An Electrochemical Biosensor based on Gold Microspheres and Nanoporous Gold for Real-time Detection of Superoxide Anion in Skeletal Muscle Tissue*

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***Abstract*— Superoxide anion (SOA) as a member of reactive oxygen species (ROS) group is involved in various physiological and pathological states. For instance, generation of SOA is known to increase with skeletal muscle contractile activity and fatigue. It is therefore important to selectively detect and accurately quantify the release of SOA within both physiological and pathological levels. We report fabrication and characterization of a cytochrome-*c* functionalized SOA biosensor built on commercially available miniaturized screen-printed electrodes made of gold microspheres. The device was first tested and calibrated in a xanthine/xanthine oxidase (XOD) system and then employed to detect SOA release from C2C12 myoblasts and myotubes upon stimulation with PMA.**

I. Introduction

Reactive oxygen species (ROS) include oxygen radicals such as the hydroxyl radical, $OH^{\bullet}$, and the superoxide anion free radical, $O_2^{\bullet-}$ (SOA), as well as non-radicals like hydrogen peroxide H_2O_2. While at rest, skeletal muscle cells are recognized to continuously give off various ROS, primarily the SOA [1]. The rate of SOA generation rises during contractile activity and to a higher extent during fatiguing exercise [1, 2]. Oxidative stress induced by higher than normal levels of ROS are followed by pathological causes such as decreased cell proliferation rate, altered gene expression, damage to lipids and proteins or DNA, and may eventually lead to tissue destruction [3-5]. Hence, in order to differentiate between physiological and pathological states of the tissue, it is essential to detect and evaluate the release of SOA in real-time and with a reasonable selectivity and sensitivity [6].

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Mitochondria are the major site of SOA generation in skeletal muscle cells. Actually, a significant fraction of the mitochondrion oxygen intake may experience one-electron reduction resulting formation of SOA [3]. Upon inflammation cells produce large amounts of SOA as part of their immunological defense mechanism. Compared with other free radicals SOA has a longer half-life and can move around within the cell targeting various organelles. However, because of being negatively charged it is relatively membrane impermeable and difficult to detect in the extracellular milieu [1].

Here we report the results of our ongoing research on the fabrication and characterization of an electrochemical biosensor for discrimination of extracellular SOA in skeletal muscle cells based on one-electron reduction of cytochrome-*c* hemeprotein (cyt-*c*). SOA causes reduction of cyt-c^{3+} to cyt-c^{2+} which is then reoxidized at the electrode surface producing currents proportional to the rate of anion generation. Among various methods for SOA detection, bioelectrodes with immobilized cyt-*c* have proven to be stable, relatively selective, sensitive and offer a low limit of detection [4, 7-9]. Films of sintered gold microspheres and nanoporous gold (NPG) were examined as the working electrode of an electrochemical cell. The device was used to detect stimulus-generated SOA in C2C12 myoblasts and myotubes. It was also shown that the NPG coated electrode offers an enhanced sensitivity over the gold microsphere coated counterpart.

II. Experimental Work

A. Sensor fabrication and calibration procedures

Screen-printed three-electrode strips with working and counter electrodes coated by gold microspheres and a silver reference electrode were purchased from DropSens Ltd [10]. Working electrodes were used as received or they were coated with a 1μm-thick film of nanopourus gold (NPG) as described previously [11]. Briefly, an $Au_{18}Ag_{82}$ layer was electrodeposited on the electrode first. Next, the silver constituent was removed by electrochemically dealloying in 1.0 M HNO_3 at room temperature, leaving the nonporous network of pure gold with an average pore size of 20 nm.

SEM micrographs taken from the surface of the original and NPG-modified working electrodes are shown in Fig. 1a and 1b respectively. After rinsing the electrodes in DI water and drying in air, cyt-*c* was attached to them by means of a

covalent binder, 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP). To realize this, the electrodes were first drop coated with 5 µl aliquots of 50 mM DTSSP and left at room temperature in clean bench for 20 minutes to dry. Next, 5 µl aliquots of 2 mM phosphate buffered saline (PBS) based cyt-*c* solution were placed on the DTSSP modified electrodes and incubated at 37 °C for two hours. After incubation, the sensors were stored at 4 °C until usage, prior to which they were rinsed again with DI water to remove unattached cyt-*c* remnants.

A custom-made fixture was built with three electrical feedthroughs to facilitate connection of guarded triax leads to the electrodes on the strip. The strip was initially secured in a transparent methacrylate wall-jet flow cell. Fig. 1c shows a snapshot of the sensor fixture. Two source measure units (SMU) of a Keithley 4200 semiconductor parameter analyzer were programmed to carry out chronoamperometric measurements. The flow cell was filled by 500 µl of the background substance; either a HEPES based saturated solution of xanthine for calibration purposes, or pure HEPES for actual SOA pickup from the C2C12 cell culture. The current drawn by the working electrode and the voltage of the reference electrode were monitored during operation, while the former was biased at 200 mV with respect to the counter electrode.

