



# Application of polyhydroxylated fullerene derivatives in hemoglobin biosensors with enhanced antioxidant capacity

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## ABSTRACT

Hemoglobin (Hb) biosensors based on polyhydroxylated fullerene derivatives were constructed through layer-by-layer assembly. Not only could fullerene derivatives provide a favorable microenvironment to realize the direct electrochemistry and electrocatalysis of Hb, but they could reduce the oxidant damage for Hb caused by the attack of H<sub>2</sub>O<sub>2</sub> as well. Moreover, the antioxidant effect was closely related with fullerene species.

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## 1. Introduction

A major problem for the application of biosensors is their performance reduction caused by the inactivation of biomolecules. In order to solve this problem, antioxidant defense systems have been used to reduce the oxidative damage [1,2]. For instance, the performance of Hb electrochemical biosensors is reduced by the damage of reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub> [4]. One such approach is to make use of natural enzymes such as superoxide dismutase (SOD), and catalase (Cat), due to their high substrate specificity and high efficiency under mild conditions [3]. However, they also have some drawbacks such as sensitivity of catalytic activity to environmental conditions, low stability due to denaturation, and difficulty in preparation and purification.

Nanomaterials with unique properties and functions have provided an innovative route in antioxidant defenses, such as Pt, Fe<sub>3</sub>O<sub>4</sub> and CeO<sub>2</sub> nanoparticles et al. [5–7]. Among various nanomaterials, water-soluble fullerene derivatives have been well-known to possess a unique capacity for scavenging of ROS [8–10]. For instance, C<sub>60</sub>(OH)<sub>x</sub> could reduce ROS-mediated excitotoxic and apoptotic death of cultured cortical neurons [8]. Gd@C<sub>82</sub>(OH)<sub>x</sub> can protect cells more efficiently than other fullerenes against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage [9]. To

the best of our knowledge, the antioxidant ability of nanomaterials in electrochemical biosensors has not been explored yet.

In the present study, polyhydroxylated fullerene derivatives were introduced into Hb electrochemical biosensors through layer-by-layer assembly. We investigated the electrochemistry and electrocatalysis of Hb biosensors constructed by C<sub>60</sub>(OH)<sub>x</sub>. In addition, we explored the antioxidant effect of C<sub>60</sub>(OH)<sub>x</sub> and other fullerene derivatives, such as C<sub>70</sub>(OH)<sub>x</sub> and Gd@C<sub>82</sub>(OH)<sub>x</sub>, trying to explore their special role in biosensors.

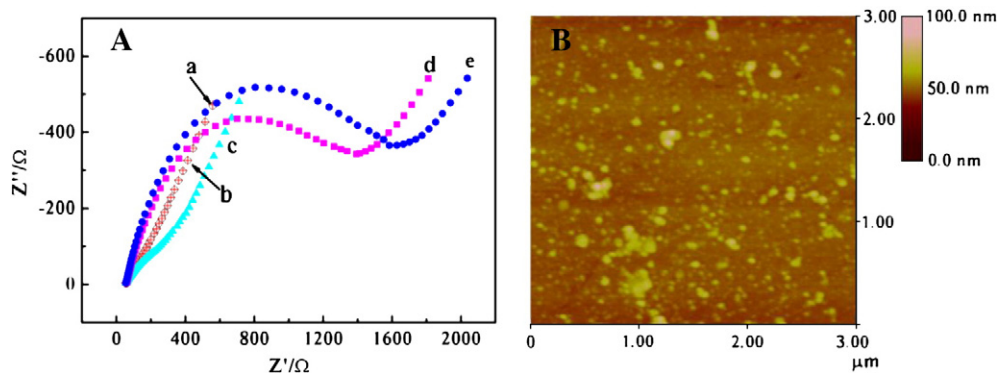
## 2. Experimental

Hemoglobin from bovine (Hb, pI=6.8), and poly(allylamine hydrochloride) (PAH) were purchased from Sigma-Aldrich. K<sub>3</sub>Fe(CN)<sub>6</sub> and K<sub>4</sub>Fe(CN)<sub>6</sub> were obtained from Beijing Chemical Plant. Highly purified hydroxylated C<sub>60</sub>(OH)<sub>x</sub>, C<sub>70</sub>(OH)<sub>x</sub>, and Gd@C<sub>82</sub>(OH)<sub>x</sub> were prepared by previously published methods [11,12]. The average hydroxyl number of each fullerene derivative was about 22. All the solutions were prepared with Millipore water. Buffers were 0.05 M sodium acetate (pH 5.0), potassium dihydrogen phosphate (pH 7.0), or boric acid (pH 9.0) solutions, all containing 0.1 M KCl.

