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Stimuli Responsive Electrodes Detect Oxidative Stress and Liver Injury

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Graphical Abstract

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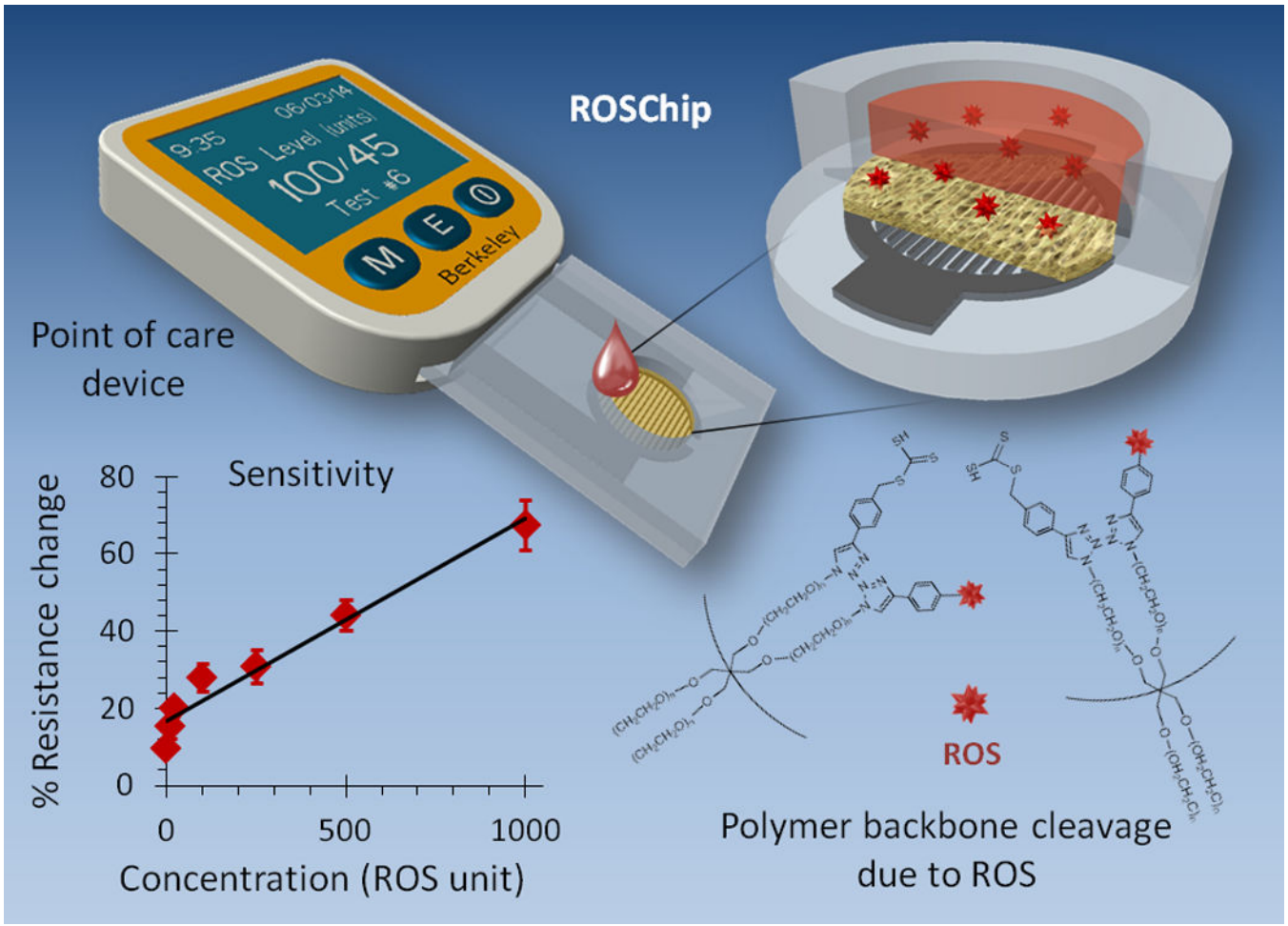

Keywords

reactive oxygen species; interdigitated microelectrodes

Reactive oxygen species (ROS) are a universal diagnostic biomarker for many human diseases. Screening for ROS in a point-of-care (POC) manner has the potential to improve the diagnosis and treatment of a wide variety of diseases, ranging from cardiovascular diseases to drug induced liver failure.[1–15] Developing POC assays for ROS and oxidative stress has been challenging because current methods for ROS monitoring are based on optical detection, which is incompatible with the restrictions inherent to POC assays, such as low cost, portability and minimal infrastructure.[5,7–9,11–13,16–22] Electrode-based biosensors are ideal for POC monitoring of oxidative stress because of the low cost of manufacturing electrodes, their small size, and the minimal instrumentation needed to make measurements from electrodes. Currently the only method available for ROS detection by electrodes is via amperometric detection of H_2O_2.[23–30] However, this strategy has been challenging to convert into a POC assay because of potential problems with sensitivity, storage and the inability to detect ROS species outside of H_2O_2 such as lipid hydroperoxides.

