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Macroporous mesh of nanoporous gold in electrochemical monitoring of superoxide release from skeletal muscle cells

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ABSTRACT

Real-time monitoring of metabolically relevant biochemicals released in minuscule amounts is of utmost diagnostic importance. Superoxide anion as a primary member of reactive oxygen species, has physiological and pathological effects that depend on its concentration and release rate. Here we present fabrication and successfully testing of a highly sensitive electrochemical biosensor featuring a three-dimensional macroporous mesh of nanoporous gold tailored to measure the dynamics of extracellular superoxide concentration. Wide and accessible surface of the mesh combined with high porosity of the thin nanoporous gold coating enables capturing the analyte in pico- to nano-molar ranges. The mesh is functionalized with cytochrome-*c* (cyt-*c*) and incorporated as a working electrode to measure the release rate of drug-induced superoxides from C2C12 cells through a porous membrane. The device displays a considerably improved superoxide sensitivity of 7.29 nA nM^{-1} cm^{-2} and a low level of detection of 70 pM. Such sensitivity is orders of magnitude higher than any similar enzyme-based electrochemical superoxide sensor and is attributed to the facile diffusion of the analyte through the well-spread nanofeatured gold skin. Superoxide generation rates captured from monolayer myoblast cultures containing about 4×10^4 cells, varied from 1.0 to 9.0 nM min^{-1} in a quasi-linear fashion as a function of drug concentration. This work provides a platform for the development of highly sensitive molecular electrochemical biosensors.

1. Introduction

Biosensors are useful in numerous applications in medicine, including biomolecular sensing, monitoring cellular interactions with their microenvironment, and in assessing free radicals such as reactive oxygen and reactive nitrogen species (ROS and RNS) (Calas-Blanchard et al., 2014; Ingebrandt, 2015; Qiu et al., 2014; Zhao et al., 2011). Detection of antioxidants, free radicals, and particularly ROS have recently attracted much attention because of their diverse physiological and pathological effects (Halliwell, 2006). Such diverse, yet contrasting functions of ROS, and superoxide as the primary member of the group in particular, makes accurate determination of these biospecies an important and challenging task.

In the context of skeletal muscle tissue, extracellular superoxides rise during contractile activity and fatigue (Kolbeck et al., 1997; Powers and Jackson, 2008; Yavari et al., 2015). Such rise is limited within physiologically safe levels and is even known to attenuate during aging (Jackson and McArdle, 2011). Superoxides at higher levels, along with other members of ROS, may trigger muscle tissue malfunctioning and damage (Jackson and McArdle, 2011; Matecki et al., 2014; Rochard et al., 2000; Sestili et al., 2009).

The longer half-life of superoxides compared to other ROS allows them to move within the cytoplasmic region targeting

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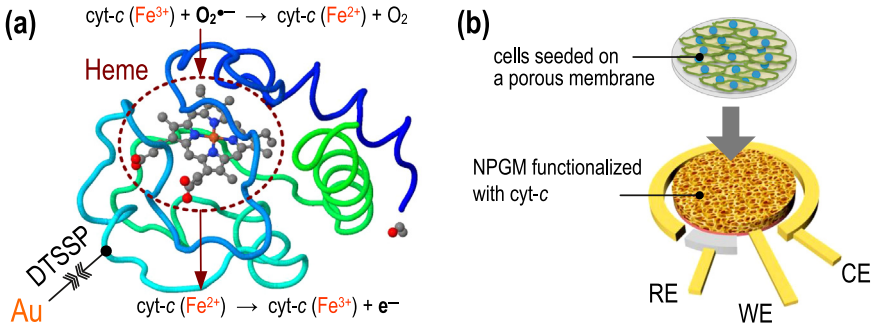

Fig. 1. Electrochemical detection of extracellular superoxides. (a) Sensing mechanism by oxidation and direct electron transfer between the redox protein (cyt-*c*) and the electrode. Fe^{3+} located at the center of the heme group is reduced by an approaching superoxide radical molecule which is in turn reoxidized at the positively biased electrode. (b) Cells cultured on a porous membrane are interfaced with the functionalized electrode. WE, CE, and RE denote the working and counter electrodes (Au), and the reference electrode (Ag), respectively.

various organelles. Nonetheless, they are negatively charged and rather impermeable through the plasma membrane making them difficult to monitor in the extracellular milieu (Murrant and Reid, 2001). The uncertainly in the spatiotemporal states of superoxides in the literature accompanied by their concurrent scarceness and volatility necessitate design of a sensitive, rapid, and noninvasive biosensor capable of targeting and detecting the substances in minuscule amounts and in real-time.

