of the change in the enzyme activity vs. the number of hydrogel beads of Batch 2 and 4. The graph suggests that the enzyme activity of six beads from Batch 2 and that of ten beads from Batch 4 were similar to the average enzyme activity (0.00058 ± 0.00006 AU/s) of the eight beads from the other three batches. These data suggest that the interbatch variability of the enzyme activity can be fixed by changing the number of beads. However, the potential problem of the interbatch variability could also be avoided by producing a large number of beads in one batch and using the same batch for the whole sets of experiments.

The enzyme activities of hydrogel-entrapped *E. coli* from three different batches were examined for a time period of two weeks. These beads were stored at 4°C prior to testing. To trace any change of the enzyme activities, we performed the hydrolysis reaction of 100 μM of paraoxon in a pH 9.0 CHES buffer, as described before. There was less than a 10% decrease

Fig. 2 (A) Graph showing the OPH enzyme activities of *E. coli* entrapped within hydrogel beads prepared in different batches. The enzyme activities were compared by tracing the absorption peak of *p*-nitrophenol, the cellular enzyme reaction product of paraoxon (100 μ M), at 400 nm in CHES buffer at pH 9.0. The enzyme reaction rate was expressed as the absorption unit/s. The error bar indicates the standard deviation of five measurements. (B) Plot of the enzyme reaction rate vs. the number of beads. The beads were chosen from Batch 2 and 4. The six beads from Batch 2 and the nine beads from Batch 4 show similar reaction rates to the eight beads from Batch 1, 3, and 5.

in the enzyme activity after one week, and a $\sim 15\%$ decrease after two weeks (Fig. S2, Supporting Information). Thus, no significant decrease in the enzyme activities was observed for at least two weeks. The stability of the intracellular enzyme activity of *E. coli* entrapped within hydrogel has been well known. Previously, it has been reported that the enzyme activity of the whole cell biosensor based on poly vinyl alcohol was stable for at least two months.¹⁵ The stability of these gel-based whole cell sensors is due to protection of the entrapped cells from compromising the cell membranes.

Paraoxon analysis using hydrogel beads

Paraoxon could be detected using the hydrogel-entrapped *E. coli* and the pH-sensitive fluorescent dye. The product generated from the OPH enzyme reaction bears an acidic -OH group, as shown in Fig. 3, and is partially deprotonated in aqueous solution, causing the pH change. We added SNAFL to the surrounding solution of hydrogel beads containing *E. coli* in

Fig. 3 Schematic diagram showing the hydrolysis reaction of organophosphates (OPs) catalyzed by organophosphate hydrolase (OPH) enzyme. Paraoxon: (1) R₁ and R₂ are ethoxy groups; (2) Y is 4-nitrophenolate.

Fig. 4 Calibration curve of the fluorescence intensity (FI) ratio of SNAFL vs. solution pH. The FI ratio was obtained by dividing the intensity at 540 nm (acidic peak) by the one at 645 nm (isosbestic point). The solution pH was continuously changed using the OPH enzyme reaction with paraoxon, which produces protons. The FI ratio and the solution pH were measured simultaneously as a function of time at the same time interval.

order to determine the pH change caused by the OPH enzyme reaction. One of the main advantages of using SNAFL as a pH sensor is that unlike many other pH-sensitive fluorescence dyes, its fluorescence intensity increases as the proton concentration increases. Since the SNAFL dye shows a pH-dependent peak at 540 nm and an isosbestic point at 635 nm, we measured the ratio of the two peaks to eliminate any errors that may be associated with an instrumental instability. To characterize the pH dependency of the FI ratio of the SNAFL dye, we traced the FI ratio and the solution pH at the same time while using the intracellular OPH enzyme reaction that produces protons. This eliminated the necessity of preparing a variety of SNAFL solutions at different pHs. Figure 4 shows the calibration curve of the FI ratio of SNAFL vs. pH.

The relationship between the two was obtained using the curve-fitting function in the Origin (Northampton, MA) software. The best fitting for this curve was a sigmoidal function,

$$R = (A_1 - A_2)/(1 + e^{(X - X_0)/dx}) + A_2. \quad (1)$$

Here, X and R represent the pH of the solution and the FI ratio of SNAFL, respectively. Other constants obtained from the curve fitting are displayed in Fig. 4. The FI ratio of the SNAFL dye changes more drastically in the basic region (pH 7.5 – 9.0)

Fig. 5 (A) Plot of the time-trace FI ratio of SNAFL in response to the OPH enzyme reaction with different concentrations of paraoxon (0–20 μM). These curves were obtained after subtracting the data collected using the *E. coli*-free hydrogel beads from the raw data collected using the *E. coli* containing hydrogel beads. The initial pH of the solution was set at 9.5, and all measurements were performed at 40°C. See the main text for detailed experimental conditions. (B) Calibration plot for the analysis of paraoxon. The average of steady-state FI values collected at between 3000 and 3600 s was plotted vs. paraoxon concentration. Each point indicates the average of three or four measurements, and the error bar indicates $\pm 1\sigma$.

than in the acidic region (below pH 7). This suggests that the optimum pH of the solution for detecting OPs should be $\sim$ pH 9.

