Micropatterned Biosensing Surfaces for Detection of Cell-Secreted Inflammatory Signals

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22.1 Introduction

Inflammation is an important process that involves immune cell activation and production of inflammatory signals. Inflammation is a natural and necessary response of the body to injury or infections and inflammatory signals play an important role in recruiting immune cells to the site of the injury to clear out pathogens. However, dysregulated inflammation can contribute to a number of pathologies including diabetes [27], liver fibrosis/cirrhosis [19], and cancer [8]. The connection between inflammation and cancer has drawn considerable attention recently [5,6]. While the causative link between inflammation and cancer is still tenuous, studies have suggested that treatment with anti-inflammatory drugs can significantly reduce cancer risk [4]. Other studies described functional impairment of T-cells in patients with malignant tumors [7]. Given the importance of inflammation in cancer formation and metastasis, the immune cells that produce inflammatory signals are likely to become either targets of anticancer therapies or diagnostic correlates of cancer progression. Importantly, histological analysis of
the presence/absence of immune cells may not be sufficiently informative with regards to pro- or anti-inflammatory effects of these cells. We envision the need to monitor function of immune cells and are developing biosensors for cell function analysis.

Macrophages are immune cells that migrate from blood vessels and position themselves in various tissues. These cells are the first to respond to injury or infection happening in the tissue/organ and produce a battery of inflammatory molecules designed to destroy the invader and to recruit other immune cell types to the site [12]. Reactive oxygen species (ROS) and cytokines are some of the inflammatory molecules produced by activated macrophages. ROS is a collective term that refers to chemical species formed by incomplete reduction of oxygen and encompasses superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (⋅OH), and peroxynitrite (ONOO$^-$). H$_2$O$_2$ is the most stable of ROS types and serves as an important indicator of ROS production and inflammation [3].

Cytokines are small proteins secreted by the immune cells to help clear out the infection by recruiting more cells to the site of the injury and by inducing proliferation of immune cells. While macrophages are known to secrete a range of cytokines, tumor necrosis factor alpha (TNF-α) is one of the most important and abundant cytokines produced by these cells. TNF-α is an inflammatory cytokine that induces apoptosis and has been implicated in helping cancer cell survival [1]. It has been proposed that TNF-α secreted by the neighboring immune cells up-regulates expression of NF-κB in cancer cells, providing survival advantage to these cells [2,21]. Thus, detection and quantification of TNF-α production by the immune cells is an important area of research since TNF-α regulation represents a likely strategy for anticancer therapy development [2].

Given the importance of H$_2$O$_2$ and cytokines as markers of inflammation, a number of bioanalytical approaches have been developed for monitoring these molecules. The methods for detection of H$_2$O$_2$ include chemiluminescence [13], fluorescence [32], and electrochemistry [18]. These methods commonly rely on the use of horseradish peroxidase (HRP)—an enzyme that catalyzes breakdown of H$_2$O$_2$. One approach for monitoring this breakdown was described by Haugland and colleagues [32] who employed Amplex Red—a small molecule that becomes fluorescent upon HRP-catalyzed reduction of H$_2$O$_2$. In contrast to enzyme-based detection of H$_2$O$_2$, cytokines are most commonly detected using monoclonal antibodies (Abs) and sandwich immunoassays [17]. While robust and sensitive, traditional cytokine immunoassays are designed to analyze an amount of cytokine present in a given physiological sample (e.g., blood). These traditional assays reveal very little about cell type and cell-to-cell heterogeneity in cytokine secretion.

Over the past several years, our laboratory has been developing strategies for isolating specific cell types from a heterogeneous sample and detecting signaling molecules secreted by these cells [9,33–35]. We are particularly interested in designing cell–biosensor interface to allow placement of cells in the immediate vicinity of sensing elements. The proximity of cells and biosensors should translate into sensitive detection of cell-secreted analytes. One approach for designing cell–biosensor interface employed by us is poly(ethylene glycol) (PEG) hydrogel photolithography whereby non-fouling biomaterial (PEG) is micropatterned to define cell adhesive and nonadhesive regions on a substrate [23]. For example, creating PEG hydrogel microwells allowed organizing cells into high-density single-cell arrays on cell culture surfaces such as glass [24,25]. In this chapter, we describe how to convert non-fouling gel into a functional/sensing layer by incorporating sensing molecules inside and around hydrogel microstructures.