Variety of techniques have been used to detect superoxides in skeletal muscle tissue (Abdel Khalek et al., 2014; Mujahid et al., 2005; Xu et al., 2010), among which the electrochemical methods are favorable because of their inherent sensitivity, fast response, and nondestructive nature (Flamm, 2014; Manning and McNeil 2011; Szilveszter 2011). The physiological concentrations of superoxides are remarkably low, at the nanomolar range, and may shrink even further by undergoing an enzymatic disproportionation reaction with superoxide dismutase (SOD). To be able to monitor the substances at these conditions, even electrochemical biosensors must be placed in a close proximity of the region of interest, making them often impractical for safe in vivo operation or to employ at the site of injury (Fujita et al., 2009; Ganesana et al., 2012; Rahman et al., 2012). Ganesana et al. (2012) recently employed a gold wire cytochrome-*c* (cyt-*c*) based sensor and measured the accumulation of superoxides in mice brain slices in micro-molar range with a sensitivity of 0.14 nA nM^{-1} cm^{-2} in culture medium. Fujita et al. (2009) used a carbon electrode functionalized with a cyt-*c*-mimic synthetic enzyme to measure superoxide concentrations in the right atria of endotoxemin rats. They integrated the amperogram over time and expressed the sensor response based on the accumulated charge resulting in a sensitivity of 0.41 μC $μM^{-1}$. Recently, Zhu et al. (2016) have utilized a porous Pt-Pd construct, decorated with SOD, and detected superoxides in living HeLa cells with a sensitivity of 1.27 nA nM^{-1}. Taking into account the importance of ROS levels in physiological and pathological contexts, it is vital to measure these species in the lowest possible amounts, necessitating devices with orders of magnitude higher sensitivities.

Sensors can be technically placed farther provided their sensitivity is enhanced by increasing the electrode surface area. Making a rough surface is one way to achieve this without compromising space. Nanoporous gold (NPG) is a spongy structure comprised of nanoscale pores and ligaments that offer effective surface areas hundreds to thousands of times larger than bulk gold films of the same volume. Such a large surface area along with the permeable network of nanopores has rendered NPG a desirable construct for the fabrication of sensitive chemical sensors (Collinson, 2013; Scanlon et al., 2012).

Recently, we have demonstrated the superior performance of NPG over screen-printed (SP) gold electrodes in superoxide biosensing (Banan Sadeghian et al., 2016). In this study, the NPG electrode functionalized with cyt-*c* displayed 14 times better sensitivity than the nonporous counterpart, having the same apparent surface area. Although the thick-film NPG offers an electrochemically-active surface area (ESA) a few hundred times larger than the nonporous SP electrode, the sensitivity of the NPG-based electrode toward superoxides does not scale up accordingly. Only a fraction of the electrode volume at the surface is exposed to the surrounding superoxide-containing medium. Nanoscale pores hinder the diffusion of the medium or cyt-*c* well into the bulk of the NPG thick-film. Hence, a large fraction of the electrode remains isolated and unmodified.

Here we present an innovative electrode architecture by wrapping a thin film of mesoporous gold over a metallic cellular matrix, thus resolving the coverage and diffusion issues at the same time and improving the electrode inherent sensitivity. The hierarchical nanofeatured network, referred to as macroporous mesh of nanoporous gold, or simply nanoporous gold mesh (NPGM), was assembled on ultralight nickel foam templates. Ni foams are commercially available, low density, permeable, but mechanically strong metallic platforms with high porosity (75–95%). They have a wide range of applications in gas and liquid filters, batteries, vibration-absorbing materials, heat exchangers, optics, etc. (Schaedler et al., 2011; Xia et al., 2015; Xiao et al., 2015). If the macroporous skeleton of the foam is covered by a mesoporous gold layer, the resulting network can offer a tremendously large surface area. In addition, because such layer is thinner and coarser than thick-film NPG, it allows the sensitive enzyme to diffuse and cover a larger fraction of the nanostructure.

Presence of Ni as a cytotoxic element, however, hinders application of the mesh as a noninvasive biosensor. Hence, the entire Ni constituent of NPGM including the core was removed chemically during the dealloying process, forming tubular ligaments. The foams were first shielded by a layer of Au to provide mechanical support for the NPG skin once the Ni core is removed. The golden construct was subsequently modified with cyt-*c*. Biosensors built on cyt-*c*-modified NPGM, were interfaced with C2C12 myoblasts through a porous membrane and used to accurately measure the rate of superoxide release in nM min^{-1} upon stimulation with phorbol 12-myristate 13-acetate (PMA). Drastically improved amperometric sensitivity, low limit of detection (LOD), and linear dynamic range over similar superoxide-sensitive enzyme-immobilized electrodes were attained. Such hierarchical nanofeatured construct can provide numerous implications in a broad range of biosensing applications where sensitivity and rapid response matter.