

Miniature Enzyme-Based Electrodes for Detection of Hydrogen Peroxide Release from Alcohol-Injured Hepatocytes

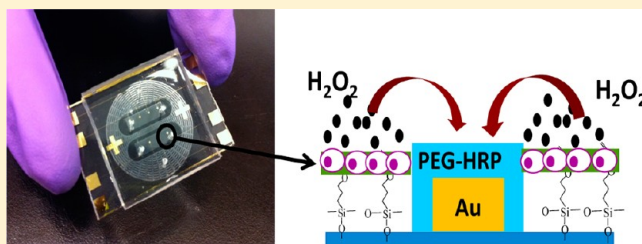
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S Supporting Information

ABSTRACT: Alcohol insult to the liver sets off a complex sequence of inflammatory and fibrogenic responses. There is increasing evidence that hepatocytes play a key role in triggering these responses by producing inflammatory signals such as cytokines and reactive oxygen species (ROS). In the present study, we employed a cell culture/biosensor platform consisting of electrode arrays integrated with microfluidics to monitor extracellular H_2O_2 , one of the major ROS types, produced by primary rat hepatocytes during alcohol injury.

The biosensor consisted of hydrogel microstructures with entrapped horseradish peroxidase (HRP) immobilized on an array of miniature gold electrodes. These arrays of sensing electrodes were integrated into microfluidic devices and modified with collagen (I) to promote hepatocyte adhesion. Once seeded into the microfluidic devices, hepatocytes were exposed to 100 mM ethanol and the signal at the working electrode was monitored by cyclic voltammetry (CV) over the course of 4 h. The CV experiments revealed that hepatocytes secreted up to $1.16 \mu\text{M}$ H_2O_2 after 3 h of stimulation. Importantly, when hepatocytes were incubated with antioxidants or alcohol dehydrogenase inhibitor prior to alcohol exposure, the H_2O_2 signal was decreased by ~ 5 -fold. These experiments further confirmed that the biosensor was indeed monitoring oxidative stress generated by the hepatocytes and also pointed to one future use of this technology for screening hepatoprotective effects of antioxidants.



Toxic effects of alcohol on the liver have long been studied.^{1,2} Excessive consumption of alcohol, resulting in alcohol hepatitis, associated with hepatosteatosis, is an early stage of alcoholic liver disease and may contribute to the progression of fibrosis, leading to cirrhosis.^{3,4} As a result of many clinical and experimental studies, it has been found that the production of reactive oxygen species (ROS) due to alcohol intake causes oxidative stress that leads to a sequence of inflammatory and fibrogenic processes. ROS are reduced or activated derivatives of oxygen [superoxide anion ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{OH}^{\bullet}$)], chemically reactive, and extremely toxic toward most biological components.^{5,6} This leads to chemical modifications (oxidation, peroxidation, nitration, etc.) of lipids, proteins, and DNA that affect the integrity of the cell and its whole metabolism.^{7,8} Much work has been focused to study the generation of ROS from Kupffer cells or liver resident macrophages;^{7,9,10} however, more recently attention has turned to the role of hepatocytes as initiators and triggers of fibrogenic and inflammatory signals produced during liver injury.^{11,12} Hepatocytes are parenchymal cells of the liver constituting 70–80% of the cell mass in this organ. There are reports describing contributions of hepatocytes to ROS production in the liver during alcohol injury;^{4,13} however, quantification of oxidative stress generated by hepatocytes has not been undertaken to the best of our knowledge.

Hydrogen peroxide (H_2O_2) is the most chemically stable ROS type¹⁴ that is produced both as the byproduct of alcohol metabolism and due to the degradation of more reactive ROS

types (e.g., superoxide anion). It is freely miscible with water, can penetrate through cell membranes readily, and is cytotoxic at high concentrations. Despite a growing interest in the detection of H_2O_2 there are few experimental tools available for real-time monitoring of H_2O_2 release from cells. There are fluorescence-based methods for detecting H_2O_2 . For example, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a cell-permeable, nonfluorescent precursor, has been frequently used as an intracellular probe that emits fluorescence upon interaction with H_2O_2 .^{15–17} Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) is another fluorescent probe that fluoresces upon interaction with H_2O_2 and may be used to monitor extracellular peroxide.^{9,10} Both intra- and extracellular fluorescent probes for detecting H_2O_2 suffer from several challenges that include photobleaching, auto-oxidation, and autofluorescence, which make quantitative measurements difficult.