For electrochemical studies, clean and rough PG electrodes were immersed in PAH solutions (1 mg mL<sup>-1</sup>, pH 5.0) for 20 min to adsorb a positively charged PAH precursor layer. The PG/PAH electrodes were then placed into C<sub>60</sub>(OH)<sub>x</sub> solutions (5 μg mL<sup>-1</sup>, pH 9.0) for 20 min and positively charged Hb solutions (2 mg mL<sup>-1</sup>, pH 5.0) for 60 min with intermediate water washing and air drying, forming PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb films. The obtained electrodes were immersed into

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**Fig. 1.** (A) EIS for (a) bare PG (+), (b) PG/PAH (○), (c) PG/PAH/C<sub>60</sub>(OH)<sub>x</sub> (▲), (d) PG/PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb (■), and (e) PG/PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb/C<sub>60</sub>(OH)<sub>x</sub> (●) in 5 mM Fe(CN)<sub>6</sub><sup>3-/4-</sup> solution with an amplitude of 5 mV and a frequency range of 0.1 Hz to 100 kHz. (B) AFM photograph of dried {PAH/C<sub>60</sub>(OH)<sub>x</sub>}<sub>5</sub> films.

C<sub>60</sub>(OH)<sub>x</sub> solutions for 20 min to add C<sub>60</sub>(OH)<sub>x</sub> layer on the top of Hb films, forming PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb/C<sub>60</sub>(OH)<sub>x</sub> films. The assembly of C<sub>70</sub>(OH)<sub>x</sub> and Gd@C<sub>82</sub>(OH)<sub>x</sub> was similar with that of C<sub>60</sub>(OH)<sub>x</sub>, forming PAH/C<sub>70</sub>(OH)<sub>x</sub>/Hb/C<sub>70</sub>(OH)<sub>x</sub> and PAH/Gd@C<sub>82</sub>(OH)<sub>x</sub>/Hb/Gd@C<sub>82</sub>(OH)<sub>x</sub> films, respectively.

A CHI 660D electrochemical workstation (CH Instruments) was used for cyclic voltammetric (CV) and electrochemical impedance (EIS) measurements. A three-electrode cell was used with an Ag/AgCl (3 M KCl) as the reference, a Pt disk as the counter, and a basal plane pyrolytic graphite (PG, Advanced Ceramics, geometric area 0.16 cm<sup>2</sup>) disk with films as the working electrode. UV–vis experiments were performed with TU-1901 spectrophotometer (Beijing, China). The microstructure and surface topography of the films were examined using a Dimension 3100 atomic force microscope (AFM) (Veeco Instruments) in the tapping mode.

### 3. Results and discussion

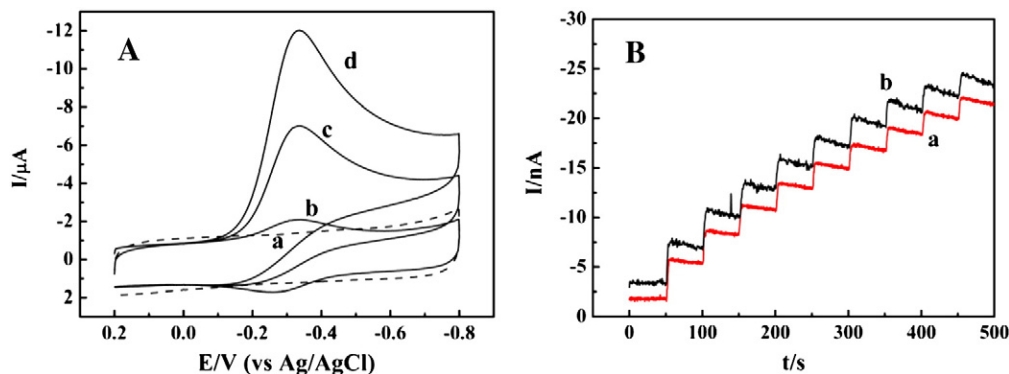
At first, we monitored and confirmed the assembly process of PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb/C<sub>60</sub>(OH)<sub>x</sub> films by EIS (Fig. 1A). The electrochemical impedance increased as layers were added, indicating that the layer-by-layer films were successfully fabricated on the electrode. It was reported that the pI of C<sub>60</sub>(OH)<sub>x</sub> is at about 1.7 [13], so it was negatively charged at pH 9.0. Under our experimental conditions, PAH (pKa ~8.5 [14]) is positively charged and Hb (pI ~6.8) has net positive surface charges at pH 5.0. Thus, the film assembly should be driven by electrostatic attraction.