In this report, we present a new strategy to develop an electrode based biosensor for enzyme free monitoring of oxidative stress, which can for the first time measure the total ROS in the blood in a POC format. The biosensor, termed ROSChip, is composed of interdigitated microelectrodes (IDMEs) coated with a thin film of a hydrogel polymer that contains ROS degradable thiocarbamate linkages in its backbone (see Figure 1). The underlying principle of the ROSChip is based on a stimuli responsive polymer coated electrode acting as an insulator, controlling the electrical properties of the electrodes.[31,32] In the ROSChip, the ROS responsive hydrogel polymer, termed RRG, acts as an electrical insulator, causing high resistance of the IDMEs in their initial state. However, exposure to ROS present in tissue specimens degrades the RRG and reduces the IDMEs resistance, which can be detected via a handheld voltage meter. We have been able to demonstrate that ROSChip can detect micromolar levels of ROS through impedance analysis, offering a cost effective alternative to optical-based assays. In addition, we have been able to demonstrate that ROSChip can detect acetaminophen (APAP) induced liver toxicity in mice by measuring blood levels of oxidative stress after drug administration, indicating that it may have the sensitivity needed to detect drug induced hepatotoxicity in a POC manner. ROSChip is the first example of an impedimetric biosensor based on ROS responsive polymer coated microelectrodes, which can directly transduce in response to ROS for POC monitoring of oxidative stress.

RRG is the key component of the ROSChip and was synthesized by reacting an alkyne functionalized bisthiocarbamate crosslinker with a 4 arm-azide polyethylene glycol (PEG). The synthesis of RRG was performed according to the scheme shown in Figure 2a and was carried out in 3 steps (see supporting information). The thiocarbamate linkage was selected for this application because it is a well-studied linkage that degrades in the presence of ROS, and its redox responsive properties have been extensively used to trigger the reversible addition-fragmentation chain transfer (RAFT) polymerizations.[33–36]

The swelling and degradation of RRG in the presence of ROS is a key part of the ROS sensing process by ROSChip. We therefore investigated the swelling and mass loss ratio of RRG in the presence of ROS. An arbitrary unit “ROS U” has been defined for ROS concentrations where one ROS U corresponds to hydroxyl radicals generated from the reaction of 1μM H_2O_2 in presence Fe_2O_3. Figure S1 demonstrates that RRG degrades in the presence of ROS. For example, the percent mass loss of RRG discs (5× 5× 1mm) after exposure to buffer containing 5000 ROS U/hour significantly increased and resulted in complete degradation of RRG within 12 hours, whereas exposure to buffer alone caused negligible mass loss. A fast response of the RRG to ROS is crucial for its application in the ROSChip. We therefore investigated the swelling behavior of RRG in the presence of ROS after 20 minutes, a timeframe suitable for POC applications. Briefly RRG discs were incubated in buffer solutions containing ROS and the swelling ratio of the RRG discs were measured. Figure 2b demonstrates that RRG responds rapidly to ROS. For example the RRG disc volume in the swollen state increased over 170 percent after 20 minutes incubation with 1000 ROS U whereas RRG swelling after exposure to buffer alone was negligible.

ROSChip is designed to detect ROS by measuring the resistance of RRG coated IDMEs and is fabricated on a plastic substrate using microfabrication technology. The ROSChip fabrication process is based on photolithography to produce a mask, nickel electrodeposition

to produce the IDMEs and hot embossing to transfer the IDMEs into a cyclo-olefin copolymer (COC) plastic substrate (see Figure 2c). In order to functionalize the IDMEs, a very small amount (5μL) of RRG pre-polymer solution was placed directly onto the IDME array and the solution was allowed to polymerize overnight at room temperature. The sensor was then sealed and stored at 4ºC until used.

