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In vivo monitoring of oxidative burst on aloe under salinity stress using hemoglobin and single-walled carbon nanotubes modified carbon fiber ultramicroelectrode

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ABSTRACT

Single-walled carbon nanotubes (SWCNTs) and hemoglobin (Hb) modified carbon fiber ultramicroelectrode (CFUME) were employed to construct a direct electron transfer based *in vivo* H_2O_2 sensor. At the low working potential of −0.1 V, Hb/SWCNTs/CFUME showed a dynamic range up to 0.405 mM with a low detection limit of 4 μM ($S/N=3$) and a high sensitivity of 1.07 log(A) $\log(M)^{-1}$ cm^{-2}. The apparent Michaelis–Menten constant ($K_{m,\ app}$) was estimated to be as low as 1.35 mM. Due to the extremely small dimension and low working potential, Hb/SWCNTs/CFUME could give directly amperometric *in vivo* monitoring of H_2O_2 in aloe leaves with salt stress for 19.5 h without the requirement of complex data processing and extra surface coatings to avoid interferences. The sharp increase of H_2O_2 level in aloe leaves with salt stress was clearly observed using Hb/SWCNTs/CFUME from 12.5 h, while in the aloe without salt stress, H_2O_2 level remained stable in the whole measurement. For further confirming the *in vivo* response of Hb/SWCNTs/CFUME, catalase (CAT) was injected into the spot adjacent to the sensor and caused rapid current decrease, which suggests the scavenging of H_2O_2. These results indicate that Hb/SWCNTs/CFUME can be a powerful tool for *in vivo* investigation of ROS.

1. Introduction

Salinity stress caused by the excess amount of salt in soil or irrigation water can adversely affect plant growth, development and productivity (Vinocur and Altman, 2005). The soil salinization has become a major limiting factor in agriculture and is widespread in many regions around world due to excessive use of fertilizer, exhausted underground water and desertification. Serious salinization of more than 50% of arable lands by the year 2050 has been expected (Ashraf and Wu, 1994). In plants, reactive oxygen species (ROS) can be induced by salinity stresses and can cause oxidative damage to proteins, DNA and lipids due to their high oxidation capability (Abbasi et al., 2007; Apel and Hirt, 2004; Giraud et al., 2008; Xu et al., 2010 2009c; Zhu et al., 2007). While ROS have also been found to play a key role as signal transduction molecules involved in mediating responses to abiotic stresses (Singha and Choudhuri, 1990). As a major ROS, hydrogen peroxide in the plant under salinity stress has been observed to gain rapid increase which can be referred to as "the oxidative burst" (Bandeoglu et al., 2004; Eltayeb et al., 2007; Singha and Choudhuri, 1990). Thus, the monitoring of hydrogen peroxide is a significant task for investigating the effect of salinity stress on plants.

Due to the importance of H_2O_2 in various biological processes, a few methods, including chemiluminescence, fluorescence and spectrophotometry, have been employed for H_2O_2 measurement (Gomes et al., 2005; Hanaoka et al., 2001; Lee et al., 1990; Nogueira et al., 2005). However, these methods are difficult for *in vivo* studying oxidative burst dynamically due to their requirement of tracers or instable chemical probes (Chen et al., 2012b). Furthermore, conventional optical probes are reactive not only to H_2O_2 but also other ROS (Sanford et al., 2010). In contrast, electrochemical techniques are more suitable for *in vivo* H_2O_2 measurement because of their simplicity, rapidity and label-free detection capability since H_2O_2 is an electroactive molecule (Chen et al., 2012a; Xu et al., 2009a, 2009b). Moreover, the sizes of electrochemical sensors can be made extremely small so that high spatial resolution measurement can be achieved (Wightman, 2006).

For electrochemical *in vivo* monitoring of biological samples, carbon fiber ultramicroelectrodes (CFUMEs) are widely used due to their extremely small dimensions, which can match the

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biological microenvironments such as cells or tissues with minimized perturbation or damage. Furthermore, good biocompatibility, easy fabrication and good electron transfer properties make CFUMEs an ideal tool for real-time *in vivo* measurement (Huffman and Venton, 2009). CFUMEs have been reported to make *in vivo* H_2O_2 measurement. Meulemans used reductive differential pulse voltammetry (RDPV) to study the production of H_2O_2 in rat corpus cavernosum after intracavernous injection of various pharmacological agents on bare CFUMEs (Meulemans, 2005). Sanford et al. used fast-scan cyclic voltammetry (FSCV) to characterize rapid H_2O_2 fluctuations at an uncoated CFUMEs in rat brain slice (Sanford et al., 2010). However, the moderate selectivity of RDPV and FSCV can complicate the analysis of *in vivo* data (Heien et al., 2004). Also, it was found that the sensitivity of naked CFUMEs could be depressed by some insulating layers formed from substances in organism (Huffman and Venton, 2009). Kulagina et al. modified CFUMEs with horseradish peroxidase (HRP) and ascorbate oxidase contained osmium-centered polypyridyl complexes for *in vivo* monitoring of H_2O_2 in the extracellular space of the rat brain (Kulagina and Michael, 2003). The osmium-centered polypyridyl complexes in the coating acted as mediators to conduct electrons between HRP and CFUMEs. However, the redox mediators used in conjunction with redox enzymes facilitate not only the electron transfer between electrode and enzyme but also various interfering reactions (Freire et al., 2003). Therefore, ascorbate oxidase was also included in the coating to prevent ascorbate from reducing the osmium complex. Unfortunately, this strategy complicated the fabrication of the sensor and may cause stability and reproducibility problems. On the other hand, in the third generation biosensors, electron transfer occurring directly between the electrode and the substrate molecule is catalyzed by the redox protein without the requirement of a mediator. Thus, this configuration usually gives higher integration between the biomolecule and electrode surface and offers better selectivity and sensitivity (Chen et al., 2012a).