SOA generated from the well known enzymatic reaction between xanthine and XOD was used for sensor calibration. To this end, 1, 3, and 5 µl aliquots of bovine milk extracted XOD, corresponding to enzymatic activities of 15, 45, and 75 units per milliliter (U ml^{-1}) were injected into pure HEPES and 0.3 mM saturated solution of xanthine in HEPES at room temperature. Fig. 2a and 2b show the amplitude of instantaneous current densities recorded from the gold microsphere electrode, operated in HEPES and saturated xanthine solution respectively. Arrows indicate the time of XOD administration. In comparing these two curves, one can see SOA is generated only in the saturated xanthine background. The 'knees' in the current density profiles as indicated by red arrows in Fig. 2b, correspond to the beginning of SOA forming reaction and can be tied to the activities of administered XOD aliquots. Enzymatic reaction resumes until a steady state is achieved.

It is well established that the steady state concentration of SOA, $[O_2^{\bullet-}]$, is proportional to the square root of XOD concentration, [XOD], [12] as in

$$J = \alpha\,[O_2^{\bullet-}] = \kappa\,[\mathrm{XOD}]^{1/2}, \qquad (1)$$

where J is the current density, α represents the ideal case where each SOA molecule reduces one cyt-*c* molecule and the resulting electron contributes to J. The apparent constant of proportionality, κ, was computed from the curve in Fig. 2b and finally the sensor transfer function was obtained by plotting $[O_2^{\bullet-}]$ versus the value of current densities at the knee points (Fig. 2c). The NPG electrode was functionalized and characterized in a similar fashion and displayed a 7-fold sensitivity improvement because of its higher electrode surface-to-volume ratio.

B. Cell culture and in vitro SOA detection

C2C12 myoblasts were seeded at a density of 10^5 cells per milliliter on plastic membranes detached from the bottom of standard 12-well cell culture plates with 400 nm wide pores. The cell laden membranes were subsequently incubated at 37 °C, in 5% CO, for two days in a growth culture medium. In order to induce formation of myotubes, the medium was switched to a differentiation medium at day two of culture and remained therein for four more days. Membranes were gently rinsed in HEPES and instantly transferred onto the cyt-*c* functionalized sensors where the working electrode was immediately biased to the desired potential. Cell viability was examined through immunostaining by calcein-AM and ethidium homodimer live/dead probes, both prior to and right after the measurement procedure. The former probe turns green in alive cells, while the latter penetrates damaged cell membranes and becomes red.

Amperometric detection of SOA release from the cell-attached membranes was carried out by the addition of phorbol 12-myristate 13-acetate (PMA), a routinely used endogenous superoxide producer. PMA stock solutions were prepared in dimethyl sulfoxide (DMSO) and administered in 650 nM dose multiples. The total concentration of PMA containing DMSO was 0.4%, well below the lethal level.

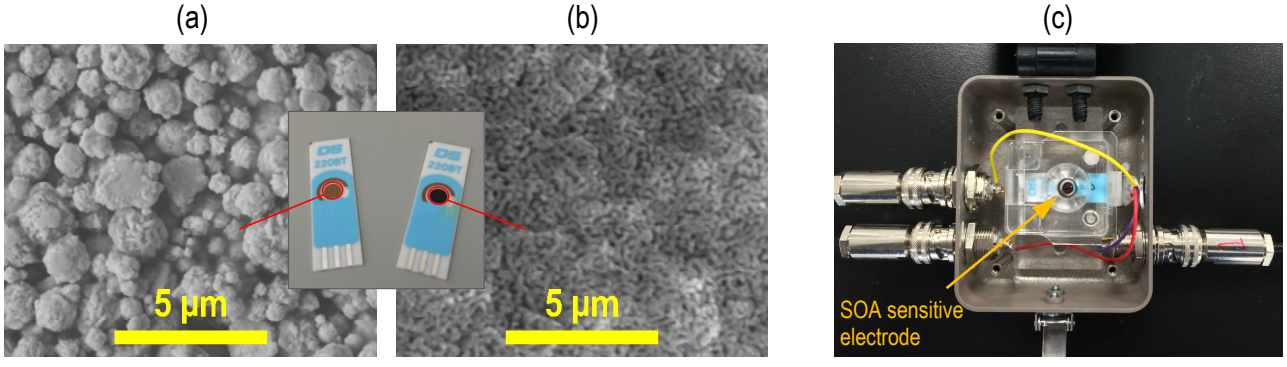

Figure 1. SEM micrographs taken from the surface of working electrodes made of (a) gold microparticles and (b) nanoporous gold film. (c) A snapshot of the sensor installed in the fixture.

