



Enzyme-containing hydrogel micropatterns serving a dual purpose of cell sequestration and metabolite detection

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ABSTRACT

The integration of sensing elements with small groups of cells is a critical step towards miniaturization of cell cultivation and analysis. This paper describes the development of an optical, enzyme-based biosensor for local detection of hydrogen peroxide (H_2O_2) secreted by stimulated macrophages. Photolithographic patterning of horseradish peroxidase (HRP)—containing poly (ethylene glycol) (PEG) hydrogel microstructures was used to create sensing structures on the glass surface. Importantly, enzyme-entrapping hydrogel micropatterns did not support protein or cell deposition and allowed to guide attachment of macrophages next to the sensing elements. Amplex Red, an organic molecule that becomes fluorescent in the presence of H_2O_2 and HRP, was either immobilized inside hydrogel elements alongside enzyme molecules or added into the cell culture media during cell activation. The production of H_2O_2 after mitogenic stimulation of macrophages resulted in appearance of fluorescence in the HRP-containing hydrogel microstructures, with fluorescence intensity being a strong function of analyte concentration. The novel cell culture system with integrated sensing elements described here may be enhanced in the future by incorporating additional biorecognition elements to enable multi-metabolite detection at the site of a cell.

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1. Introduction

Traditional approaches for measuring cellular metabolism involve cultivation of cells in Petri dishes or micro-titer plates, followed by periodic collection of cells or culture media for off-chip analysis. These conventional methods require a large number of cells and/or reagents, reveal little about metabolism dynamics and are not amenable to multiplexing. Considerable emphasis has been placed on developing new bioanalytical methods for monitoring analytes at the site of the cells as opposed to the bulk solution. Wightman's group pioneered the use of microelectrodes and developed fast scan cyclic voltammetry to monitor secretion of neurotransmitters from individual cells. (Troyer et al., 2002; Wightman, 2006) Kennedy and co-workers have published a series of reports describing amperometric measurements of glucose consumption in individual islets of Langerhans. (Jung et al., 1999; Jung et al., 2000; Kauri et al., 2003) This group reported the existence of glucose concentration gradients at the distance of 10–20 μm from the cells. Others have described development of biosensors for *in situ* detection of nitric oxide released by endothelial cells. (Borgmann et al., 2006; Isik and Schuhmann, 2006; Isik et al., 2007)

Despite the considerable success in developing bioanalytical approaches to monitor cellular activity, integration of cells and biosensors remains a challenge. Micromanipulators or scanning probe approaches are frequently employed to position biosensors (e.g. microelectrodes) at the site of the cells, complicating the experiments and making them operator-dependent. In other cases cells seeded onto surfaces containing biosensors attach indiscriminately and eliminate the possibility of monitoring a well-defined, small population of cells.

Microfabrication techniques, originally developed for microelectronics industry, provide ideal means of miniaturization of both biosensors and cell cultures. (Voldman et al., 1999; Folch and Toner, 2000; Park and Shuler, 2003) Microfabrication has previously been employed to create integrated microsensor-cell culture platforms for on-chip monitoring of acidification (pH) and oxygen consumption. (Park and Shuler, 2003; Sin et al., 2004) Reports describing the use of microfabricated sensors for detection of extracellular lactate and insulin have also appeared. (Cai et al., 2002; Cheng et al., 2006) Despite the use of microfabrication the methods for simple and effective integration of cells and miniature biosensors remain limited.

The present study explored the use of microfabricated biomaterial to define sites for cell attachment next to miniature biosensors. The biomaterial, PEG, is known to resist attachment of proteins or cells and is commonly employed to render surfaces non-fouling.

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Previously, we have demonstrated that micropatterning PEG hydrogels in a process similar to photolithography can be used to control placement of single cells on the surface with micrometer-scale resolution. (Revzin et al., 2003; Revzin et al., 2005) Importantly, in addition to excellent non-fouling properties, PEG hydrogels has been shown to be an excellent matrix for encapsulation of functional biorecognition elements such as enzymes. (Pishko et al., 1991) While multiple reports described the use of microfabricated PEG hydrogel structures for biosensing in general, (Revzin et al., 2001; Seong et al., 2003; Koh and Pishko, 2005; Allcock et al., 2006; Zguris and Pishko, 2006; Lee et al., 2008), relatively few of these reports focused on the detection of cellular metabolites. (Zguris and Pishko, 2006; Lee et al., 2008) Therefore, the goal in this study was to leverage dual functionality of PEG hydrogel microstructures as non-fouling surfaces and matrices for entrapment of enzymes in order to juxtapose cells with biosensors and subsequently detect extracellular metabolites. For our studies hydrogen peroxide (H_2O_2) was chosen. This metabolite is associated with oxidative stress and appears during inflammation as one of the reactive oxygen species (ROS). Given the central role of inflammation in a number of diseases including cancer, (Halliwell, 2007) alcohol liver injury (Albano, 2006) and diabetes, (Simmons, 2006) it is important to detect ROS in general and H_2O_2 in particular.