The most sensitive electrical parameters for the ROSChip were obtained by measuring its resistance, capacitance, impedance magnitude and phase in the presence of ROS over various frequencies. 5μL of buffer solutions spiked with ROS were placed directly on the surface of the IDMEs. ROSChip was powered by applying a very small amount of voltage (10mV) in the frequency range of 100 Hz to 100 kHz and the IDMEs electrical parameters were measured with an impedance analyzer. The results indicated that the resistance of the ROSChip measured at the frequency of 10kHz was the most sensitive parameter with the lowest variability (see supporting information).

ROSChip was utilized to measure ROS in fetal bovine serum (FBS). 10μL of FBS spiked with ROS was added to 40μL of buffer solutions on the surface of the IDMEs and the sensor resistance was monitored in real-time for 20 minutes. Figure 2d demonstrates that ROSChip can detect ROS linearly in the concentration range of 10–1000 ROS U. Thus, ROSChip is sensitive to biologically relevant concentrations of ROS and has the potential to replace photometric laboratory tests such as d-ROMs and FORT for POC monitoring of oxidative stress-related diseases. [16][37]

Additionally, in order to determine the ROSChip's detection mechanism, scanning electron microscopy (SEM) and surface profilometry of IDMEs after exposure to ROS in serum were performed. Figure 2e shows the SEM images of the RRG after exposure to buffer containing 1000 ROS U which indicated the appearance of micro/macro pores on the surface of RRG, whereas the surface of RRG after exposure to buffer only maintained its properties. In addition, Figure 2f demonstrates that the thickness of the RRG coating, measured by a non-contact 3D optical profilometer, decreases after exposure to serum spiked with 1000 ROS U. For example the average thickness of the polymer reduced from about 100μm to 80μm after 20 minutes of exposure to ROS due to RRG degradation, resulting in reduction in IDMEs' resistance. In contrast the average thickness of RRG in buffer only remained unchanged. The results from SEM and profilometry measurements indicated that exposure to ROS significantly changes the insulating properties of RRG and facilitates the diffusion of ions into the IDMEs which decreases the ROSChip resistance.

ROSChip has numerous clinical applications due to the central role of oxidative stress testing in preventative medicine. For example, the early detection of oxidative stress in drug-induced liver injury (DILI) can significantly improve treatment outcomes by enabling drug dose modifications at an early stage in order to prevent the disease progression. We therefore investigated if ROSChip could detect hepatotoxicity in mice. All animal experiments were performed in accordance with the policies of the animal ethics committee of the University of California at Berkeley. Mice were given an intraperitoneal injection of APAP and the control group of mice received the same volume of PBS. 24 hours after the injection of APAP, the mice were euthanized and the blood was collected by cardiac puncture and

analyzed by ROSChip. In addition to verify that the ROSChip responds to ROS, the serum concentration of lipid hydroperoxides were measured using the thiobarbituric acid reactive substances assay (TBAR), and the effect of antioxidants on the ROSChip signal was also investigated. Furthermore, the alanine aminotransferase (ALT) activity in the serum was measured and histological assessment of liver tissue was performed to determine if the ROSChip measurements could be used as a surrogate biomarker for liver toxicity.

Figure 3a demonstrates that ROSChip can detect oxidative stress as a result of liver injury. For example, ROSChip generated approximately a 60% reduction in IDME resistive response after 20 minutes exposure to APAP treated mice blood serum, in comparison to healthy mice, which only generated about 20% reduction in ROSChip resistance. Moreover, the ROSChip response could be suppressed by the addition of the antioxidant enzyme catalase, due to scavenging of ROS. In addition, the ROSChip measurements were in agreement with the TBARs oxidative stress assay. For example Figure 3b demonstrates that mice with DILI have a higher concentration of malondialdehyde (MDA) (by a factor of ~1.7), in comparison to control mice, indicating higher levels of lipid hydroperoxides. The results from the ROSChip measurements also correlated with the ALT blood levels in the mice, demonstrating that ROSChip has the potential to be used as a digital biosensor for the detection of oxidative stress and DILI in a POC manner.