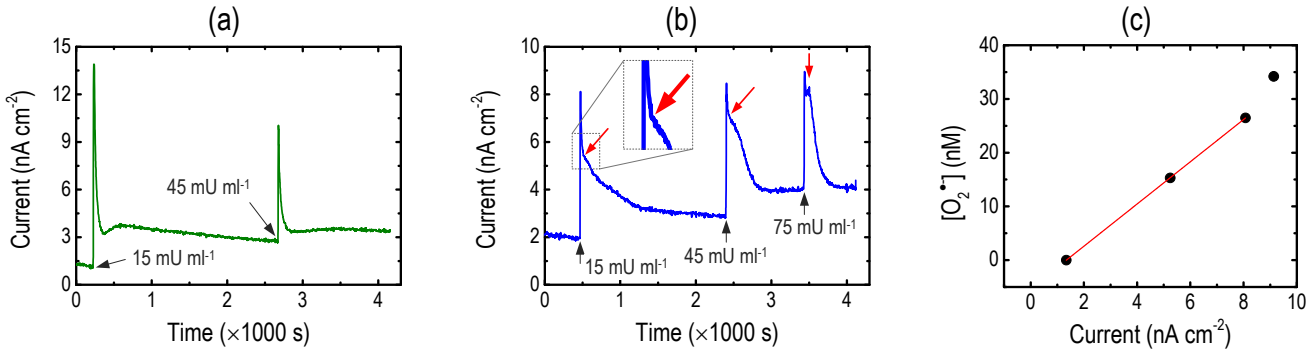

Figure 2. Instantaneous response of the SOA sensor to the addition of XOD aliquots into (a) pure HEPES, and (b) 0.3 mM solution of xanthine in HEPES backgrounds. (c) biosensor transfer function obtained by operating the sensor in the xanthine/XOD system.

III. Results and Discussions

Fig. 3a shows the simultaneous recording of the time course of SOA production by stimulated C2C12 myoblasts. After a stable baseline was achieved, 1 µl aliquots of (i) pure HEPES, (ii) DMSO (4 vol%), and (iii) PMA (650 nm) were injected into the flow cell. It can be seen that the first two injections did not perturb the baseline, while PMA induced a steady increase in the current that plateaued after about ten minutes. In average, the rate of increase during the initial 200 seconds following the administration of PMA corresponds to an SOA generation rate of 0.55 nM min^{-1}. This value is close to the rate of SOD release recorded within the first minute of fatiguing exercise (0.7 nM min^{-1}) [2]. Similar experiment was carried out using the membranes containing C2C12 myotubes at day four of differentiation. Fig. 3b shows the recorded current density after eliminating the baseline offset. In this case, addition of 650 nM PMA was not sufficient to induce a tangible SOA signal (not shown in this figure), however, administration of 1300 nM PMA triggered the C2C12 tissue to generate an SOA pulse that lasted about 200 seconds. No SOA signal was detected upon stimulation of the myotubes at day 7 of differentiation by the same amount of PMA.

Fig. 3c shows fluorescence images taken from immunostained C2C12 myoblasts stained after running the the SOA inducing experiment and measurement procedure in HEPES at room temperature for 45 minutes. Apparently, cell viability was not affected by DMSO or PMA.

IV. Conclusion

Cyt-*c* functionalized electrodes were sensitive to PMA-induced superoxide produced by C2C12 tissues cultured on porous membranes. C2C12 myoblasts displayed a relatively constant SOA generation rate after the drug was administered. Calibration procedure demonstrated NPG based sensors are about 7 times more sensitive than the as-received sensor. Ongoing work is focused on accurate quantification of SOA release using NPG based sensors.

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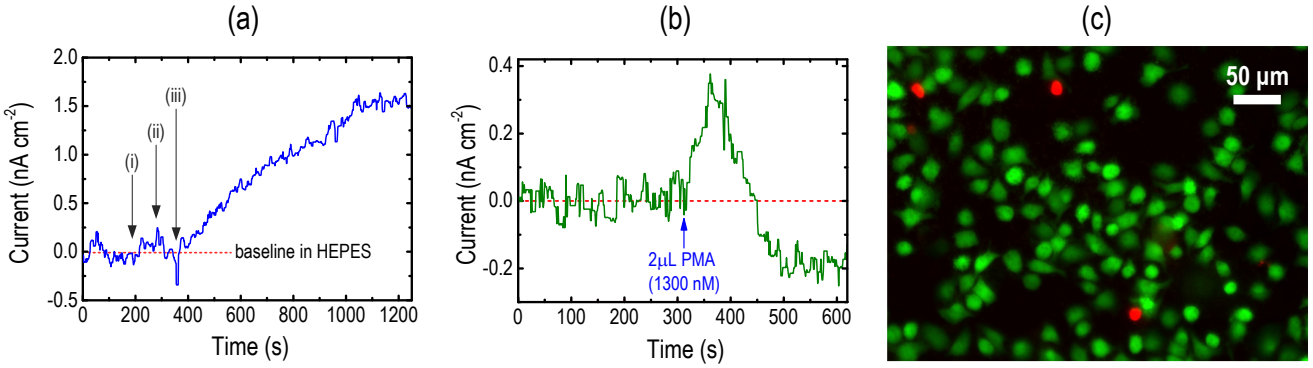

Figure 3. (a) Amperometric measurement of SOA generated by C2C12 myoblasts upon addition of 650 nM PMA at point (iii), and (b) response of C2C12 myotubes to 1300 nM PMA. (c) Fluorescence images obtained from immunostained C2C12 myoblasts to evaluate post-expertiment cell viability.

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