In the present work, HRP-containing hydrogel microstructures were photolithographically patterned on glass substrates to create cell culture surfaces capable of detecting H_2O_2 . When incubated with micropatterned surfaces, macrophages attached exclusively on the exposed glass regions and did not adhere to sensing microstructures. This allowed to juxtapose cells and biosensors, and enabled detection of macrophage-secreted H_2O_2 in the adjacent hydrogel structures. The present work focused on the proof-of-concept demonstration of metabolite detection from a small group of cells. Monitoring metabolic activity of a small cell population has relevance in tissue engineering or cancer research where detecting behavior of cells that are in contact/communication with their neighbors is important. Other applications, for example those related to phenotyping of immune cells, may require the need for single cell resolution and high-throughput screening. Importantly, the photolithographic patterning process described here has been employed to sequester single cells into high-density cell arrays (Revzin et al., 2003; Revzin et al., 2005) and, therefore, may be used in the future for high-throughput analysis of single cell function.

2. Materials and methods

2.1. Materials

Poly (ethylene glycol) diacrylate (PEG-DA, MW 575), 2-hydroxy-2methyl-propiophenone (photoinitiator), 99.9% toluene, hydrogen peroxide (35 wt% solution in water), and horseradish peroxidase (HRP), Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma–Aldrich (St Louis, MO). Amplex Red reagent was obtained from Invitrogen (Carlsbad, CA). 3-acryloxypropyl trichlorosilane was from Gelest, Inc. (Morrisville, PA). Dimethyl sulfoxide (DMSO) was from Pierce (Rockford, IL). Phosphate buffered saline (PBS, 0.1 M, pH 7.4) without calcium and magnesium was from Fisher Scientific and used to prepare aqueous solution. J774 Macrophages cell line was purchased from American Type Culture Collection (ATCC). All other chemicals were used without further purification.

2.2. Fabrication of PEG hydrogel microstructures

Amplex Red reagent was dissolved in analytically pure DMSO to reach the concentration of 5 mM and was stored in a desiccant-

loaded jar at -20°C . HRP stock solution was prepared by dissolving 10 mg HRP in 1 mL PBS to make the final concentration at 10 mg/mL. Pre-polymer PEG hydrogel solution contained PEG-diacrylate (DA) (MW 575) and 2% (v/v) photoinitiator (2-hydroxy-2-methyl-propiophenone). In this study, enzymes or probe reagents were incorporated into PEG hydrogel precursor solution and immobilized onto glass surface using PEG hydrogel photolithography. Two pre-polymer solutions were prepared: HRP-PEG-DA precursor solution was prepared by mixing HRP stock solution (10%, v/v) with PEG-DA prepolymer solution (90%, v/v); Amplex Red/HRP/PEG-DA precursor solution was prepared by mixing Amplex Red stock solution (1%, v/v) with HRP-PEG-DA prepolymer solution (99%, v/v). The final concentrations of Amplex Red and HRP in the mixture PEG-DA precursor solution were 50 μM and 0.1 mg/mL, respectively.

The layout of the micropattern was drafted using AutoCAD (Autodesk Inc.) and was then converted into a transparency-based photomask (CAD Art Services, Portland, Oregon). The pattern was later transferred from a transparency onto a chrome-coated soda-lime plate using standard photolithography and chrome etching protocols.

Before PEG gel immobilization, standard (75 mm $\times$ 25 mm) glass slides were modified with silane for PEG attachment following a protocol reported by us earlier. (Revzin et al., 2003) Sensing PEG hydrogel microstructures were fabricated according to previously reported protocols. Briefly, Amplex Red/HRP/PEG-DA polymer solution was spin-coated on the surface at 600 rpm for 5 s and exposed to UV with intensity 38 mW/cm² for 1.2 s for cross-linking. After exposure, surfaces were immersed in DI water for 10 min to remove unpolymerized PEG. The height of hydrogel microstructures could be varied from 5 to 100 μm by controlling the spin-coating rate, spin-coating time, and solution viscosity. Hydrogel features with minimal resolution of 15 μm are routinely patterned in our lab.

2.3. Cell seeding onto micropatterned surfaces

Mouse macrophage cells (J774A) were cultured at 37°C with 5% CO_2 in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were grown in suspension culture in 50 mL bioreactor tubes (Techno Plastic Products) on a rolling apparatus (Stovall). The cells were passaged two times a week by centrifuging and re-suspending in fresh culture media.