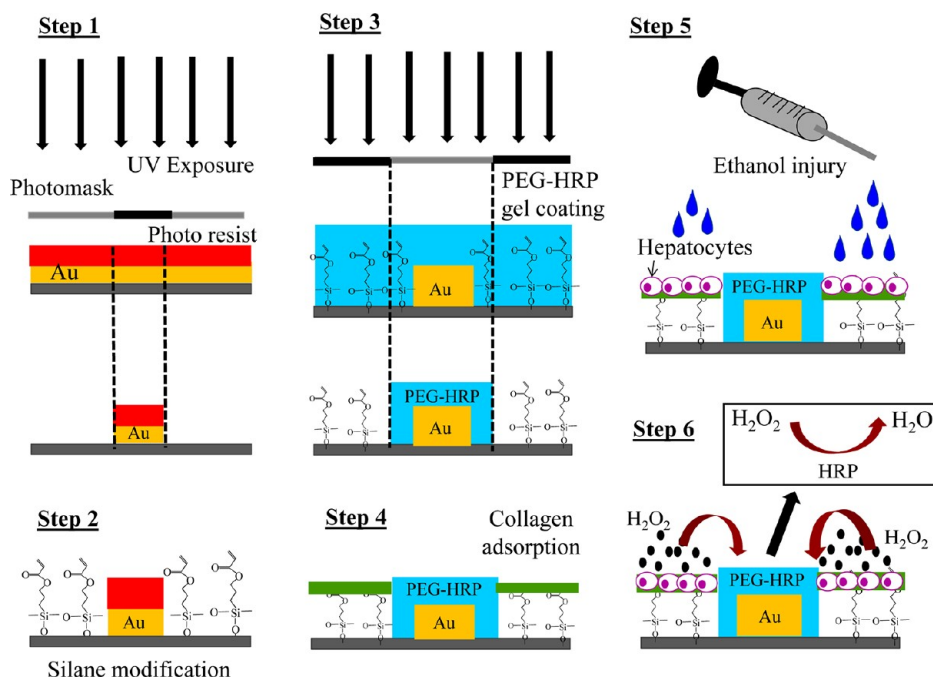
Electrochemical measurements may be used for sensitive detection of electroactive species and often provide a viable alternative to fluorescence-based detection. Several reports have described electrochemical biosensors for in vitro and in vivo detection of H_2O_2 and other ROS produced by cells.^{7,18–20} Amatore et al. studied the release of ROS and reactive nitrogen species from macrophages that were cultured in a detection

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Scheme 1. Steps 1–3. Fabrication of PEG–HRP Microstructures on Top of Au Electrodes to Create Enzyme-based Electrodes. Steps 5–6. Culture and Injury of Hepatocytes Followed by Detection of Extracellular Peroxide



chamber containing a three-electrode system and were stimulated by the microinjection of a calcium ionophore.⁷ Cheah et al. developed a microfluidic device for heart tissue perfusion with real-time electrochemical monitoring of ROS release.¹⁸ Previously, our laboratory has reported on the development of enzyme-carrying miniature electrodes²¹ and have employed these electrodes for the detection of H_2O_2 production from macrophages.¹⁹

In the present work, we wanted to extend the use of miniature enzyme-based biosensors to measure H_2O_2 generated by primary hepatocytes during alcohol injury. The sensor was fabricated by depositing horseradish peroxidase (HRP)-containing poly(ethylene glycol) diacrylate (PEG-DA) hydrogel on top of miniature gold electrodes (see Scheme 1). The primary hepatocytes were seeded onto the biochip surface, attaching and spreading around the gel-covered electrodes. The cells and electrodes were then integrated into a microfluidic device and injured in situ by alcohol exposure. Production of H_2O_2 by injured hepatocytes was monitored over the course of 4 h using cyclic voltammetry (CV) and was found to reach $\sim 1 \mu M$. Pretreatment of hepatocytes with antioxidants prior to alcohol insult was shown to significantly diminish the levels of extracellular H_2O_2 . To the best of our knowledge, this study is one of the first to attempt quantification of extracellular oxidative stress generated by injured hepatocytes. A microsystem with electrochemical biosensors integrated at the site of cells inside the microfluidic channels may become an important tool for toxicology studies and screening effectiveness of liver protective therapeutics.

MATERIALS AND METHODS

Chemicals and Reagents. PEG-DA (MW 575), PEG-DA (MW 258), 2-hydroxy-2-methyl-propiophenone (photoinitiator), 99.9% toluene, H_2O_2 , HRP, and glutaraldehyde (Glu) were purchased from Sigma, U.S.A. Chromium (CR-4S) and gold etchants (Au-5) were purchased from Cyantek Corpo-

ration (Fremont, CA). Positive photoresist (S1813) and its developer solution (MF-319) were bought from Shipley (Marlborough, MA). (3-Acryloxypropyl)trichlorosilane was from Gelest, Inc. (Morrisville, PA). 5-(and 6)-Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCFDA), N-acetyl cysteine (NAC), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and 4-methylpyrazole were purchased from Sigma, U.S.A. Phosphate-buffered saline (PBS 0.1 M, pH 7.4) solution was purchased from Fisher Scientific. Poly(dimethylsiloxane) (PDMS) and silicone elastomer curing agent were purchased from Dow Corning (Midland, MI).

A 10 mg mL^{-1} solution of HRP was prepared in PBS solution of pH 7.4. 2% (v/v) solution of Glu was prepared in deionized water. The prepolymer hydrogel enzyme solution was prepared by adding $20 \mu L$ of HRP solution, $2 \mu L$ of Glu, and 2% (v/v) solution of the photoinitiator in $25 \mu L$ of PEG-DA (MW 575 and 258). Two different PEG-DAs were used to improve attachment of the hydrogels to the substrate. We found that attachment of hydrogel constructs made out of a 1:1 mixture of PEG 575 and 258 was better than PEG 575 alone. While attachment properties of PEG 258 alone were excellent, the nonfouling properties were compromised. The mixture of two PEG types allowed us to improve adhesion of gel constructs while retaining nonfouling properties. The prepolymer hydrogel enzyme solution was stirred overnight to react off all the aldehyde groups of Glu to the primary amines of the enzyme molecules.

Different concentrations of H_2O_2 and 100 mM ethanol were prepared in 0.1 M PBS solution of pH 7.4.