In one strategy, glass attachment sites inside PEG hydrogel microwells were modified with cytokine-specific Abs (e.g., anti-TNF-α) in order to capture cells and detect cell-secreted cytokine molecules in the same microwells. This approach allows establishing production of TNF-α by concrete individual macrophages and will enable cell-by-cell analysis of heterogeneity in cytokine release by these cells. Importantly, in addition to preventing cell attachment, PEG hydrogels provide an excellent matrix for entrapment of enzymes [11,22]. Therefore, hydrogel microstructures not only are useful for guiding cell attachment but also may contain enzymes (biorecognition molecules) and may serve as sensing elements [31]. To highlight this, we describe the development of HRP-carrying hydrogel microstructures that can be used for detection of H$_2$O$_2$ produced by macrophages. Finally, as a future application of micropatterned cell–biosensor surfaces, we describe PEG hydrogel microwells that may be used for detecting both TNF-α and H$_2$O$_2$ secreted by the same cells.
22.2 Materials and Methods

22.2.1 Materials

PEG diacrylate (PEG-DA, MW 575) (Catalog #437441), 2-hydroxy-2-methyl-propiophenone (photoinitiator) (Catalog #H55103), 99.9% toluene (Catalog #34866), hydrogen peroxide (35 wt% solution in water) (Catalog #349887), HRP (Catalog #P6782), phorbol 12-myristate 13-acetate (PMA) (Catalog #P1585), and lipopolysaccharide (LPS) (Catalog #L2630) were purchased from Sigma-Aldrich (St. Louis, MO). Amplex Red reagent (Catalog #A12222) and Alexa Fluor-546 Streptavidin (Catalog #S11225) were obtained from Invitrogen (Carlsbad, CA). 3-Acryloxypropyl trichlorosilane (Catalog #SIA0199.0) was purchased from Gelest, Inc. (Morrisville, PA). Dimethyl sulfoxide (DMSO) (Catalog #20688) was from Pierce (Rockford, IL). PBS (0.1 M, pH 7.4) without calcium and magnesium (Catalog #MT-21-031-CM) was obtained from Fisher Scientific and used to prepare aqueous solution. J774 Macrophages cell line (Catalog #HB-197) was purchased from American Type Culture Collection (ATCC, Manassas, VA). Anti-mouse TNF-α (Catalog #AFC-410-NA), biotinylated anti-mouse TNF-α (biotinylated TNF-α) (Catalog #BAF410), and recombinant TNF-α (Catalog #410MT) were purchased from R&D Systems (Minneapolis, MN). Neutravidin fluorescein FITC conjugated (Catalog #31006) was from Pierce. Silicone gaskets were purchased from Grace Bio-Labs (Bend, OR). Chromium etchant (CR-4S) and gold etchant (Au-5) were from Cyantek Corporation (Fremont, CA). Positive photoresist (AZ 5214-E IR) and developer solution (AZ300 MIF) were bought from Mays Chemical (Indianapolis, IN). Poly(dimethylsiloxane) (PDMS) and its curing agents (MSDS #01064291) were purchased from Dow Corning (Midland, MI). SU-8 (Catalog # Y 131269) was from MicroChem Corp (Newton, MA). All these chemicals were used as received without further purification.

22.2.2 Methods

22.2.2.1 Silane Modification of Glass Substrates to Ensure Attachment of Hydrogel Microstructures

Standard 3×1 in. glass slides were cleaned by immersion in 3:1 mixture of sulfuric acid and hydrogen peroxide. Right before silane modification, the clean glass substrates were exposed to O₂ plasma (YES-R3, San Jose, CA) for 3 min at 300 W. Then they were immediately placed into a glass dish containing toluene and moved into an N₂-filled glove bag. Two percentage (v/v) of 3-acryloxypropyl trichlorosilane was added to the dish inside the bag. Silane self-assembly was allowed to proceed for 1 h under N₂ atmosphere, after which the substrates were removed, rinsed in fresh toluene, and dried using N₂ gas. The substrates were then placed in an oven for 3 h at 100°C to cure the silane layer. This silanization procedure has been used by us previously for anchoring PEG hydrogel microstructures to glass substrates [23,25].