Prior to cell seeding, glass slides with HRP-containing hydrogel micropatterns were diced into 0.5 in. $\times$ 0.5 in. pieces using a diamond scribing pen and these smaller glass pieces were placed into P60 Petri dishes. 1 mL of cell suspension at 10^6 cells/mL was introduced into a Petri dish, allowing cells to sediment and interact with the micropatterned surface. Silicone gaskets (diameter: 6 mm; depth: 1 mm) were used to decrease the required volume of cell suspension to 50 μL . After 30 min of incubation cell suspension was aspirated and replaced with fresh DMEM. In the process of cell seeding, macrophages were able to attach only on silane-modified glass regions, becoming localized next to but not on top of enzyme-carrying hydrogel microstructures (see Fig. 1).

2.4. Detection of macrophage-secreted H_2O_2 using hydrogel microstructures

In order to induce production of H_2O_2 macrophages residing near HRP-containing PEG hydrogel structures were exposed for to PMA, a mitogenic stimulant dissolved in cell culture, for 1 or 3 h. In order to detect presence of the secreted metabolite

50 μM Amplex Red was added into cell culture medium after cell activation. As result, HRP-carrying hydrogel structures were expected to have a fluorescence signal corresponding in intensity to concentration of secreted H_2O_2 . Appearance of the fluores-

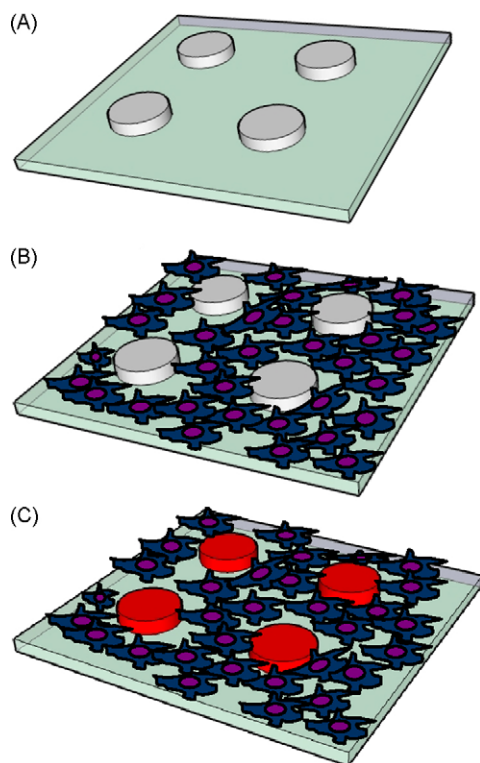


Fig. 1. Culturing cells with sensing hydrogel micropatterns. (A) HRP-containing PEG hydrogel micropatterns are fabricated on glass substrate. (B) Encapsulation of enzyme molecules does not diminish cell-resistive properties of PEG hydrogel. When incubated with micropatterned substrates macrophages attach next to sensing hydrogel structures. (C) Macrophages release H_2O_2 upon mitogenic stimulation. Release of this metabolite in the presence of Amplex Red results in an appearance of fluorescence signal in the adjacent PEG hydrogel structures.

cence signal in the hydrogel microstructures was monitored using a microscope as described below and was determined to occur rapidly (on the scale of minutes). In most of the experiments reported here, fluorescence images of sensing hydrogel structures were acquired 5 min after introducing Amplex Red into the Petri dish containing cells and hydrogel micropatterns. The calibration curves were constructed to convert fluorescence intensity signal into analyte concentration. In order to construct a calibration curve, HRP-containing hydrogel micropatterns without cells were exposed to H_2O_2 ranging in concentration from 0 to $20 \mu M$ and corresponding fluorescence signals were recorded as described below. All experiments were performed in triplicate ($n=3$) for statistical significance. In order to account for experimental variability that was likely due to oxidation of Amplex Red under ambient conditions, fluorescence signal in response to $1 \mu M$ H_2O_2 was used to normalize other data points of the calibration curve. Therefore, an experiment for detecting oxidative burst from macrophages consisted of two components performed in parallel: (1) detection of H_2O_2 release from macrophages using sensing hydrogel microstructures and (2) sensor response after exposure to a standard of $1 \mu M$ H_2O_2 and $50 \mu M$ of Amplex Red. In order to determine concentration of cell-secreted metabolite, fluorescence signal recorded from the cell cultures was normalized (divided) by the fluorescence signal generated from $1 \mu M$ H_2O_2 reference point. This normalized fluorescence value was then compared to a calibration curve that was created using the same normalization technique.

A Zeiss 200M epi-fluorescence microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY) equipped with an AxioCam MRm (CCD monochrome, $1300 \text{ pixels} \times 1030 \text{ pixels}$) was used in order to detect fluorescence signal from hydrogel microstructures.