Isolation of Primary Hepatocytes. Primary hepatocytes were isolated from adult female Lewis rats weighing 125–200 g (Charles River Laboratories, Boston, MA), using a standard two-step collagenase perfusion procedure.²² Typically, 100–200 million hepatocytes were obtained with viability >90% as determined by trypan blue exclusion. Primary hepatocytes were maintained in Dulbecco's modified Eagle's medium (DMEM)

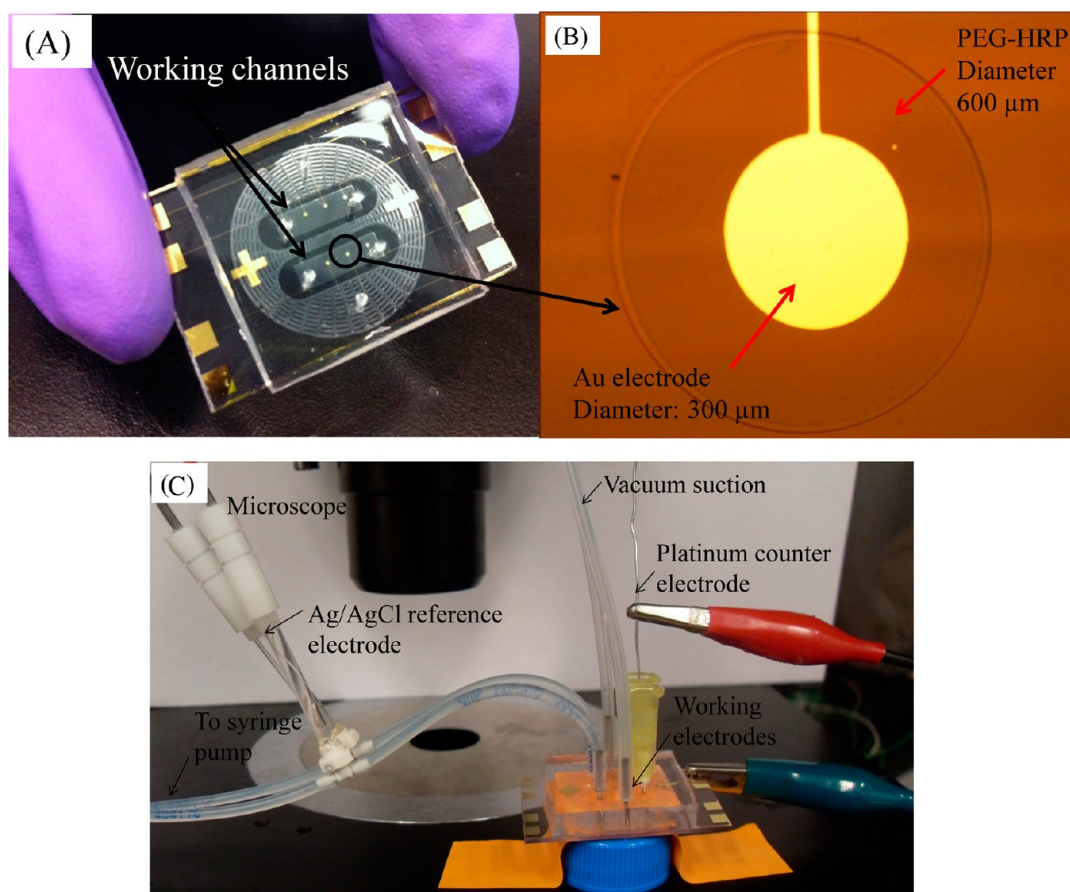


Figure 1. (A) PDMS covered sensing chip containing eight micropatterned Au electrodes (four in each working channel). (B) An image of a PEG–HRP polymerized Au electrode. (C) Microfluidic device for on-chip electrochemical experiments containing flow-through Ag/AgCl as reference (connected to the outlet), Pt wire as counter (in the inlet), and patterned Au as working electrodes.

supplemented with epidermal growth factor, glucagon, hydrocortisone sodium succinate, recombinant human insulin, 200 units mL^{-1} penicillin, 200 mg mL^{-1} streptomycin, and 10% fetal bovine serum.

Micropatterning of Au Electrodes on Glass. The micropatterned Au arrays were prepared using a previously described standard photolithography and metal-etching method.²³ Briefly, glass slides with dimension of $7.5 \text{ cm} \times 2.5 \text{ cm}$ sputtered with Cr (15 nm) and Au (100 nm) were purchased from Lance Goddard Associates (Santa Clara). A protective layer of a positive photoresist was spin-coated (2000 rpm for 30 s) on top of the Au-coated slide and soft-baked at 115°C for 1 min. The electrode array pattern made in AutoCAD was formed on the slides with a mask aligner, and the final patterned slides were obtained using the wet etching method. In this way, an array of eight electrodes of circular shape ($300 \mu\text{m}$ diameter) was obtained for fabricating the microfluidic platform.

Formation of HRP-Carrying Hydrogel Microstructures on Au Electrode Arrays. The micropatterned glass slides, containing photoresist on top of Au surface, were treated with oxygen plasma for 10 min and incubated in 0.05% solution of (3-acyloxypropyl)trichlorosilane in toluene for about 1 h under nitrogen atmosphere to obtain a self-assembled monolayer of silane on the glass regions. The photoresist layer protected the Au region of the slide from the silane modification. These slides were then sonicated in acetone to remove the photoresist from Au regions. The prepolymer

hydrogel solution (PEG–HRP–Glu containing 2% photo-initiator) was coated onto the Au electrodes of the patterned slides. These slides were exposed to UV radiation ($60 \text{ mJ}/\text{cm}^2$) using an Omnicare 1000 light source for 3.5 s through an aligned photomask on top of the electrodes. As a result liquid prepolymer was cross-linked into hydrogel microstructures formed on top of Au electrodes.