22.2.2.2 Fabricating Hydrogel Microstructures with Integrated Biorecognition Molecules

The layout of the micropattern was drafted using AutoCAD (Autodesk Inc.) and was then converted into a transparency-based photomask (CAD Art Services, Portland, OR). The pattern was later transferred from a transparency onto a chrome-coated soda lime plate (Nano Film Company, Westlake Villaget, CA) using standard photolithography and chrome-etching protocols. Briefly, positive resist (AZ 5214-E IR) was spin-coated at 800 rpm for 10 s followed by 4000 rpm for 30 s, resulting in formation of a 4 μm thick layer of photoresist. The photoresist-coated substrate was soft-baked on a hot plate (make/model) at 100°C for 105 s, then placed in contact with a photomask and exposed to 365 nm, 10 mW/cm² UV source for 55 s using Canon PLA-501F mask aligner. The substrate was then placed into a developer solution (AZ300 MIF) for 4 min. After the development step, the substrate was immersed in chrome-etching solution (1:1 v/v mixture of CR-4S etchant with H₂O) for 2 min.
Figure 22.1 shows a step-by-step process for fabricating hydrogel microstructures on glass substrates. The first step in the process involved spin-coating photosensitive prepolymer solution onto the substrate using a spinner from Headway Research Inc. (Garland, TX). This prepolymer was prepared by dissolving a photoinitiator—2% (v/v) (2-hydroxy-2-methyl-propiophenone)—in PEG-DA (MW 575). To fully mix the PEG-DA with the photoinitiator, the mixture was stirred for 15 min prior to use. When fabricating H$_2$O$_2$-sensing hydrogel structures, HRP solution (10 mg/mL in 1× PBS) was added to the PEG prepolymer to make it 10% v/v. In cases when fluorogenic reagent Amplex Red was to be integrated into hydrogel structures, it was added to the HRP–PEG prepolymer solution to make it 1% v/v in prepolymer.

After adding sensing molecules (HRP and Amplex Red) into the PEG prepolymer, this solution was spin-coated at 600 rpm for 5 s and then exposed to UV (60 mW/cm$^2$) from Omnicure 1000 (EXFO, Mississauga, Ontario, Canada) for 1.2 s. This resulted in free-radical polymerization/cross-linking of exposed regions of the prepolymer. After exposure, surfaces were immersed in distilled water for 2 min to remove the unpolymerized PEG and dried with N$_2$. This photopatterning process, described in Figure 22.1, resulted in physical entrapment of HRP and Amplex Red molecules inside the hydrogel microstructures.

In order to immobilize cytokine-sensing Abs, glass slides with micropatterned hydrogel structures were immersed in 0.2 mg/mL solution of anti-TNF-α, supplement with Tween 20 (0.005%) for 1 h. Ab molecules were physically adsorbed onto silanized glass regions but did not deposit on non-fouling PEG hydrogel structures.

### 22.2.2.3 Seeding Cells on Micropatterned Biosensing 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 resuspending in fresh culture media.
Figure 22.1B shows the scheme of seeding cells onto surfaces micropatterned with PEG hydrogels. Prior to cell seeding, glass slides with HRP-containing hydrogel micropatterns were diced into 0.5 × 0.5 in. pieces using a diamond scribing pen. These smaller glass pieces were placed into P35 Petri dishes. Fifty microliters of cell suspension at 10^6 cells/mL concentration 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 limit the cell suspension volume to 50 μL. After 30 min of incubation, the cell suspension was aspirated and replaced with fresh DMEM. In the process of cell seeding, macrophages were able to attach onto silane-modified glass regions, becoming localized next to but not on top of enzyme-carrying hydrogel microstructures. This particular macrophage cell line did not require adhesive ligands for attachment to silane-modified glass. When working with less “sticky” cells, we have precoated micropatterned surfaces with extracellular matrix (ECM) proteins (e.g., collagen (I) or fibronectin) to promote cell attachment to glass [14,25].