During image acquisition objectives, camera and fluorescence filters were computer controlled through a PCI interface. A time-lapse function was set up to capture images at the rate of one frame per minute. Image acquisition and fluorescence analysis were carried out using AxioVision software (Carl Zeiss MicroImaging, Inc. Thornwood, NY). In this work, fluorescence associated with Amplex Red reagent was detected using excitation $550/25 \text{ nm}$ /emission $605/70 \text{ nm}$ filter. The excitation light source was a 120 W mercury lamp (X-cite 120, EXFO, Mississauga, Ontario, Canada). To acquire quality fluorescence images with right-scaled intensity and to avoid photobleaching, neutral density filters (ND) were inserted in the excitation light path based on the sample brightness. Usually, the same ND filter was utilized to a group of experiments requiring the intensity comparison. In cases when ND filters had to be modified the intensity was converted to a consistent scale based on their attenuation. All images were recorded under a $10\times$ objective.

Production of H_2O_2 by activated macrophages was also verified using a standard microplate reader. In these experiments, $200 \mu L$ of macrophage suspension at concentration of one million cells/mL were added into wells of a 96-well plate. The cells were then activated with PMA dissolved in DMEM for 1 or 3 h. Cells without stimulant served a negative control. After the desired incubation time at $37^\circ C$ with $5\% CO_2$, $2 \mu L$ of 5 mM Amplex Red were added into each well. After 5 min interval plates were analyzed using a fluorescence microplate reader (with excitation/emission wavelength of $563 \pm 5 \text{ nm}/587 \pm 5 \text{ nm}$) (Safire²™, Tecan Inc).

3. Results and discussion

We are reporting a strategy for intimate integration of cells with miniature biosensors. In this approach, enzyme-containing PEG hydrogel microstructures were photolithographically patterned on glass substrates to define regions conducive to cell adhesion. When seeded on the micropatterned surfaces, macrophages attached on glass domains in the immediate vicinity of sensing hydrogel structures (see Fig. 1). Entrapment of HRP in hydrogel microstructures allowed to detect oxidative burst (H_2O_2 production) of activated macrophages.

3.1. Non-fouling properties of enzyme-carrying hydrogel microstructures

Surface micropatterning with PEG hydrogels has been used extensively to create well-defined substrates for engineering cellular microenvironment. (Revzin et al., 2003; Suh et al., 2004) In parallel, hydrogel microstructures carrying enzymes or cells have been proposed for use as biosensors. (Koh et al., 2002; Koh et al., 2003; Koh and Pishko, 2005) The goal of the present study was to demonstrate that hydrogel microstructures can serve a dual purpose of defining and detecting cellular microenvironment. Fig. 2 shows representative HRP-containing PEG hydrogel micropatterns after incubation with $10 \mu M$ of H_2O_2 . As seen from Fig. 2A, dimensions of the hydrogel biosensors could be controlled from 20 to $500 \mu m$ diameter by photolithographic patterning. A compilation of focal plane slices presented in Fig. 2B underscores three-dimensionality of the hydrogel structures (thickness $\sim 42 \mu m$) and the uniform distribution of the enzyme molecules within these sensing structures.

It should be noted that in experiments described in Figs. 2 and 3, Amplex Red was entrapped inside hydrogel microstructures alongside HRP molecules. However, we found that encapsulated Amplex Red molecules became auto-fluorescent over time in cell culture media, likely due to degradation of this reagent into fluorescent byproducts (e.g. resorufin). Therefore, when detecting cell-secreted

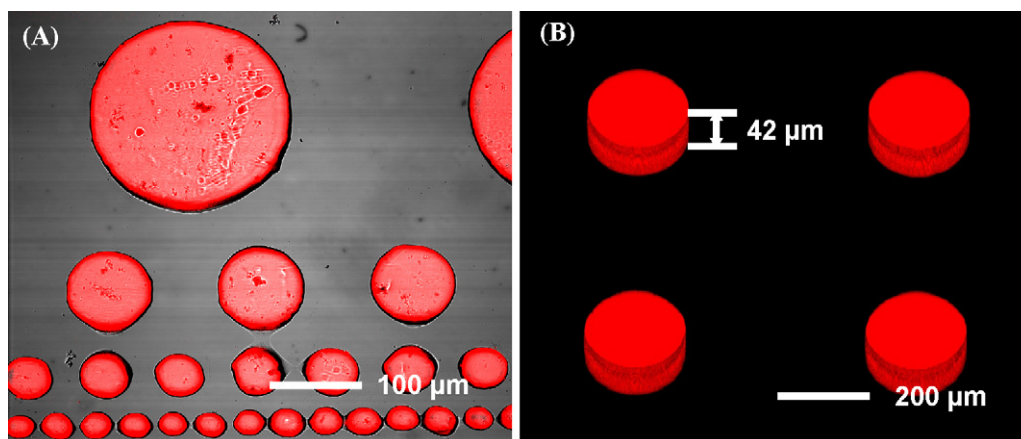


Fig. 2. (A) HRP-containing PEG hydrogel micropattern challenged with 10 μM H_2O_2 in the presence of Amplex Red. (B) Z-stack image of hydrogel micropatterns underscoring three-dimensionality of the structures and uniform distribution of HRP-molecules inside the gel.

