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REVIEW

Progress on Electrochemical Determination of Superoxide Anion

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Abstract: Superoxide anion ($O_2^{\bullet-}$) is the primary species of the reactive oxygen species (ROS), resulting from the metabolism of O_2 in the living organisms. Its dynamic changes can provide broad implications in physiological and pathological conditions. Therefore, the analytical methods that can determine $O_2^{\bullet-}$ real-time and in vivo are receiving more and more attention. In light of these requirements, electrochemical approaches are the most promising candidate techniques in analysis of $O_2^{\bullet-}$ due to their simplicity, direct measurements, and ease of miniaturation. This review summarizes the recent progress on electrochemical analysis of $O_2^{\bullet-}$ taking into consideration toward the key issues: design of the electrolyte/electrode interface, direct electron transfer of enzymes, and applications in live cells and animals.

Key Words: Superoxide anion; In vivo; Electrochemical analysis; Direct electron transfer; Enzymes; Reveiw

1 Introduction

As the active intermediates of metabolism, $O_2^{\bullet-}$ maintains a relatively stable state in the cells/organisms. Cells/Organisms rely on antioxidant ability of cytochrome *c* (Cyt. *c*) and superoxide dismutase (SOD) to turn $O_2^{\bullet-}$ into harmless substances and keep the dynamic balance by self-repair. This series of process have significant effect on cell proliferation, apoptosis, injury, and play a central role in cell signal transduction. Depending on concentration, location and context, $O_2^{\bullet-}$ can be either “friends” or “foes”. When cells are stimulated or diseases mutate, excessive $O_2^{\bullet-}$ generation leads to apoptotic and necrotic cell death and pathogenesis of a panel of clinically distinct disorders including neurodegeneration, atherosclerosis, diabetes and cancer, which further affect the physiological and pathological functions[1–5]. Therefore, it is undoubtedly of great significance to realize the determination of $O_2^{\bullet-}$ in vivo.

However, it is still an analytical challenge to detect the local concentration of $O_2^{\bullet-}$ real-time and in vivo, especially in the biological systems, because of low concentration, high reactivity, and short lifetime. Electrochemical methods have drawn extensive attention because they have more advantages such as direct detection, high sensitivity, measurement in vivo, and so on. Among them, electrochemical sensor based on enzyme is the most attractive[6–11].

2 Design of electrolyte/electrode interface and direct electron transfer of enzymes

2.1 Design of electrolyte/electrode interface

The key problem to electrochemical analysis for $O_2^{\bullet-}$ is how to rationally design the electrolyte/electrode interface to improve and enhance the analytical performance of sensors[12–16]. However, the active centers of enzyme usually are buried deeply inside because of the large size of enzyme molecule. Thus, the electron transfer process would become difficult due to the longer distance between elctroactive centers and electrode surface. To solve this problem, the second-generation sensors adopt mediator confined on the electrode to facilitate the electron transfer. However, the

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disadvantages such as loss of mediators and interference, limit the extensive application of sensors relied on mediator. Therefore, to construct the selective and sensitive third-generation for $O_2^{\bullet-}$, much effort has been paid for direct electron transfer of enzyme. By optimizing the microstructure of electrode surface and the interfacial reactions, it would make the electroactive centers in enzyme molecule exposed more close to the electrode, and then promote the direct electron transfer between proteins and electrode surface.

2.1.1 Molecular design

Of these hitherto reported categories of electron transfer of Cyt. *c* and SOD, the one accomplished by electron transfer promoters is the most attractive because of its less dependence on electrode surface, higher electron transfer rate and potential utilization for the development of the third generation of $O_2^{\bullet-}$ biosensors.