Enzyme-carrying hydrogel microstructures were made larger ($600 \mu\text{m}$) than Au electrodes ($300 \mu\text{m}$) so that acryl group of the silane could cross-link to the PEG–DA providing more stability to the enzyme–hydrogel onto the glass surface (see Figure 1). The UV-exposed PEG–DA precursor gets polymerized and subsequently cross-links to the silane layer on the glass substrate, while the unexposed regions were later washed from the surface using deionized water. To seed primary hepatocytes around the PEG–HRP electrodes, the micropatterned glass slides were incubated in collagen (0.2 mg mL^{-1}) solution for about 30 min and were kept at 37°C in an incubator. The collagen gets adsorbed onto silane-modified glass regions and not on the gel-coated Au electrode regions of the surface due to the nonfouling properties of PEG. Subsequently, primary rat hepatocytes were seeded for about 24 h at 37°C onto these surfaces and then enclosed inside microfluidic channels to commence on-chip detection experiments. Scheme 1 (steps 1–6) describes the stepwise fabrication of enzyme–hydrogel electrodes and subsequent cell attachment.

Integration of Sensing Surfaces with Microfluidic Channels for Electrochemical Detection of H_2O_2 . PDMS-based microfluidic channels were designed and fabricated according to procedures described by us previously.²⁴ The microfluidic device consisted of two channels of dimension $10\text{ mm} \times 3\text{ mm} \times 0.1\text{ mm}$ and a volume of $3\text{ }\mu\text{L}$ with the inlets and outlets punched with a blunt 16-gauge needle (Figure 1A). Along with the two fluidic channels, a network of auxiliary channels was fabricated on the same PDMS surface and was used for suctioning the device onto glass substrates carrying cells and electrodes.

All the electrochemical measurements were done inside the microfluidic device connected with a precision syringe pump (Harvard Apparatus, Boston, MA) for generating controlled flow. A CHI instrument (842B, CH Instruments, Austin, TX) was employed for the voltammetric experiments. The electrochemical cell consisted of a flow-through Ag/AgCl (3 M KCl) reference electrode inserted at the outlet, a platinum wire counter electrode placed in the inlet of the microfluidic device, and Au working electrodes positioned inside the fluidic channels (Figure 1C). The solutions were made in 0.1 M PBS for all the electrochemical measurements. The contact pads of the sensing Au arrays were connected to a home-built multiplexing setup capable of collecting CV spectra at prescribed time intervals from individual members of the electrode array.

Cyclic voltammetry was used to characterize responses of PEG–HRP electrodes to H_2O_2 . In order to create a calibration curve, known concentrations of H_2O_2 prepared in PBS were infused into the microfluidic device containing enzyme-based electrodes. The cyclic voltammograms (CVs) were taken from 0.7 to -0.7 V at the scan rate of 50 mV/s . The calibration curve was constructed by plotting the absolute value of reduction current at -0.4 V versus H_2O_2 concentration.

To analyze H_2O_2 production from alcohol-injured cells, primary hepatocytes were seeded onto the surface of the biochip. The surface was pretreated with 0.2 mg mL^{-1} of collagen (I) in order to promote hepatocyte attachment. Subsequently, hepatocytes were suspended in cell culture medium at a concentration of $10^6\text{ cells mL}^{-1}$ and incubated at $37\text{ }^\circ\text{C}$ over the surface of the biochip for 1 h. The unattached cells were then washed away using PBS, and the remaining cells were allowed to recover for at least 12 h prior to assembling the PDMS channels on top of the biochip. The injury experiments were commenced by injecting 100 mM ethanol into the fluidic channel. CV measurements were made every 15 min during the 4 h alcohol stimulation experiment.

Fluorescence-Based Detection of Peroxide. Hepatocytes were seeded into the wells of a 96-well plate precoated with 0.2 mg/mL collagen (I). After 24 h of incubation with cells, certain wells were incubated with NAC ($0.6128\text{ }\mu\text{M}$) or Trolox C ($0.1997\text{ }\mu\text{M}$) for 24 h, or 4-MP (2 mM) for 1 h, or left unprotected by antioxidants. After antioxidant incubation, the cells were washed and then incubated with CM-DCFDA ($10\text{ }\mu\text{M}$) for 45 min. After washing away the residual CM-DCFDA not taken up by the cells, ethanol (100 mM) was added to the protected and unprotected cells and their fluorescence was measured every 10 min for 3 h using plate reader (Tecan Safire 2) employing excitation/emission of $485\text{ nm}/530\text{ nm}$. Plates were stored in a tissue culture incubator at $37\text{ }^\circ\text{C}$ when not in use.