In conclusion, in this report we present a new portable digital biosensor, based on ROS-responsive interdigitated microelectrodes, which can detect oxidative stress in a POC manner. ROSChip has the potential to be utilized for monitoring oxidative stress in routine clinical practice and therefore can have a significant impact on preventative medicine, given the central role of oxidative stress in the pathogenesis of human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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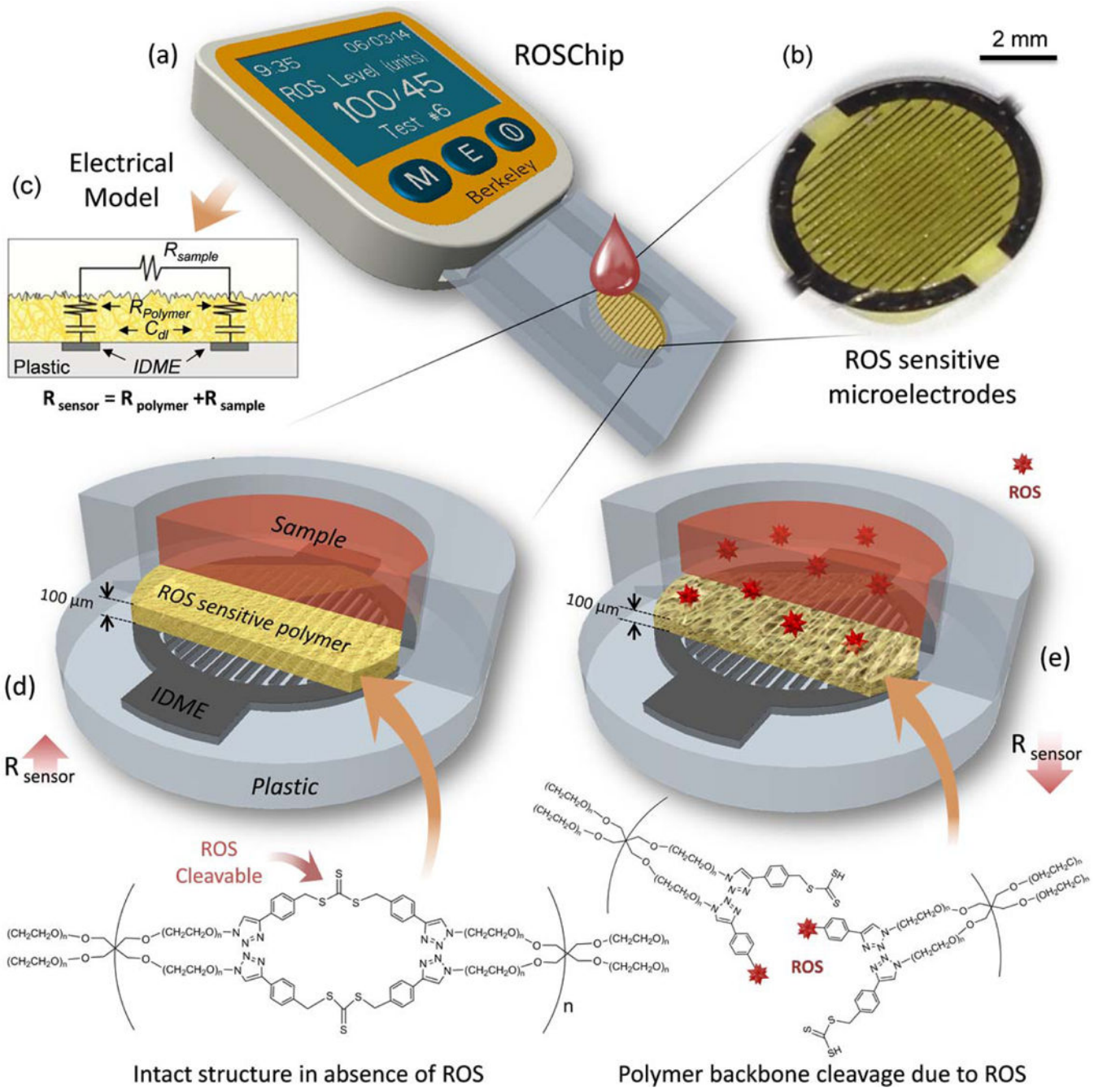

Figure 1: ROSChip is an oxidative stress point of care biosensor based on a ROS responsive electrodes