### 22.2.2.4 Detection of Macrophage-Secreted H₂O₂ Using Hydrogel Microstructures

As described in Section 22.2.2.2, Amplex Red, a reagent that fluoresces during HRP-catalyzed breakdown of H₂O₂, can be impregnated into PEG microstructures. However, we found this approach to be impractical for cell sensing because of oxidation/autofluorescence and leaching out of this reagent. Therefore, when detecting cell-secreted H₂O₂, Amplex Red added into cell culture media. Prior to cell detection experiments, Amplex Red was dissolved in analytically pure DMSO to reach a concentration of 5 mM and was aliquoted and stored in a desiccant-loaded jar at −20°C. One aliquot was used per experiment for quality assurance due to the easy oxidation of Amplex Red. In order to induce secretion of H₂O₂, macrophages residing around HRP-containing PEG hydrogel structures were exposed for 1 h to a mitogen (PMA) dissolved in cell culture media. In order to visualize cellular production of H₂O₂ and its breakdown by HRP inside gel microstructures, 50 μM Amplex Red was added into the cell culture medium after cell activation (Figure 22.1B). As a result, HRP-carrying hydrogel structures were expected to become fluorescence with signal intensity corresponding to concentration of secreted H₂O₂. The appearance of fluorescence signal in the hydrogel microstructures was monitored using a fluorescence microscope as described later 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 H₂O₂ ranging in concentration from 0 to 20 μM and corresponding fluorescence signals were recorded as described later. All experiments were performed in triplicate (n = 3) for statistical significance. In order to account for variability in fluorescence signal from one experiment to the next—an artifact of autooxidation of Amplex Red under ambient conditions—the fluorescence response to 1 μM H₂O₂ 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 H₂O₂ release from macrophages using sensing hydrogel microstructures, and (2) sensor response after exposure to a standard of 1 μM H₂O₂ and 50 μM of Amplex Red.

In order to determine concentration of a cell-secreted product, fluorescence signal recorded from the cell cultures was normalized (divided) by the fluorescence signal generated from 1 μM H₂O₂ reference point. This normalized fluorescence value was then compared with a calibration curve that was created using the same normalization technique. A Zeiss 200M epifluorescence microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY) equipped with an AxioCam MRm (charge-coupled device [CCD] monochrome, 1300 × 1030 pixels) was used in order to detect fluorescence signal from hydrogel microstructures. 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 nm/emission 605 ± 35 nm filter (550 ± 25 nm describes a band-pass filter with optimal excitation of 550 nm and bandwidth of 50 nm).
22.2.2.5 Integrating Hydrogel Micropatterns inside Microfluidic Devices

It is beneficial to integrate micropatterned biosensing surfaces inside a microfluidic device to permit easy reagent exchange and to perform experiments in a small volume. A PDMS-based microfluidic device was fabricated using standard soft-lithography approaches and is described in detail elsewhere [34]. Briefly, a transparency photomask (CAD Art Services, Bandon, OR) was generated based on AutoCAD drawing of the device. This photomask was then employed to micropattern SU-8 on a 4 in. silicon wafer in order to create a negative replica fluidic network. PDMS was mixed 10:1 with a curing agent, poured onto a Si wafer containing SU-8 features and cured for 12 h at 60°C. The elastomer with embedded channel architecture was released and inlet–outlet holes were punched with a blunt 16-gauge needle. This microfluidic device consisted of two flow chambers with width × length × height dimensions of 3 × 10 × 0.1 mm and a network of independently addressed auxiliary channels. The volume of each flow chamber was ∼3 μL. The auxiliary channels were used to apply negative pressure (vacuum suction) to the PDMS mold and reversibly secure it on top of the micropatterned substrate.

Silicone tubing (1/32 in. I.D., Fisher) was used to connect a 10 mL syringe to the outlet through a metal insert cut from a 20-gauge needle. A blunt, shortened 20-gauge needle carrying a plastic hub was positioned at the inlet. The pressure-driven steady flow was generated by a precision syringe pump (Harvard Apparatus, Boston, MA).

22.2.2.6 Detection of Macrophage-Secreted TNF-α in Hydrogel Microwells

For analysis at single-cell level, arrays of hydrogel microwells with 30 μm in diameter were fabricated on glass. These microwells were made comparable to the size of single macrophages (∼15–20 μm in diameter) to ensure capture of individual cells. For TNF-α detection, anti-TNF-α Ab molecules were physically adsorbed onto microwell arrays. The walls of microwells were composed of non-fouling PEG hydrogel and Ab molecules deposited selectively on the glass attachment sites at the bottom of the microwells. Macrophages express receptors for Fc domains on Ab molecules and therefore readily attach inside the microwells. These micropatterned surfaces were enclosed inside a PDMS microfluidic device. Prior to the introduction of cells, a PDMS device containing fluidic and vacuum channels was sterilized by UV for 30–45 min in a tissue culture hood. 0.5 mL of 1 × 10⁶/mL of macrophages were concentrated by centrifugation and resuspended in phenol red-free DMEM media. The final concentration of the cells was ∼20 million/mL. To remove air bubbles inside the microfluidic chamber, sterilized 1× PBS was introduced first and was followed by 50 μL of the concentrated cell solution injected at a flow rate of 20 μL/min. Flow was stopped for 15 to 20 min for cell attachment. Unbound cells were washed away by applying 1× PBS at a flow rate set at 50–100 μL/min for 5 min. The whole procedure was observed using a microscope (Nikon Inc., Melville, NY). After cell seeding, 1 μg/mL of LPS diluted in phenol red-free DMEM was introduced into the channel to stimulate macrophages to produce TNF-α. Once the LPS solution was introduced into the flow chamber, the flow was stopped and a surgical clamp was used to secure the outlet tubing and eliminate convective mixing. The sample was kept in a tissue culture incubator for 3 h.