RESULTS AND DISCUSSION

Fabrication and Electrochemical Characterization of Enzyme-Based Biosensors. PEG is a biocompatible polymer known for its excellent nonfouling properties. This biomaterial has been used widely to minimize unwanted protein adsorption and cell attachment in tissue engineering and biosensor development.^{10,21,25,26} In addition to possessing excellent nonfouling properties, PEG-based hydrogels also provide a hydrated environment that helps retain high levels of activity in entrapped enzymes.^{27,28} As shown in Figure 1B, the PEG–HRP sensor was fabricated by polymerizing enzyme–hydrogel on top of micropatterned Au electrodes. The glass slides were modified with acryl silane to promote attachment of hydrogel micropatterns (Scheme 1). HRP is an oxidoreductase enzyme that catalyzes the reduction of H_2O_2 according to eq 1 below.²⁹



The CV measurements were carried out to characterize the enzyme–hydrogel electrodes. Given that H_2O_2 is an electroactive compound we wanted to assess the oxidation/reduction of this compound at the electrodes containing or lacking HRP. Figure 2A shows CVs of the hydrogel electrodes in PBS solution in absence (Figure 2A, curve i) and presence of HRP (Figure 2A, curve ii) in the PEG hydrogel. The incorporation of redox enzyme in the hydrogel matrix results in increased current response in the positive and negative potential range (Figure 2A, curve ii) as compared to the response for only hydrogel electrode (Figure 2A, curve i). This is attributed to the incorporation of electrochemically active enzyme into PEG gel. Subsequently, an increase in reduction current was obtained upon the addition of H_2O_2 into the microfluidic channel containing PEG–HRP microelectrodes indicating enhanced reduction of H_2O_2 (diffused into the hydrogel) by HRP (Figure S1, Supporting Information). Conversely, the microelectrodes made with pure PEG did not show much change in current when challenged with H_2O_2 (Figure S2, Supporting Information). This validates that the HRP augments the reduction of H_2O_2 . About a 60-fold increase in the reduction current was found with the PEG–HRP as compared to the PEG-only electrode.

Calibration of the prepared enzyme sensor was performed by infusing known concentrations of H_2O_2 into the microfluidic device containing PEG–HRP microelectrodes and monitoring the reduction current using CV in the range of 0.7 to -0.7 V . The injected H_2O_2 diffuses into the porous hydrogel matrix and reacts biochemically with the HRP present inside the hydrogel (Figure 2B). The reduction current was found to increase with increasing H_2O_2 concentration. Figure 2C illustrates the calibration plot (reduction current versus H_2O_2 concentration at -0.4 V) for detection of exogenous H_2O_2 . The low operating potential (-0.4 V) obviates interference due to other species such as ascorbic acid, uric acid, etc.³⁰ The lowest concentration of H_2O_2 that could be detected by each sensing electrode of area 0.070714 mm^2 was found to be $0.2\text{ }\mu\text{M}$. The sensitivity of the sensor was calculated to $27.5\text{ nA }\mu\text{M}^{-1}\text{ mm}^{-2}$. The stability of the enzyme–hydrogel electrode was tested at a regular interval of 1 week by infusing a $50\text{ }\mu\text{M}$ concentration of H_2O_2 in the microfluidics channel containing the PEG–HRP sensor. The biochip was stored in PBS at $4\text{ }^\circ\text{C}$ when not in use. The value of reduction current was monitored each time and plotted against number of weeks. The reduction current did not change