ROSChip is an electrode based biosensor which is composed of interdigitated microelectrodes (IDMEs) coated with a thin film of a ROS sensitive hydrogel polymer, termed RRG, that contains ROS degradable thiocarbamate linkages in its backbone. The RRG acts as an electrical insulator, causing high resistance of the IDMEs in their initial state. However, exposure to radical oxidants present in the sample result in RRG degradation which decreases the IDMEs resistance that can be detected via a handheld voltage meter. (**a**) ROSChip is a plastic cartridge designed for the POC monitoring of oxidative stress. (**b**) A

picture of IDMEs coated with the RRG. (**c**) A simplified serial circuit to quantify ROSChip responce. C_{dl} represents the capacitance behavior of the sensor which is directly associated with the RRG properties. $R_{polymer}$ and R_{sample} represent the total resistance of the sensor (R_{sensor}) (**d**) The RRG polymer remains intact in the absence of ROS, its electrical insulating properties are maintained and the total resistance of the sensor is high. (**e**) The total resistance of the sensor reduces in the presense of ROS as the RRG thiocarbamate linkages break and the polymer degrades.

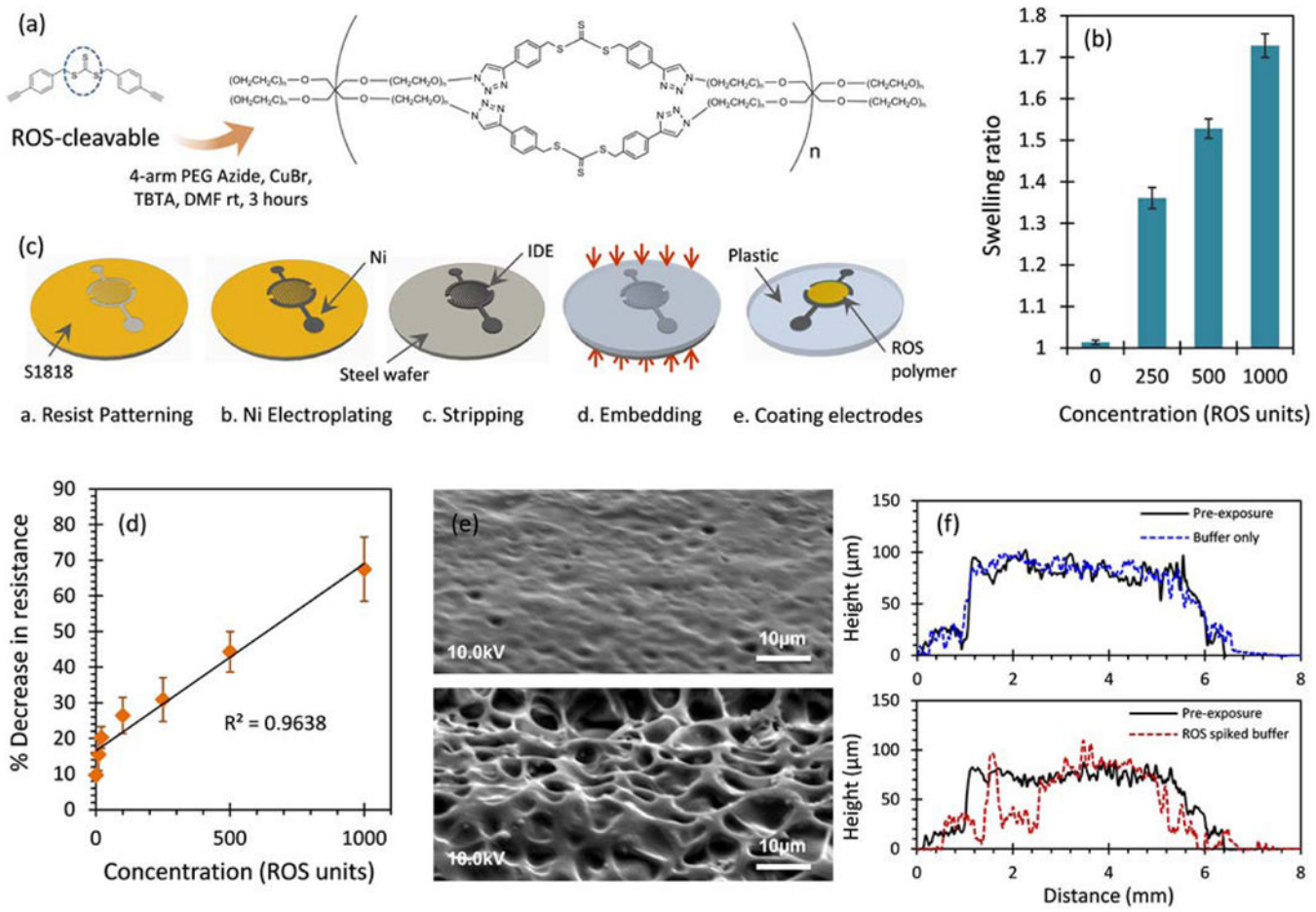

Figure 2: ROSChip is fabricated on a plastic substrate and can detect the presence of hydroxyl radicals in the sample.