Hemoglobin (Hb) is one of the heme proteins which contains iron centered porphyrins as their prosthetic groups and easily undergo oxidation and reduction over a wide range of potentials, which are varied by the polypeptide environment around heme groups (Chapman, 1982). Due to the redox capability of Hb, it has been widely used to construct various biosensors (Chen et al., 2012). On the other hand, carbon nanotubes (CNTs) have been widely used for chemical and biological sensing applications due to their high surface to volume ratio, high conductivity and electrocatalytic activity (Patolsky et al., 2004; Wang et al., 2004). In electrochemical sensors, CNTs could increase the sensitivity by promoting electron transfer to biomolecules and to alleviate surface fouling effects by biomolecules (Cai and Chen, 2004; Musameh et al., 2002; Wang et al., 2009). Thus, to construct direct electron transfer based biosensors, CNTs and Hb modified CFUMEs are particularly attractive for *in vivo* H_2O_2 monitoring. In this

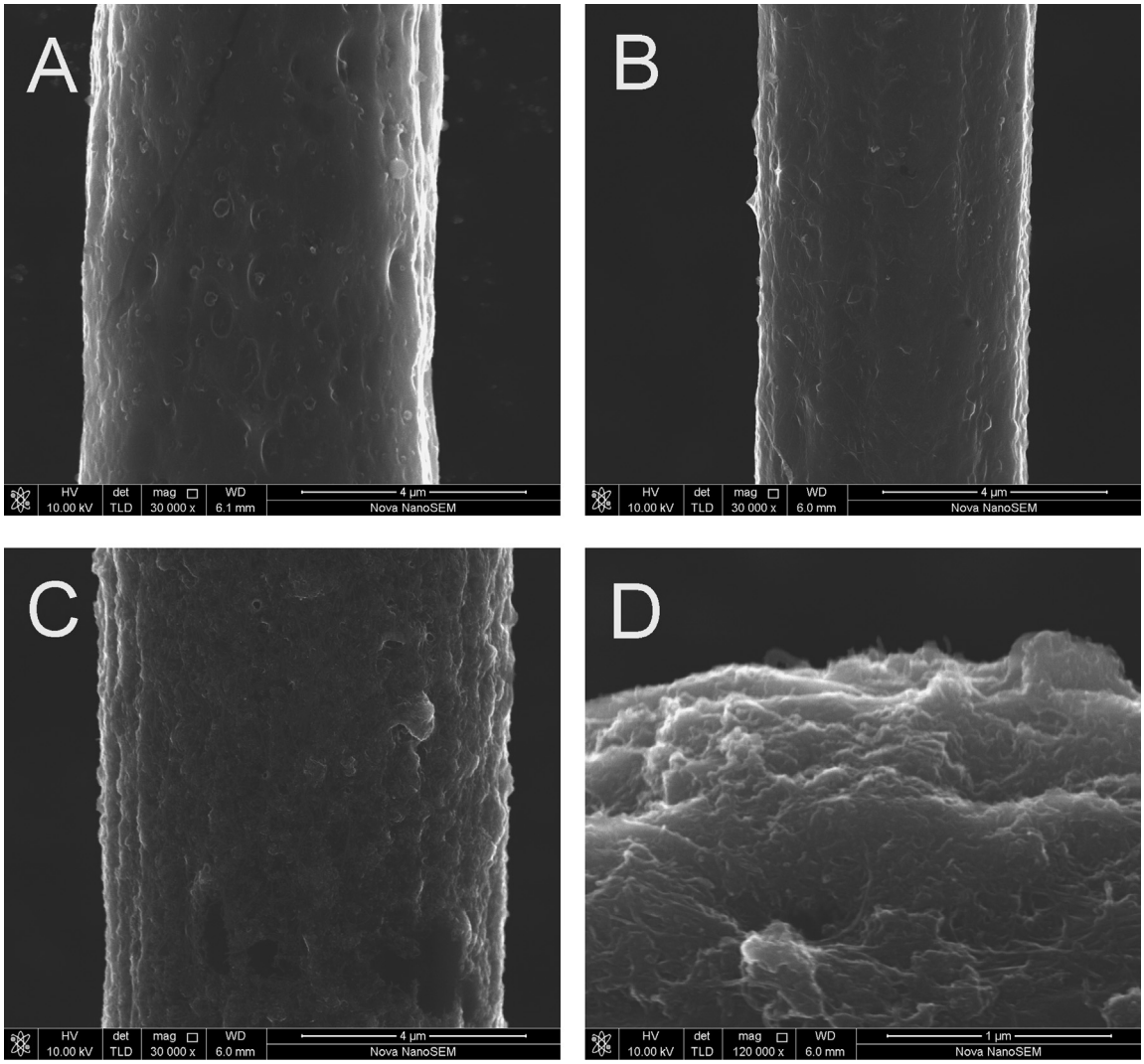

Fig. 1. SEM images of bare CFUME (A), SWCNTs/CFUME (B), Hb/SWCNTs/CFUME (C) and tip of Hb/SWCNTs/CFUME (D).