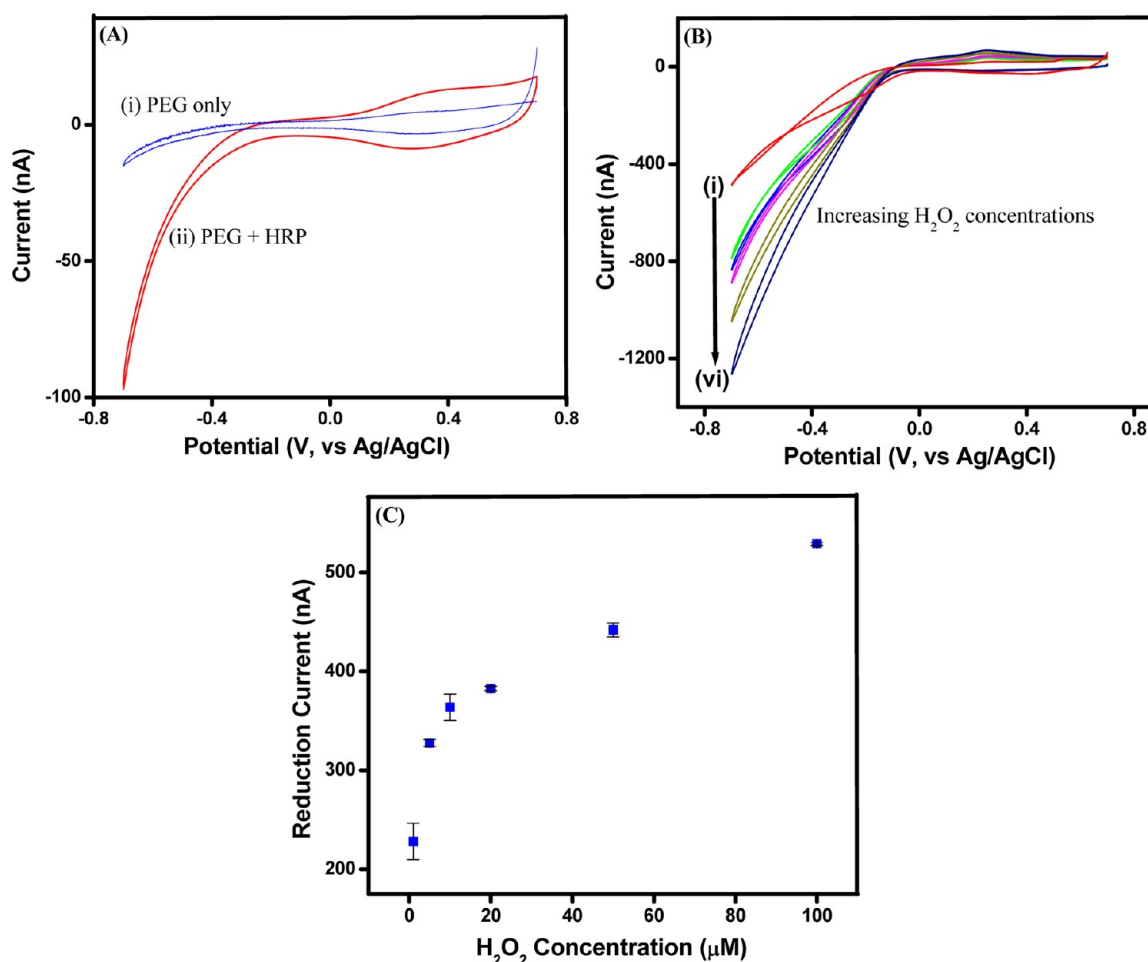


Figure 2. (A) CV plots of (i) PEG and (ii) PEG-HRP polymerized onto miniature Au electrodes in the potential range of 0.7 to -0.7 V at the scan rate of 50 mV/s. The better current obtained with HRP-incorporated PEG is attributed to the redox property of the enzyme. (B) CV plots of the PEG-HRP electrode as a function of H_2O_2 injected into the microfluidic device: (i) 1, (ii) 5, (iii) 10, (iv) 20, (v) 50, and (vi) 100 μM . The reductive current increases with increasing concentration of H_2O_2 . This indicates enhanced enzymatic reaction between HRP and H_2O_2 . (C) Calibration plot obtained for the PEG-HRP electrode showing the absolute value of the reduction current as a function of different H_2O_2 concentrations (current values at -0.4 V).

over the course of 4 weeks (Figure S3, Supporting Information). This verifies stable entrapment of enzyme molecules inside the gel constructs. The stability of enzyme electrodes was also tested at 37 °C under cell culture conditions. We did not observe changes in sensor response over 2 days under cell culture conditions. This stability was sufficient to conduct ethanol injury experiments. The stability of the enzyme at varied temperatures is attributed to the biocompatible environment provided by the hydrated gel matrix. There are several reports in the literature confirming stability of HRP up to ~ 50 °C when encapsulated in gel matrixes.^{31,32}

Electrochemical Detection of Hepatic H_2O_2 Released During Alcohol Injury. To conduct alcohol injury experiments the hepatocytes were seeded onto collagen (I)-coated surfaces containing enzyme electrodes. The cells captured inside the biochip were kept in a tissue culture incubator at 37 °C for 24 h. As seen in Figure 3, parts A and B, which shows images of hepatocytes taken immediately after seeding and 12 h post seeding, cells formed a monolayer with bright cell borders, prominent nuclei, and cuboidal morphology indicative of the hepatic phenotype. It is of note that hepatocytes did not attach on top of the electrodes due to the nonfouling properties of

PEG. We found that, in cases when nonfouling properties were compromised and hepatocytes attached to the gel/electrode region, the sensor's response was compromised (results not shown). An estimated 50 000 cells were cultured inside each channel during the peroxide detection experiment.

The cells were stimulated by infusing 100 mM ethanol into the microfluidic channel. This level of ethanol is pathophysiological and is similar to the levels observed during acute alcohol injury.^{17,33} The resultant release of H_2O_2 was detected with CV by sequentially addressing H_2O_2 sensing electrodes located inside the microfluidic channel. Measurements were made every 15 min for up to 4 h using a home-built multiplexer coupled with automated data collection. The flow inside the fluidic channel was stopped prior to conducting electrochemical measurements. An increase in reduction current of H_2O_2 was observed after ~ 60 min of incubation with 100 mM ethanol, pointing to the production of H_2O_2 from hepatocytes injured with alcohol. The reduction current was found to saturate after 3 h. Figure 3C shows representative response of hepatocytes to 100 mM ethanol. When testing less than 100 mM concentration of ethanol we did not observe a change in the reduction current over time. The H_2O_2 produced from hepatocytes was determined to be ranging from 0.29 μM