(**a**) RRG is the key component of the ROSChip and was synthesized by reacting an alkyne functionalized bisthiocarbamate crosslinker with a 4 arm-azide polyethylene glycol (PEG). (**b**) The swelling ratio of the RRG discs after 20 minutes incubation with hydroxyl radicals significantly increased whereas RRG swelling after exposure to buffer alone was negligible. (**c**) The fabrication workflow of ROSChip on a plastic substrate. For detailed description of the fabrication process, please see supplementary information.(**d**)ROSChip was utilized to measure ROS in fetal bovine serum (FBS). ROSChip resistance decreased linearly in the concentration range of 10μM-1mM. (**e**) Representative SEM images of the IDMEs coated with RRG after 20 minutes exposure to buffer only and buffer containing 1000 ROS.U. Significant changes in the RRG morphology and the appearance of micro/macro pores on its surface were observed after exposure to hydroxyl radicals. In contrast RRG remained intact after exposure to buffer only. (**f**) Corresponding surface topography scans performed by optical profilometer indicated that the average thickness of the coated IDMEs decreased from about 100μm to 80μm in the presence of 1000 ROS U. In contrast the thickness of the RRG coated IDMEs remained unchanged after 20 minutes exposure to buffer alone.

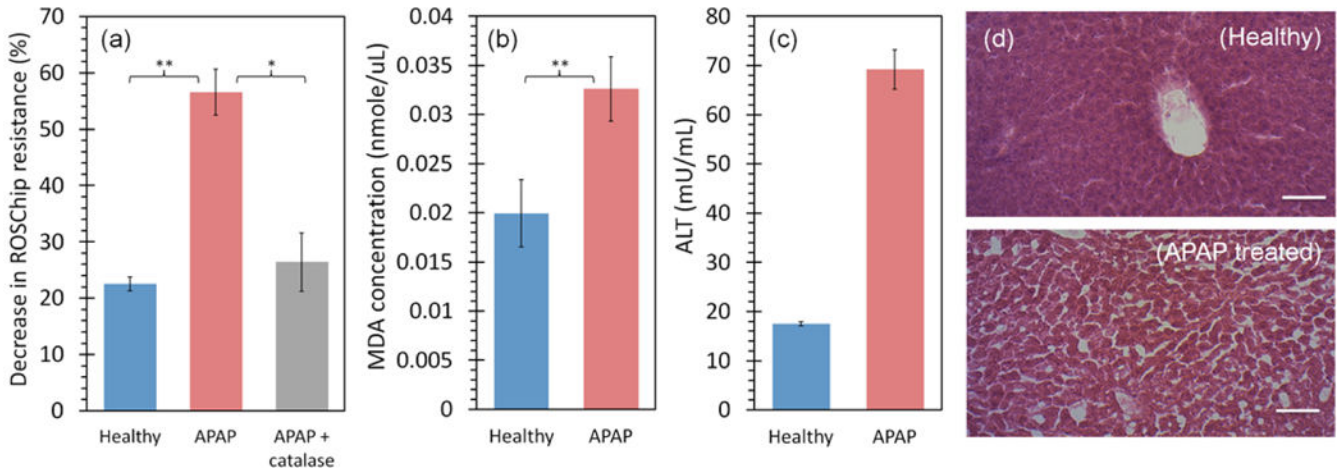

Figure 3: ROSChip can detect drug induced liver injury (DILI)

ROSChip can detect acetaminophen (APAP) induced liver injury in mice by measuring blood ROS levels after drug administration, indicating its potential to detect drug induced hepatotoxicity in a POC manner. (**a**) ROSChip was capable of detecting drug induced hapatotoxicity by measuring circulationg ROS. The results indicated a 60% reduction in ROSChip resistive response after exposure to APAP induced mice serum compared to a 20% reduction in healthy mice. The addition of 50μM catalase to APAP treated mice serum suppressed the ROSChip response. (**b**) TBARs assay of APAP treated mice serum compared to control mice indicated higher levels of MDA in APAP treated mice. (**c**) Blood ALT levels in APAP induced mice compared to control mice. (**d**) Histology analysis from the liver sections indicating significant necrosis in mice treated with APAP.