Surface topography of the PAH/C<sub>60</sub>(OH)<sub>x</sub> films has also been studied by AFM. “Island-like” domains, forming a rough surface, could be observed in the image (Fig. 1B). This might originate from the aggregation of C<sub>60</sub>(OH)<sub>x</sub>, as they are prone to aggregate in aqueous solution

[9]. Previous works have shown that nanoparticle films have better porosity than polyelectrolyte films, which is more beneficial to protein immobilization and electron transfer [15]. Rough C<sub>60</sub>(OH)<sub>x</sub> films would provide a good microenvironment to realize the immobilization and electron transfer of Hb.

When a PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb film electrode was immersed into a pH 7.0 buffer containing no Hb, a pair of well-defined and nearly reversible CV peaks at approximately  $-0.30$  V vs. Ag/AgCl (3 M KCl) was observed (Fig. 2A, curve b), which is the characteristic redox couples of Hb heme Fe(III)/Fe(II). In contrast, a PAH/C<sub>60</sub>(OH)<sub>x</sub> film modified PG electrode in pH 7.0 buffer gave no CV response in the same position (Fig. 2A, curve a). The peak-to-peak separation ( $\Delta E_p$ ) was 64 mV at  $0.2$  V s<sup>-1</sup>, which indicated the nearly reversible direct electron transfer of Hb. Based on the direct electrochemistry, the PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb film electrodes could be used to catalyze the reduction of oxygen. When 10 mL of air was passed through a pH 7.0 buffer by a syringe, a significant increase in reduction peak accompanied by the disappearance of the HbFeII oxidation peak was observed (Fig. 2A, curve c). Increasing the amount of oxygen to 20 mL, the reduction peak current (Fig. 2A, curve d) continued to increase correspondingly. The results indicated that C<sub>60</sub>(OH)<sub>x</sub> could successfully immobilize Hb onto the electrode and provide a suitable microenvironment for Hb to exchange electrons with electrode.

Hb can also be employed to catalyze the reduction of H<sub>2</sub>O<sub>2</sub>, due to its peroxidase activity. Amperometry was employed to investigate the electrocatalytic activity of the PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb modified PG electrodes towards H<sub>2</sub>O<sub>2</sub> (Fig. 2B, curve a). The stepped increase of the amperometric current was observed after each addition of H<sub>2</sub>O<sub>2</sub> to the solution, indicating that the immobilized Hb exhibited electrocatalytic activity towards the reduction of H<sub>2</sub>O<sub>2</sub>. However, when the electrode was transferred to another blank buffer for CV scans after



**Fig. 2.** (A) CVs at  $0.2$  V s<sup>-1</sup> in 5 mL of pH 7.0 buffers for (a) PAH/C<sub>60</sub>(OH)<sub>x</sub> films, (b) PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb films, (c) PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb films after 10 mL of air was injected, and (d) PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb films after 20 mL of air was injected. (B) Amperometric response of (a) PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb and (b) PAH/C<sub>60</sub>(OH)<sub>x</sub>/Hb/C<sub>60</sub>(OH)<sub>x</sub> films at  $-0.1$  V in pH 7.0 buffers with an increment of  $4$  μM H<sub>2</sub>O<sub>2</sub> every 50 s.

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