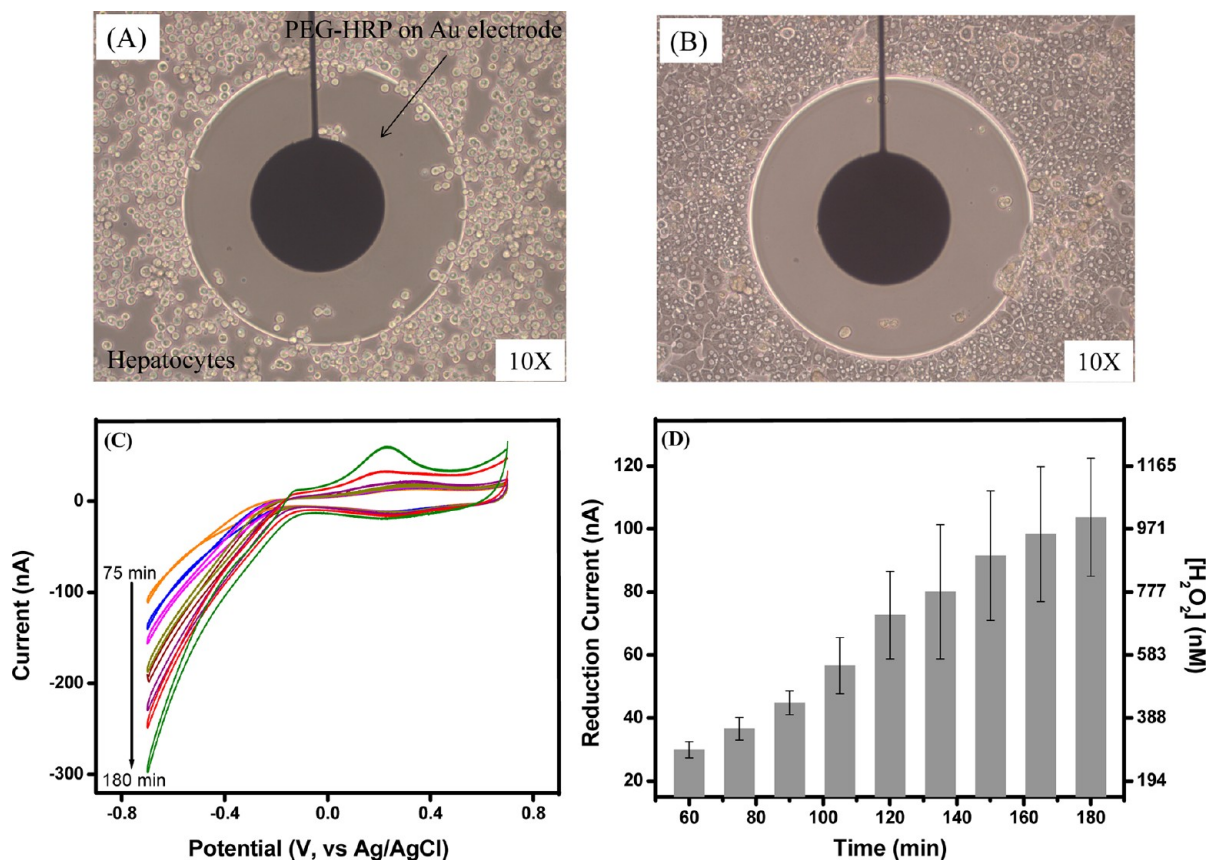


Figure 3. Sensor surface (A) just after hepatocyte seeding onto the collagen-modified silane region around the PEG–HRP electrode and (B) after incubation of the sensor chip at 37 °C in an incubator for 24 h. (C) CV measurements taken from hepatocytes exposed to 100 mM ethanol. Increase in reduction current with time indicates enhanced cellular production of H_2O_2 . (D) Plot showing enhancement in reduction current (at -0.4 V) due to cellular production of H_2O_2 .

after 1 h to $1.16 \mu\text{M}$ after 3 h (Figure 3D). Thus, the rate of H_2O_2 release from $\sim 12\,000$ stimulated hepatocytes around each PEG–HRP sensing electrode was found to be 6.5 nM min^{-1} from 1 to 3 h. It is worth noting that the response time for detection of extracellular peroxide by hepatocytes is likely a function of the biosensor sensitivity. Our laboratory has previously demonstrated that sensitivity may be improved by conjugating enzymes with Au nanoparticles.³⁴ It is therefore conceivable that more sensitive biosensors will enable earlier detection of H_2O_2 from hepatocytes and may enable cellular responses to lower alcohol concentrations.

A number of control experiments were carried out to ensure that the signal observed in Figure 3C was indeed due to extracellular H_2O_2 . Exposure of enzyme electrodes to 100 mM ethanol did not cause a change in reduction current. Similarly, cultivation of hepatocytes around enzyme electrodes in the absence of alcohol did not result in an electrochemical signal (results of both experiments not shown). This suggests that the generation of the electrochemical signal required the presence of hepatocytes and injury with alcohol.

It may be noted that most of the electrochemical biosensors reported in literature are used to monitor ROS production from immune cells^{7,18,30,35} but not from hepatocytes. The prepared electrochemical enzyme–hydrogel-based sensor is able to detect H_2O_2 in a wide range of concentrations and shows sensitive detection of H_2O_2 secreted from alcohol-injured hepatocytes in PBS without any external redox indicator.

Effects of Antioxidants on Detection of H_2O_2 . Development and screening efficacy of antioxidants is an important part of liver toxicology and liver therapeutics. To demonstrate potential utility of our biosensor for liver toxicology studies we assessed the effects of commercial antioxidants such as NAC³⁶ and Trolox³⁷ on oxidative stress production in alcohol-injured hepatocytes. These antioxidants are known to protect the cells against oxidative stress possibly by the scavenging activity.³⁷ The purpose of these investigations was to monitor the protective effect of these antioxidants on hepatocytes toward production of ROS. These experiments also served as additional controls for verifying that the electrochemical signals measured in the vicinity of injured hepatocytes were due to oxidative stress.

The hepatocytes cultured around enzyme electrodes were incubated with either $0.6128 \mu\text{M}$ NAC or $0.1997 \mu\text{M}$ Trolox in DMEM for 24 h prior to alcohol injury. The extra antioxidant molecules were washed out by infusing 0.1 M PBS into the microfluidic channels. Cells were then exposed to 100 mM ethanol, and electrochemical measurements were performed as described in the previous section of this paper. Figure 4 compiles the reduction current values from experiments where hepatocytes were protected with antioxidants prior to injury versus a control scenario where hepatocytes were injured in the absence of antioxidant treatment. This set of data clearly demonstrates that antioxidant treatment caused a dramatic decrease in the reduction current in the electrochemical signal measured at the sensing electrode. Importantly, peroxide signal