After 3 h stimulation with LPS, micropatterned surfaces were taken out from the incubator for cytokine staining. First, the LPS solution was washed away with 1× PBS and flow channels were infused and incubated with biotinylated anti-TNF-α for 1 h followed by incubation with either streptavidin–Alexa 546 (red fluorescence) or neutravidin–FITC (green fluorescence) for 30 min. Cells were fixed with 4% PFA for 15 min and stained with DAPI for 5 min to visualize cell nuclei. Between each step, the sample was washed with 1× PBS for 5 min to remove the previous reagent. All staining and washing steps were performed inside a microfluidic device at room temperature. The fluorescently labeled cytokine was visualized and imaged with an A Zeiss 200M epifluorescence microscope described in the preceding section.

To demonstrate detection of both H₂O₂ and TNF-α from the same micropatterned surface, HRP molecules were incorporated into the walls of hydrogel microwells as described earlier. The glass attachment pads of the microwells were modified with anti-TNF-α Ab molecules. Macrophages were seeded on these surfaces as stimulated with LPS as described before to commence TNF-α production. This protocol did
not elicit sufficient production of $\text{H}_2\text{O}_2$ to cause a change in fluorescence of hydrogel microstructures. To demonstrate detection of two inflammatory markers from the same micropatterned surface, $1\mu\text{M}$ of exogenous $\text{H}_2\text{O}_2$ and $50\mu\text{M}$ of Amplex Red solution were added to the sample. Secreted TNF-α molecules were visualized with neutravidin–FITC (green signal) whereas $\text{H}_2\text{O}_2$ appeared as red fluorescence.

22.3 Results and Discussion

22.3.1 Non-Fouling Properties of Enzyme-Carrying Hydrogel Microstructures

To demonstrate sensing of $\text{H}_2\text{O}_2$, HRP/Amplex Red-carrying hydrogel microstructures were challenged with known concentration of this analyte. Figure 22.2A shows an array of hydrogel microstructures (30–500μm diameter) fluorescing in response to incubation of $10\mu\text{M}$ $\text{H}_2\text{O}_2$. This image highlights the possibility of fabricating sensors of various sizes to meet the need of different experiments. For example, as shown later in this chapter, the ability to control dimensions of hydrogel microstructures was used to capture individual cells inside the microwells and to detect cell-secreted TNF-α. As shown in Figure 22.2B, the HRP/Amplex Red molecules were uniformly distributed inside the hydrogel structures with uniform fluorescence signal emanating from different $z$-plane slices of these sensing 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 the incorporation of enzymes does not adversely affect non-fouling properties of PEG hydrogels, an HRP-containing PEG precursor solution was photopatterned on glass substrates and incubated with macrophages or fibroblasts. As shown in Figure 22.3A and B, macrophages and fibroblasts selectively attached on glass regions with limited or no adhesion observed on PEG domains that contained enzyme HRP. The introduction of exogenous $10\mu\text{M}$ $\text{H}_2\text{O}_2$ into culture media resulted in the appearance of an optical (fluorescence) signal from sensing hydrogel microstructures. In addition, a multistep PEG fabrication process could be employed to micropattern enzyme-carrying as well as enzyme-free hydrogel structures on the same surface. Figure 22.3C demonstrates one example where polymer spin-coating and photopatterning were performed twice using two different prepolymer solutions to create sensing PEG structures integrated into a nonsensing PEG layer. The nonsensing PEG layer can serve as a negative control in metabolite detection experiments.