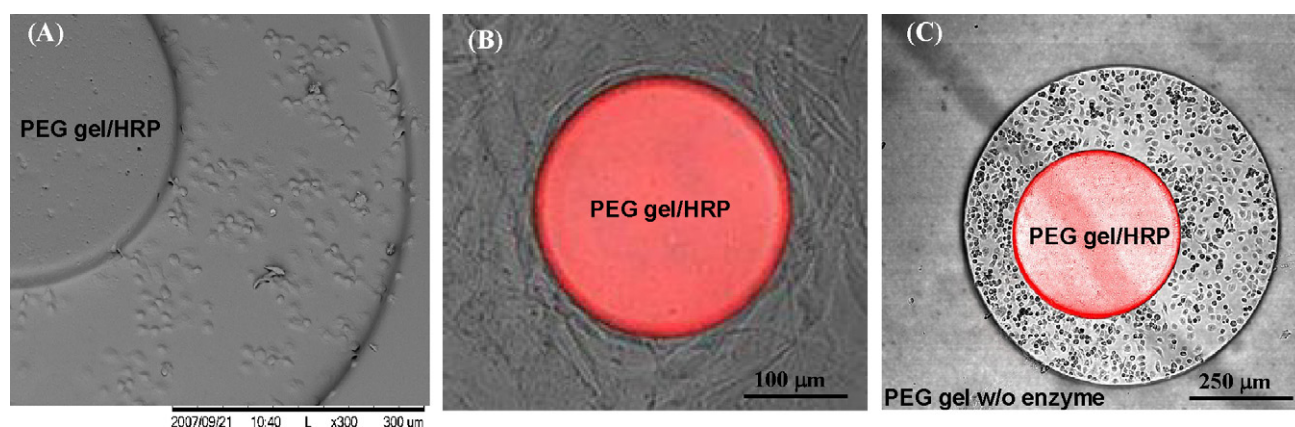


Fig. 3. (A) SEM image of HRP-containing hydrogel micropattern after incubation with macrophages. The cells attached exclusively on exposed glass regions and did not bind to hydrogel microstructures. (B) Attachment of 3T3 fibroblasts around HRP-containing hydrogel microstructure 250 μm in diameter. Addition of 10 μM H_2O_2 and 50 μM Amplex Red resulted in appearance of fluorescence in the gel. This image highlights dual role of hydrogel structures as sensing elements and non-fouling biomaterials. (C) Two sets of hydrogel microstructures fabricated in alignment on the same surface. HRP-containing cylindrical elements 250 μm in diameter were fabricated inside an array of enzyme-free hydrogel wells 1 mm in diameter. After addition of 10 μM H_2O_2 signal appeared only from HRP-containing gel structures. Both sets of microstructures were non-fouling and macrophage attachment was seen only on glass regions.

H_2O_2 , Amplex Red was added into cell culture media prior to imaging of the HRP-carrying hydrogel structures.

Effective juxtaposing of small groups of cells with sensing PEG hydrogel micropatterns requires that enzyme-entrapping hydrogel structures remain non-fouling. To demonstrate that incorporation of enzymes did not adversely affect non-fouling properties of PEG hydrogels, HRP-containing PEG precursor solution was photopatterned on glass substrates and incubated with macrophages or fibroblasts. These cell types are known to robustly produce matrix proteins and then attach on variety of surfaces via endogenous matrix proteins. As shown in Fig. 3A,B, upon interaction with glass substrates containing HRP-PEG micropatterns, macrophages and fibroblasts selectively attached on glass regions with limited or no adhesion observed on PEG domains. Importantly, introduction of exogenous H_2O_2 at 10 μM concentration into culture media resulted in optical (fluorescence) signal appearing from sensing hydrogel microstructures and pointed to retention of enzymatic activity of entrapped HRP. In addition, a multi-step PEG fabrication process could be employed to micropattern enzyme-carrying as well as enzyme-free hydrogel structures on the same surface.

Fig. 3C demonstrates one example where two different prepolymer solutions and two photomasks were used to create sensing HRP-containing PEG structures integrated into non-sensing (enzyme-free) PEG layer. As seen from Fig. 3C, only circular

(250 μm diameter) HRP-carrying hydrogel element shows fluorescence response when challenged with H_2O_2 . The enzyme-free PEG hydrogel layer shows no fluorescence and may be used as a negative control in metabolite detection experiments. Overall, Fig. 3 highlights the non-fouling nature of enzyme-carrying hydrogel microstructures and points to the possibility of integrating cells with sensing hydrogel micropatterns.