Next, we examined whether the *E. coli*-containing beads can quantitatively indicate the presence of OPs by testing different concentrations of paraoxon solutions (0–100 μM). Figure 5A is representative data showing the FI ratio change of the SNAFL dye in response to various concentrations of paraoxon solutions ranging from 0 to 20 μM (Fig. S3, in Supporting Information for the full range of paraoxon concentration). The FI ratio given in Fig. 5A was displayed after the background signal was subtracted from the raw data. Each curve was collected over a time period of 3600 s. The FI ratio increased slowly in the beginning, but increased rapidly after 600 s. At $\sim$ 3000 s, the signals were stabilized. As shown in Fig. 5A, the steady state

signal increased with the increase of paraoxon concentration. This trend is attributed to the dependency of the amount of protons produced on the paraoxon concentration. For a quantitative analysis, the average of FI ratio values obtained between 3000 and 3600 s vs. the paraoxon concentration was plotted, as shown in Fig. 5B. The error bar indicates $\pm 1\sigma$ (standard deviation) of three or four measurements. The FI ratio showed a linear relationship to the paraoxon concentration up to 20 μM with a detection limit of 3 μM, but no longer beyond 20 μM. However, a nonlinear curve-fitting function made it possible to detect paraoxon as high as 80 μM.

The slow response time of hydrogel-entrapped *E. coli* resulted from the diffusion barriers by hydrogel. According to a previous report, the diffusion coefficient of tetrahydroamine (M_w : 444 g/mol), which has a similar order of molecular weight of paraoxon (M_w : 275 g/mol), within the hydrogel constructed from 20% PEG-DA in water, is $\sim 4 \times 10^{-7}$ cm²/s.²⁴ From the diffusion equation ($t = L^2/D$; D , diffusion coefficient; L , diffusion distance; t , diffusion time) the diffusion time of paraoxon into a 0.6 mm radius bead is estimated to be 9000 s. Since our data shows that the signal steady state was established within 3000 s, the diffusion coefficient of paraoxon in our experimental condition should be at least $3 \times$ larger than $\sim 4 \times 10^{-7}$ cm²/s. This faster diffusion coefficient of paraoxon in our experimental condition is probably due to the temperature effect on the diffusion coefficient. Considering that the Fickian diffusion coefficient is linearly proportional to the temperature,²⁶ our result is not surprising, because we measured it at 40°C.

The detection limit of this sensor is similar to those of the spectrofluorometric method using OPH-enzyme coated beads (8 μM),⁷ and the potentiometric method using *E. coli* with surface-expressed OPH enzymes (2 μM),¹³ but higher than that of using OPH-SNAFL conjugate immobilized within hydrogel (0.16 μM).²⁴ Considering that the OPH-SNAFL conjugate is not a whole cell enzyme, but a purified enzyme, our whole-cell sensor showed a comparable detection limit to other types of whole-cell sensors. When the activity of the used hydrogel-entrapped cells was examined, as described in the earlier section, we discovered that the cells lost >80% of their OPH activity. This suggests that the sensor cannot be reused.

As discussed above, the main drawbacks of the whole-cell sensor reported here was a long analysis time. It took more than 30 min to analyze one sample. This slow response time makes this sensor look less attractive than other whole-cell OP sensors. The diffusion time of small molecules within the gel can be significantly reduced by shrinking the hydrogel size. A previous report showed that *E. coli* could be entrapped within a 20-μm thick hydrogel micropatch, which was fabricated in a microfluidic channel using photolithography.²⁰ The diffusion time of small molecules into this gel is 900-times faster than that into the 0.6 mm hydrogel bead. This will improve the signal response time enormously. We plan to implement the hydrogel component in microfluidic channels, as shown in a previous report.²⁷

The slow response time can be further improved by eliminating the diffusion barrier formed by cell membranes. Previous reports have shown that the cellular surface-expressed OPH enzyme could increase the hydrolysis rate of an organophosphate by 7× compared with the intracellular OPH enzyme. Thus, the introduction of *E. coli* having surface-expressed OPH within the hydrogel could improve the reaction rate,²⁸ but a reduction of the gel size, as suggested above, will mainly solve the issue of the response time. Other than the OP sensor, the hydrogel beads containing *E. coli* can be applied to detoxify soils or water contaminated by OPs. The slow response time will not be a

critical issue for environmental remediation.

The selectivity of this type of whole-cell sensor for analytes can be another issue. Although the OPH enzyme shows specificity towards organophosphates, many other enzymes present in the cells can incorrectly respond to other components in the real sample. Problems associated with poor selectivity can be reduced by inactivating particular enzymes or leaking small molecular-weight cofactors through permeabilized cell membranes, which minimizes other side reactions.¹⁶

Conclusions

We demonstrated that hydrogel-entrapped *E. coli*, in which the OPH enzyme was expressed, could detect paraoxon quantitatively. This whole-cell sensor based on photo-crosslinkable hydrogel showed a stable intracellular enzyme activity for at least two weeks. The use of a pH-sensitive fluorescence dye and a photo-crosslinkable hydrogel will enable us to create a microfluidic array OP sensor that is compatible with a fluorescence microscope. We anticipate that this will allow us to detect OPs in a fast manner by running calibration standards and unknown samples simultaneously.

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Supporting Information

Detailed procedures for cell culture and for preparing for hydrogel precursor solution are included along with Figs. S1 – S3. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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