22.3.2 Characterization of $\text{H}_2\text{O}_2$-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 $\text{H}_2\text{O}_2$ [28];
therefore, the 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 the detection process. We noticed that the fluorescence intensity of Amplex Red changed over time even though the concentration of analyte was held constant (see Figure 22.4). This behavior was attributed to continuous production of red-fluorescent oxidant resorufin in the HRP-catalyzed reaction. Similar behavior of Amplex Red was reported previously [10]. We chose to record fluorescence intensity after 5 min of interaction between the sensing hydrogel structures and Amplex Red. This time frame was long enough to achieve ∼40% of maximum signal strength [32]. To reduce the detection time, a 5 min time point was also frequently chosen by other groups in the study of Amplex Red [16, 26].

The fluorescence signal was also concentration dependent. Figure 22.5A and B 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 Figure 22.5C, indicating that the concentration-dependent response could be derived from the micropatterned PEG hydrogel structures. It should be noted that when constructing calibration curve

FIGURE 22.3 (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 of H₂O₂ and 50 µM of Amplex Red resulted in appearance of fluorescence in the gel. This image highlights the 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 500 µm in diameter were micropatterned inside an array of enzyme-free hydrogel wells 1 mm in diameter. After the addition of 10 µM of H₂O₂, the signal appeared only from HRP-containing gel structures. Both sets of microstructures were non-fouling and macrophages attachment was seen only on exposed glass regions.

FIGURE 22.4 Time-dependent increase in fluorescence intensity of hydrogel microstructures in the presence of 5 or 20 µM H₂O₂ and 50 µM Amplex Red.
shown in Figure 22.5C, 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.

22.3.3 Detecting H₂O₂ Released from Activated Macrophages Using Sensing Hydrogel Microstructures

To investigate the possibility of detecting endogenous H₂O₂, macrophages were cultured on surfaces next to sensing hydrogel microstructures and were stimulated with a mitogen, PMA, for 1 h. This protocol was expected to result in oxidative burst and release of H₂O₂ from macrophages. Figure 22.6A and B shows representative bright field/fluorescence images of hydrogel microstructures after 1 h PMA stimulation and without stimulation. Analysis of fluorescence intensity from these images, presented in Figure 22.6C, shows that 1 h of PMA activation of macrophages led to a more than threefold increase in signal observed in hydrogel-sensing elements, compared to macrophages not exposed to this stimulant, and around twofold increase compared to cells with 3 h stimulation. While some fluorescence is observed in Figure 22.6B, this should be considered a background signal that is most likely due to autooxidation.
of Amplex Red into fluorescent resorufin in the ambient environment. 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 (data not shown). These results highlight the connection between activation of macrophages and the appearance of $\text{H}_2\text{O}_2$ signal in the adjacent hydrogel biosensors. The fluorescence signal detected in HRP-containing hydrogel micropatterns was converted to an analyte concentration using the calibration curve presented in Figure 22.5C. The results of this experiment presented in Figure 22.6C point to 1 and 0.55 $\mu$M of $\text{H}_2\text{O}_2$ production in the cases of 1 and 3 h stimulation, respectively.

### 22.3.4 Creating Microdevices for Capture and Analysis of Single Macrophages

In the results described in preceding sections of this chapter, we demonstrated that sensing hydrogel microstructures were non-fouling and allowed placement of tens to hundreds of macrophages in defined locations on the glass surface. However, PEG hydrogel photolithography offers a precise control of bio-interfacial properties so that placement of single cells in desired locations of the surface is achievable [24]. We envision functional analysis at the single-cell level as a prerequisite to better understanding of heterogeneity of cancer cell and immune cell populations. Our laboratory is currently developing biosensors for detecting $\text{H}_2\text{O}_2$ at a single-cell level and has already demonstrated detection of cytokine release from single cells [35]. The strategy of detecting cell-secreted cytokines, shown in Figure 22.7A, involves capturing macrophages inside microwells that contain cytokine-specific Abs. Upon release, cytokines become bound next to the secreting cells and can be visualized using standard sandwich immunoassay approaches (see Figure 22.7A). This allows us to make a direct connection between individual cells and levels of secreted cytokines. Micropatterned sensing surfaces were integrated into microfluidic devices (see, e.g., Figure 22.7B) to permit ease of cell seeding, activation and to carry out immunoassays inside a small volume (~3 $\mu$L per channel).