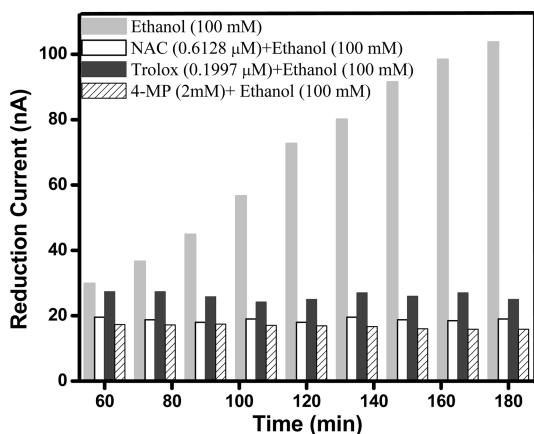


Figure 4. Peroxide production in hepatocytes injured with alcohol in the presence or absence of antioxidants (NAC, Trolox, 4-MP). The hepatocytes protected with antioxidants show negligible change in current when stimulated with 100 mM ethanol.

from antioxidant-treated cells remained constant over time ($\sim 0.2 \mu\text{M}$), whereas signal from unprotected hepatocytes increased over time ranging from $0.29 \mu\text{M}$ at 1 h to $1.16 \mu\text{M}$ at 3 h.

We also evaluated the effect of 4-MP—a small-molecule inhibitor of alcohol dehydrogenase¹¹—on the generation of oxidative stress in hepatocytes. Alcohol dehydrogenase is accountable for breaking down the ethanol into acetaldehyde, and activity of this enzyme is connected to generation of H_2O_2 .³⁸ As shown in Figure 4, the treatment of hepatocytes with 2 mM 4-MP for 1 h decreased the levels of peroxide measured by our enzyme-based electrodes.

Detection of H_2O_2 Using Fluorescence Spectroscopy.

Fluorescence-based detection of H_2O_2 was used to corroborate results of electrochemical sensing. These experiments utilized CM-DCFDA, a cell-permeable intracellular dye.^{39,40} In the presence of H_2O_2 this dye is converted into its fluorescent form and thus can be used as an intracellular probe to monitor oxidative stress. The hepatocytes were seeded into the wells of a 96-well plate and treated with antioxidants and alcohol in a manner similar to that described previously in this paper. The cells with no ethanol produced the least amount of fluorescence (Figure 5, curve i). Conversely, the cells stimulated with 100 mM ethanol and not protected with antioxidants produced the highest fluorescence signal (Figure 5, curve v). Additionally, lower levels of fluorescence were found in case of hepatocytes incubated with optimized concentrations of antioxidants (Figure 5, curves ii–iv). This is attributed to the effect of antioxidants that prevent the release of H_2O_2 and thus can protect the liver cells from alcohol injury. There are significant differences between fluorescence and electrochemical detection; the former is intracellular, whereas the latter is extracellular; the former is more qualitative, whereas the latter is quantitative. However, fluorescence measurements confirmed that hepatocytes injured by alcohol produced intracellular peroxide and that the levels of this ROS could be decreased by treatment with antioxidants.

CONCLUSIONS

This study describes the use of miniature enzyme-based electrodes integrated with microfluidics to enable the detection of H_2O_2 from hepatocytes injured with alcohol. This biosensor had a detection limit of $0.2 \mu\text{M}$ H_2O_2 with linear range

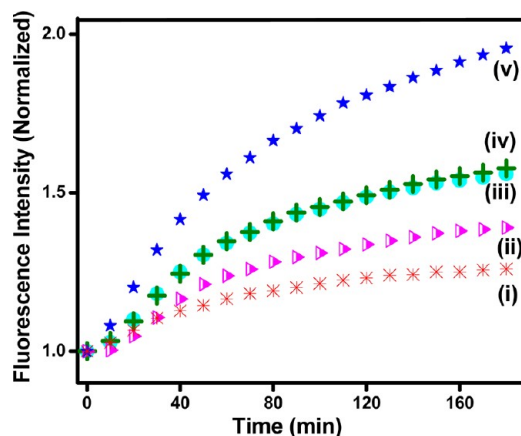


Figure 5. Time-dependent increase in fluorescence intensity for hepatocytes containing CM-DCFDA with (i) no ethanol, (ii) 4-MP (2 mM) treatment plus ethanol (100 mM) (iii) Trolox (0.1997 μM) treatment plus ethanol (100 mM), (iv) NAC (0.6128 μM) treatment plus ethanol (100 mM), and (v) only ethanol (100 mM). The hepatocytes stimulated with ethanol show enhanced fluorescence with time, while hepatocytes protected with NAC and Trolox show reduced fluorescence indicating the protective effect of these antioxidants. The hepatocytes treated with 4-MP show a further reduced fluorescence change.

extending to $100 \mu\text{M}$. A microsystem utilizing enzyme-based electrodes was used to detect hepatic production of H_2O_2 that ranged from $0.29 \mu\text{M}$ at 60 min to $1.16 \mu\text{M}$ after 3 h. Due to the encapsulation of enzyme inside the gel the sensor shows excellent stability. To demonstrate utility of our platform for liver toxicology studies, the hepatocytes were pretreated with antioxidants prior to alcohol insult and were observed to generate significantly lower levels of oxidative stress as result. The functionality of this microsystem may be enhanced in the future by integrating additional biosensors for inflammatory molecules produced by liver cells during injury.

ASSOCIATED CONTENT

Supporting Information

Performance of hydrogel (PEG-DA) electrodes containing and lacking enzyme (HRP); stability of HRP inside the PEG-DA hydrogel matrix. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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