3.2. Characterization of sensing hydrogel microstructures

In our initial experiments Amplex Red was entrapped inside hydrogel microstructures along with HRP. However, Amplex Red is irreversibly oxidized during the HRP catalyzed breakdown of H_2O_2 (Towne et al., 2004), therefore, immobilized probe was expected to lose its ability to sense an analyte over extended periods of time. An alternative approach was to introduce soluble Amplex Red into cell culture media during detection. Fig. 4 shows a set of experiments where HRP-containing hydrogel micropatterns were exposed to 5 and 20 μM H_2O_2 in the presence of 50 μM Amplex Red. As seen from these data, fluorescence intensity of sensing elements challenged with a constant concentration of analyte increased as a function of time. Similar behavior of Amplex Red was reported previously (Kim et al., 2005). Based on the data presented in Fig. 4, we chose to record fluorescence intensity after 5 min of interaction

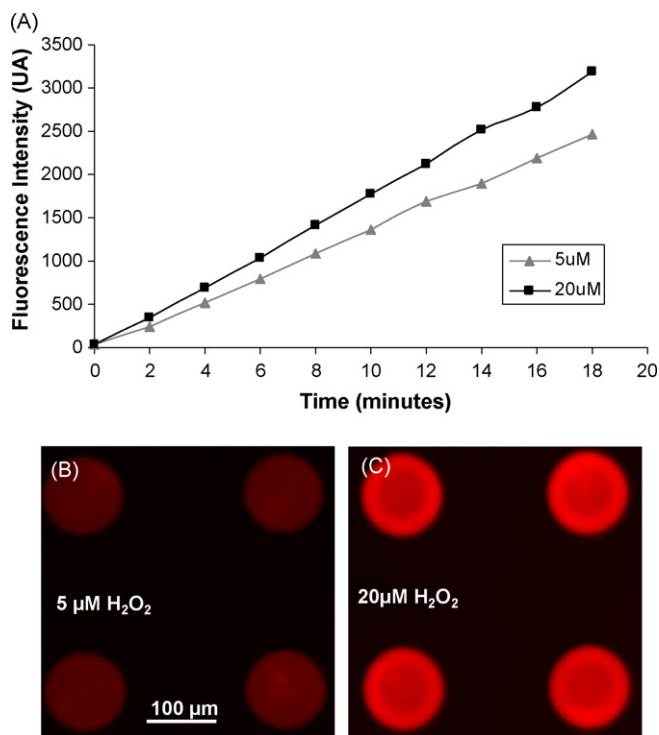


Fig. 4. (A) Time-dependent increase in fluorescence intensity of hydrogel microstructures in the presence of 5 or 20 μM H₂O₂ and 50 μM Amplex Red. (B and C) Fluorescence images of hydrogel microstructures after 5 min incubation with 5 μM H₂O₂ (B) and 20 μM H₂O₂ (C) in the presence of Amplex Red. When sensing for cell-secreted H₂O₂ images were collected 5 min after introduction of Amplex Red into cell culture medium.

between sensing hydrogel structures and Amplex Red. This time was short enough to potentially capture dynamics of H₂O₂ secretion by live cells, yet was long enough to result in a strong signal (~40% of maximum). Others have reported using similar time frame for detecting fluorescence signals based on Amplex Red oxidation (Rupcich and Brennan, 2003; Li and Jin, 2006; Zhou et al., 1997). This point is underscored by Fig. 4B,C that shows a difference in fluorescence signal after incubation of hydrogel sensors with 5 vs. 20 μM of H₂O₂ for 5 min.

The calibration curve of (H₂O₂) vs. fluorescence intensity was constructed in order to quantify the amount of metabolite produced by macrophages. The fluorescence signal increased linearly over the range of 0–20 μM as shown in Fig. 5, indicating that the concentration-dependant response could be derived from the micropatterned PEG hydrogel structures. Similar detection range was reported by Zhou et al., for a system where HRP and Amplex Red were present in solution (Zhou et al., 1997). It should be noted that when constructing calibration curve shown in Fig. 5 absolute fluorescence intensity signals were normalized by the sensor signal due to 1 μM H₂O₂. We found this normalization procedure necessary in order to account for experiment-to-experiment differences in absolute fluorescence signals. When detecting endogenous H₂O₂ from macrophages, there was always a control experiment where the same hydrogel micropatterns without cells were exposed to 1 μM H₂O₂ providing a reference point. This allowed us to compare normalized signal generated from cell-secreted metabolite to the normalized calibration curve.