Given that the size of macrophages used in the present study ranged from 15 to 20 $\mu$m diameter, we fabricated arrays of 30 $\mu$m diameter wells. These wells consisted of non-fouling hydrogel walls and silane-modified glass bottom that supported cell attachment (Figure 22.8A). As can be seen in Figure 22.8A, anti-TNF-α Ab immobilized onto the silanized glass bottom was visualized by sequential incubation of 500 ng/mL of recombinant TNF-α, anti-TNF-α–biotin, and neutravidin–FITC. Fluorescence emanated from glass attachment sites inside the microwells and no fluorescence was seen on non-fouling hydrogel
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walls. Figure 22.8B seeding macrophages into 30\(\mu\)m diameter PEG wells resulted in sequestration of single cells. As described by us in previous publications, this surface micropatterning/cell-seeding process allows forming large-scale single-cell arrays [24,25].

22.3.5 Detecting TNF-\(\alpha\) from Single Macrophages

In order to detect the cytokine, anti-TNF-\(\alpha\) Ab was immobilized at the bottom of hydrogel microwells by physical absorption. Macrophage attachment did not require precoating of the surfaces with adhesive ligands as these cells were able to attach onto silanized glass or Ab-containing surfaces. However, in case cell attachment needs to be promoted, microwells may be modified with ECM proteins or cell-specific Abs as described by us in several preceding reports [14,15]. To extend the strategy of capturing single cells and then detecting secreted proteins to other cell types, adhesion-promoting ECM proteins/Ab can be mixed with a detection Ab and co-immobilized inside microwells.

After seeding macrophages onto arrays of microwells, we proceeded to detect TNF-\(\alpha\) released from single cells. The microdevice (hydrogel microwells integrated into PDMS microfluidic channels) was kept in the incubator for 3 h after stimulant LPS was introduced into the chamber. Stimulation was performed in tissue culture incubator (37\(^\circ\)C, 5% CO\(_2\)) as the cells were found to be more responsive to stimulants under physiological conditions [20]. An identical vacuum system was set up inside the incubator to ensure the complete sealing of the PDMS device on top of the sample.

After LPS activation, microwell arrays with captured macrophages were stained with anti-TNF-\(\alpha\)–biotin followed by streptavidin–Alexa 546. Staining was performed in situ, inside the microfluidic channels. Figure 22.8C and D shows representative bright field and fluorescence images of macrophages after activation with LPS. As seen from Figure 22.8C, macrophages had a “healthy” phenotype—cells were spread out inside the microwells. Importantly, fluorescence microscopy (Figure 22.8D) revealed a strong red fluorescence associated with anti-TNF-\(\alpha\)–biotin/streptavidin–Alexa 546 staining. This fluorescence signal was due to TNF-\(\alpha\) production by macrophages as it only appeared in the wells occupied by macrophages and only when macrophages were activated by a stimulant LPS. While not demonstrated here, a calibration curve of TNF-\(\alpha\) concentration vs. fluorescence intensity may be constructed in order to quantify the amount of cytokine secreted by single cells [35].
Integrating Biosensors for $\text{H}_2\text{O}_2$ and TNF-$\alpha$ Detection into the Same Platform

In this chapter, we have demonstrated two separate biosensing strategies based on PEG hydrogel microstructures: (1) entrapping enzyme molecules within the gel structures for $\text{H}_2\text{O}_2$ detection, and (2) using hydrogel micropatterns to adsorb anticytokine Abs onto glass regions inside the microwells. To demonstrate flexibility of hydrogel micropatterning, we fabricate arrays of PEG microwells where hydrogel walls contained entrapped HRP and the glass attachment sites contained anti-TNF-$\alpha$ Abs (see cartoon in Figure 22.9A for description of this biosensor). Therefore, the same hydrogel micropatterns were expected to detect both small ROS molecules and cytokines. In a proof of concept experiment, macrophages were seeded into sensing hydrogel microwells, stimulated with LPS for 3 h and stained for secreted TNF-$\alpha$. Figure 22.9A shows that the microwells contained macrophages (nuclei stained with DAPI) and that the cytokine signal is associated with the presence of macrophages in the wells.