The self-assembled monolayer (SAM) of thiols and disulfides confined on gold electrodes can efficiently facilitate the electron transfer of the Cyt. *c* and SOD, and the combination of such a well-promoted electron transfer properties with the inherent biological catalytic activity of the proteins toward $O_2^{\bullet-}$ substantially make it possible to construct the third-generation biosensors for $O_2^{\bullet-}$. Ohsaka *et al*[17] demonstrated that the direct electrochemical redox reaction of Cu, Zn-SOD was clearly observed at a gold electrode modified with a SAM of cysteine in phosphate buffer solution containing SOD, which did not happen at the bare electrode. In this case, it is easier for SOD to be stably confined on the cysteine-SAM electrode, suggesting the self-assembly of cysteine formed a thermodynamic stable molecular layer. And then, Ohsaka's group[18] realized the direct electron transfer of three kinds of SODs (Cu, Zn-SOD, Fe-SOD and Mn-SOD) on the 3-mercaptopropionic acid (MPA)-modified electrodes. The electron transfer process was investigated in the system of self-assembled molecular monolayer at the interface, and further feedback information for the deeper design of molecules and molecular assembly of function.

As commonly used means of selective binding of His-tagged protein, nitrilotriacetic acid/histidine-tag (NTA/HT) technique has become the most successful histidine binding template, and it can immobilize the enzyme firmly and further promote the process of electron transfer. Martin *et al*[19] utilized this technique to act as a generic template for the fabrication of oriented protein films on surfaces. The Acbztacn ligand stabilized the ligand/metal interaction through the macrocyclic effect, reducing the complexities associated with metal leaching, and indicating that the Acbztacn/Ni complex was very stable. NTA/HT technique was first employed to facilitate the electron transfer of SOD by Tian's group, and it was found that the direct electron transfer of SOD was greatly enhanced at NTA-modified electrode with a high rate constant of (24 ± 1.1) s^{-1}. Furthermore, the excellent analytical performance of sensor in this case successfully offered an electrochemical approach to in vivo monitoring of $O_2^{\bullet-}$ in a rat brain during cerebralischemia/reperfusion processes[20].

2.1.2 Nanomaterials

In biological models, the quantitative detection of $O_2^{\bullet-}$ concentration is very difficult due to its fleeting existence and low concentration. Therefore, an increase in proteins loading and a more efficient electron transfer may be considered for the development of a new $O_2^{\bullet-}$ sensor surface. With the development of nanostructured materials, a burst of research activity has been paid to nanostructured particles. Different from bulk materials and single molecules, nanomaterials with unique physical and chemical properties, especially the biocompatibility and the stability, are excellent matrix for proteins, and receive the widespread application in sensing.

Natan *et al*[21] observed the direct, reversible cyclic voltammetry of Cyt. *c* in solution at SnO_2 electrodes modified with uncoated sub-monolayers of 12-nm-diameter colloidal Au particles without any pretreatment or polishing steps. The colloidal particles behaved as an ensemble of closely-spaced but isolated microelectrodes. However, when the size of electrode features increased through particle aggregation, Cyt. *c* electrochemistry became quasi-reversible or irreversible, showing the importance of nanometer-scale morphology in protein electrochemistry, and well-defined, Au colloid-based substrates held promise as substrates for biological measurements on metal surfaces. In addition, a layer-by-layer route to prepare nanoporous Au film materials on transparent ITO substrates was first reported by alternatively assembling Au and Ag nanoparticles with 1,5-pentanedithiol as a cross-linker, followed by that Ag nanoparticles were dissolved at room temperature in $HAuCl_4$ solution[22]. Cyt. *c* adsorbed onto the nanoporous Au film still maintained its enzymatic activity toward H_2O_2, and its electron transfer at electrode surface was greatly facilitated with an electron transfer rate constant of 3.9 s^{-1}. Meanwhile, this sensor exhibited good selectivity, stability, wider dynamic detection linear range from 1.0×10^{-5} M to 1.2×10^{-2} M, and detect limits down to 6.3×10^{-6} M.

Zhao *et al*[23] studied the interface behavior and biocatalytic activity of SOD immobilized the electrode modified using carbon nanotubes with cyclic voltammetry. The large numbers of defects in the lattices of multi-walled nanotubes surfaces provided a higher local electron density. They contributed to the high surface activation, which could benefit the electron transfer between the enzyme and the nanotubes. On the other hand, the special nanostructure of CNT might act as a "molecular wire", leading electrons to the redox centers of SOD. Both the factors stand a good chance for the direct