As seen from Fig. 5, there appear to be two linear regions in the calibration curve associated with lower (inset) and higher concentration of the analyte. There could be several possible explanations for this behavior. Higher concentrations of hydrogen peroxide may result in changes of enzymatic activity due to HRP poisoning. In addition, Amplex Red is a highly complex molecule that is oxidized

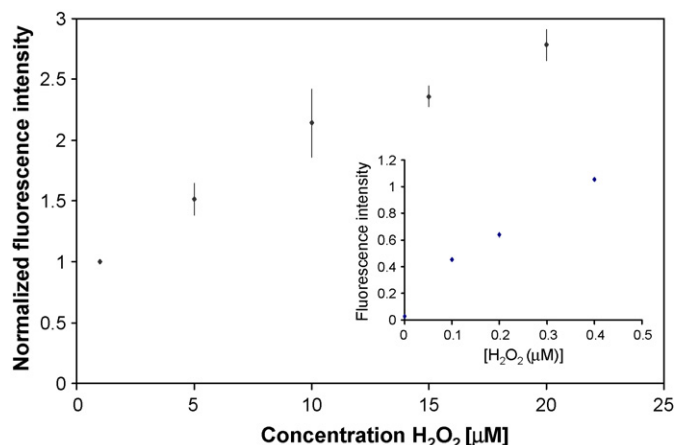


Fig. 5. Calibration curve correlating fluorescence intensity of the hydrogel microstructures to concentration of H₂O₂. Because of experiment-to-experiment variability in optical signal, all fluorescence intensity values were divided (normalized) by the signal for 1 μM H₂O₂. Inset shows signal vs. analyte relationship for low concentrations of H₂O₂.

into a fluorescent intermediate Resorufin that can in turn be further oxidized into a non-fluorescent reagent – Resazurin. (Towne et al., 2004) showed that oxidation of fluorescent Resorufin into a non-fluorescent reagent is not observed in the range of 0.01–~5 μM of H₂O₂ but becomes quite prominent beyond 10 μM. Therefore, it is also possible that the smaller slope of high H₂O₂ concentration range is due to the conversion of fluorescent Resorufin into a non-fluorescent byproduct. This behavior is accounted for by the calibration curve, and while important and noteworthy, does not detract from our ability to detect H₂O₂.

Activity and stability of the entrapped enzyme is a prerequisite to a functional biosensor. Previous study showed that protein lost 60% of its activity during the first minute of continuous exposure to UV light at 20 mW/cm² and 80% during the first 100 s (Sirkar and Pishko, 1998). In our study, a UV source of comparable intensity was used in short 1.2 s exposure burst. Therefore, we expect that most of entrapped HRP molecules remain enzymatically active after photopolymerization. It should also be noted that relatively short PEG-DA oligomers (MW575) contribute to formation of a highly cross-linked hydrogel with small (angstrom-scale) pore size that can prevent leaching out of the entrapped enzyme molecules (Mellott et al., 2001; Russell et al., 2001). To further ensure retention of enzyme molecules inside the gel microstructures these molecules can be acrylated using well established protocols (Kim et al., 2005) and covalently bound to the PEG hydrogel network upon UV exposure.

3.3. Detecting H₂O₂ production by macrophages using sensing hydrogel microstructures

To investigate the possibility of detecting endogenous H₂O₂, macrophages were introduced into the HRP-containing PEG hydrogel microstructures and stimulated with a mitogen, PMA, for either 1 or 3 h. This protocol was expected to result in oxidative burst and release of H₂O₂ from macrophages. Upon activation of cells, Amplex Red was introduced into cell culture medium and the fluorescence signal appearing in sensing hydrogel structures was recorded. Fig. 6A shows a representative brightfield/fluorescence image of unactivated macrophages cultured with hydrogel micropatterns in the presence of Amplex Red. While some fluorescence is observed, this should be considered as non-specific background signal that is most likely due to auto-oxidation of Amplex Red into fluorescent resorufin in the ambient environment. This point is