**FIGURE 22.8** (A) An array of 30 $\mu$m diameter PEG hydrogel microwells. The deposition of anti-TNF-$\alpha$ Ab onto the silanized glass bottom was visualized by sequential incubation of 500 ng/mL of recombinant TNF-$\alpha$, biotinylated anti-TNF-$\alpha$, and neutravidin–FITC. (B) Single macrophages attaching inside microwells. Note that the gel surface is rough because of entrapped HRP molecules. (C) Higher magnification bright field image showing macrophages captured inside the PEG hydrogel microwells and stimulated with PMA for 3 h. (D) The same set of microwells stained with biotinylated anti-TNF-$\alpha$ Ab and streptavidin–Alexa 565 shows red fluorescence inside the wells. This fluorescence signal is due to cell-secreted TNF-$\alpha$. Note that nuclei of cells are stained with DAPI (brighter gray color) and that presence of cytokine signal is associated with the presence of macrophages in the wells.

**22.3.6 Integrating Biosensors for $\text{H}_2\text{O}_2$ and TNF-$\alpha$ Detection into the Same Platform**

In this chapter, we have demonstrated two separate biosensing strategies based on PEG hydrogel microstructures: (1) entrapping enzyme molecules within the gel structures for $\text{H}_2\text{O}_2$ detection, and (2) using hydrogel micropatterns to adsorb anticytokine Abs onto glass regions inside the microwells. To demonstrate flexibility of hydrogel micropatterning, we fabricate arrays of PEG microwells where hydrogel walls contained entrapped HRP and the glass attachment sites contained anti-TNF-$\alpha$ Abs (see cartoon in Figure 22.9A for description of this biosensor). Therefore, the same hydrogel micropatterns were expected to detect both small ROS molecules and cytokines. In a proof of concept experiment, macrophages were seeded into sensing hydrogel microwells, stimulated with LPS for 3 h and stained for secreted TNF-$\alpha$ (neutravidin–FITC used for fluorescence labeling). As shown in Figure 22.9A, microwells with cells emitted green fluorescence due to secreted TNF-$\alpha$. Figure 22.9A shows that the microwells contained macrophages (nuclei stained with blue dye DAPI) and that the cytokine signal was associated with specific cells. As shown by staining cell nuclei with DAPI, macrophages are captured almost exclusively inside the glass attachment sites of hydrogel microwells. To demonstrate that the same microwells may also be used for detecting $\text{H}_2\text{O}_2$, 1 $\mu$M of exogenous $\text{H}_2\text{O}_2$ and 50 $\mu$M of Amplex Red solution were added to the sample. As seen from Figure 22.9B, hydrogel microwells emitted red fluorescence in response to stimulation with $\text{H}_2\text{O}_2$. While this result stops short of detecting endogenous $\text{H}_2\text{O}_2$ in addition to TNF-$\alpha$, it points out a future strategy for detecting two inflammatory markers secreted by single cells.
This chapter describes fabrication of sensing hydrogel microstructures that can be used to both define cell attachment and detect cell-secreted molecules. Entrapment of HRP molecules inside the gel allowed detecting H$_2$O$_2$ release by macrophages cultured nearby. Creating hydrogel microwells and modifying glass attachment sites with anti-TNF-α allowed capturing single macrophages and detecting cytokine molecules secreted by these cells. Finally, we demonstrated micropatterned surfaces that can be used for detection of both H$_2$O$_2$ and TNF-α inflammatory signals produced by macrophages.

While we demonstrate the possibility of integrating biosensors for enzyme-based detection of a small molecule (H$_2$O$_2$) and antibody-based sensing of a cytokines (TNF-α) into the same micropatterned surface, the differences in function of biorecognition molecules would need to be reconciled in the future. Enzymes are constantly turning over, providing temporal information about substrate breakdown and signal generation. On the other hand, antibody-based immunoassays provide end-point measurements and reveal little about the change in cytokine signal over time. Replacing antibody molecules with aptamer-beacons [29,30] would allow real-time detection of cell-secreted cytokines or other proteins so that affinity sensors will function in a similar way as enzyme-based biosensors. In addition, the number of analytes detected from single cells will be expanded in the future.

The overarching goal of the studies described in this chapter is to enable local detection of cell function with novel micropatterned sensing surfaces. Analysis of cell function (i.e., what the cell produces) is different from standard approaches of phenotyping cells based on morphology or cell surface marker expression. The sensor-cell platform described in this chapter can be used to analyze two inflammatory signals, H$_2$O$_2$ and TNF-α, that play important roles in cancer formation and progression. The platform described here may be used for screening efficacy of anticancer drug therapies. In addition, we see the ability to detect and quantify cell-secreted products with single-cell resolution as an important step toward improved, more nuanced diagnosis of cancer and toward the development of personalized anticancer therapies.

**REFERENCES**