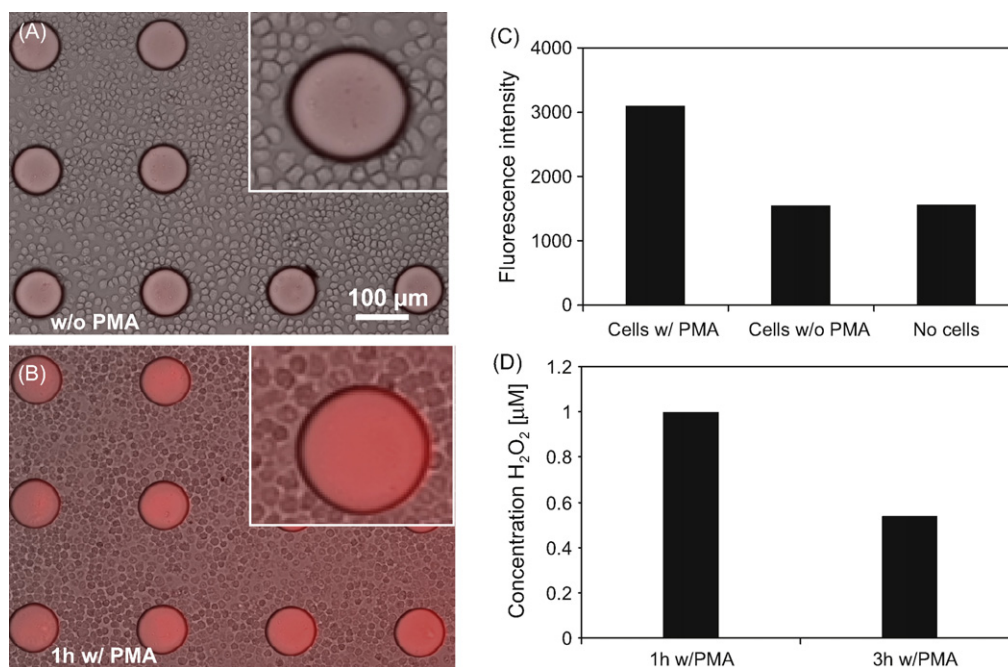


Fig. 6. (A and B) Brightfield/fluorescence images of macrophages adherent around HRP-containing PEG microstructures without (A) and with (B) PMA incubation. Exposure of cells to PMA was expected to trigger H₂O₂. (C) Fluorescence signal from sensing hydrogel elements incubated with macrophages (+) PMA, macrophages (–) PMA and no cells. These results show that minimal or no H₂O₂ release was observed in unactivated macrophages. (D) Using calibration curves presented in the previous Figure, concentration of macrophage-secreted H₂O₂ was determined after 1 h and 3 h of incubation with stimulant PMA.

underscored by the image of macrophages after 1 h incubation (Fig. 6B) that clearly shows higher fluorescence intensity in the hydrogel microstructures. Analysis of fluorescence intensity from these images, presented in Fig. 6C, shows that PMA activation of macrophages led to more than 2-fold increase in signal observed in hydrogel sensing elements, compared to macrophages not exposed to this stimulant. In addition, incubation of HRP-containing hydrogel micropatterns with Amplex Red but without cells resulted in fluorescence signals that were comparable to signals from unactivated macrophages (Fig. 6C). Images in Fig. 6A,B were taken 1.5 h after incubation so that cells did not have time to spread out and remained rounded.

Cellular secretion of H₂O₂ and the resultant fluorescence signal varied from one experiment to the next due to variability in the numbers and health of the macrophages from one experiment to another. However, the signal from stimulated cells normalized (divided) by signal from non-stimulated was consistent for three separate experiments ($n=3$) 2.1 ± 0.1 pointing to repeatability of the proposed sensing scheme. These results highlight the connection between activation of macrophages and the appearance of H₂O₂ signal in the adjacent hydrogel biosensors. In addition, standard spectrofluorometric analysis employing soluble HRP/Amplex Red (Zhou et al., 1997) was used to validate production of H₂O₂ by macrophages exposed to stimulant and lack of H₂O₂ secretion when the stimulant was absent.

Interestingly, we noted a difference in levels of H₂O₂ production by macrophages exposed to PMA (stimulant) for 1 vs. 3 h. The fluorescence signal detected in HRP-containing hydrogel micropatterns was converted to analyte concentration using a calibration curve presented in Fig. 5. The results of this experiment presented in Fig. 6D point to ~2 fold higher H₂O₂ production (1 vs. 0.55 μM) in the case of shorter stimulation. While this observation is counter-intuitive at first sight, it may be explained by a transient nature of oxidative burst whereby a high initial H₂O₂ production is followed by attenuation of levels of this metabolite. We are currently carrying out experiments to further test dynamics of oxidative burst of macrophages upon activation.

Overall, hydrogel micropatterns allowed to detect changes in levels of macrophage-secreted H₂O₂ *in situ*, in the Petri dish, without the need for media collection followed by off-line analysis. Placement of miniature biosensors next to cells, demonstrated in this manuscript, is envisioned as a step toward real-time monitoring of metabolites produced by small groups of cells or individual cells.

4. Conclusion

In this paper PEG hydrogel micropatterns were employed for the dual purpose of controlling cell attachment and detecting secreted cellular products. The enzyme-carrying hydrogel structures were found to retain their non-fouling properties and did not support attachment of macrophages or fibroblasts. This enabled precise and reproducible placement of cells adjacent to the biosensing structures and contributed to intimate integration of cells with biosensors. In a proof-of-concept experiment, HRP-containing hydrogel micropatterns were used to detect oxidative burst (H₂O₂ release) from activated macrophages. The non-fouling and sensing hydrogel micropatterns described here may be used in the future to detect metabolic activity of single cells arranged into high-density array format. In addition, the multi-step hydrogel photopatterning process may be used in the future to immobilize multiple sensing elements at the site of the cells, enabling detection of multiple metabolites in parallel.

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