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Oxygen carrying capability evaluation based on direct electrochemistry of highly loaded hemoglobin spheres

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ABSTRACT

Hemoglobin-based oxygen carriers (HBOCs) are widely researched as blood substitute and mainly produced based on templates. As reported, their optimal size should be preferably less than 1 μm and more than 100 nm, but porous calcium carbonate ($CaCO_3$) is the most common template to synthesis HBOCs and their average size is larger than 3 μm. Therefore, in this study, we employed manganese carbonate ($MnCO_3$) instead of $CaCO_3$ for the production of much smaller multilayer hemoglobin (Hb) sphere with glutaraldehyde (GA) as the crosslinking agent. According to our previous work, the obtained $\{Hb/GA\}_n$ nanospheres were also immobilized on chitosan modified glassy carbon electrode (GCE) to evaluate their oxygen-carrying ability with electrochemical techniques. By comparison, $\{Hb/GA\}_5$ demonstrates a higher sensitivity to oxygen, stronger carrying-oxygen ability and more stable properties than $\{Hb/GA\}_1$. The present work suggests potential applications of HBOCs as blood substitute.

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1. Introduction

Hemoglobin-based oxygen carriers (HBOCs) have been diffusely prepared to address the problems of blood shortage and disease infection caused by blood transfusion [1,2]. Hemoglobin, Hb, has the function to carry and transport oxygen, while cannot be used as blood substitute without the protection of cytomembrane. The reason is that the non-matrix Hb can turn into dimer to bring in kidney toxicity and high colloid osmotic pressure [3]. Up to date, researchers have exploited various methods to blocking the leakage of free Hb from HBOCs in blood vessel [4–6].

Nitric oxide, NO, arises from the blood vessel endothelial cells, is an important vasodilator, causing vasodilation and preventing blood pressure from continuing rise [7]. Stroma-free Hb and nano-sized HBOCs can cross the gaps between the endothelial cells and bind to the NO receptor of the smooth muscle tissue. Meaningful research has shown that vasoconstriction and hypertension are inversely proportional to the size of the HBOCs [8]. As a result, synthesis of HBOCs with the diameter larger than 100 nm would be helpful to avoid HBOC extravasation through the endothelial gaps. Besides, particles with the size from 1 μm to 3 μm can be strongly phagocytosed [9], and particles larger than 5 μm can block microcirculation at higher concentrations [10]. Considering all these factors, the size of HBOCs should be preferably more than 100 nm and less than 1 μm.

Templates, such as calcium carbonate ($CaCO_3$), are usually chosen for the fabrication of hemoglobin based oxygen carriers with glutaraldehyde (GA) as the cross-linking reagent [11,12]. For example, our previous work has fabricated HBOCs with the help of $CaCO_3$ and applied various electrochemical techniques to study their oxygen-carrying performance [13,14]. Based on cyclic voltammetry (CV) and differential pulse voltammetry (DPV), our result presents that the oxygen-carrying ability depends on the multilayer number [14]. However, the large size of $CaCO_3$ based HBOCs remains a challenge due to aforementioned size effect.

As a result, in this study, in order to obtain HBOCs with their size precisely less than 1 μm and more than 100 nm, manganese carbonate ($MnCO_3$) were used to replace $CaCO_3$ to construct different Hb-based nanospheres (denoted as $\{Hb/GA\}_n$) through layer by layer (LbL) assembly method. According to our previous work [14–20], we applied amperometric current-time curve, differential pulse voltammetry (DPV) and cyclic voltammetry (CV) to search the optimal layer number for the fabrication of HBOCs and explore the relationship between the size of HBOCs and oxygen-carrying capability.

Abbreviations: HBOCs, hemoglobin-based oxygen carriers; Hb, hemoglobin; CV, cyclic voltammetry; DPV, differential pulse voltammetry; GCE, glassy carbon electrode; SCE, saturated calomel electrode; SEM, scanning electron microscopy; EIS, electrochemical impedance spectroscopy; PBS, phosphate buffered saline.

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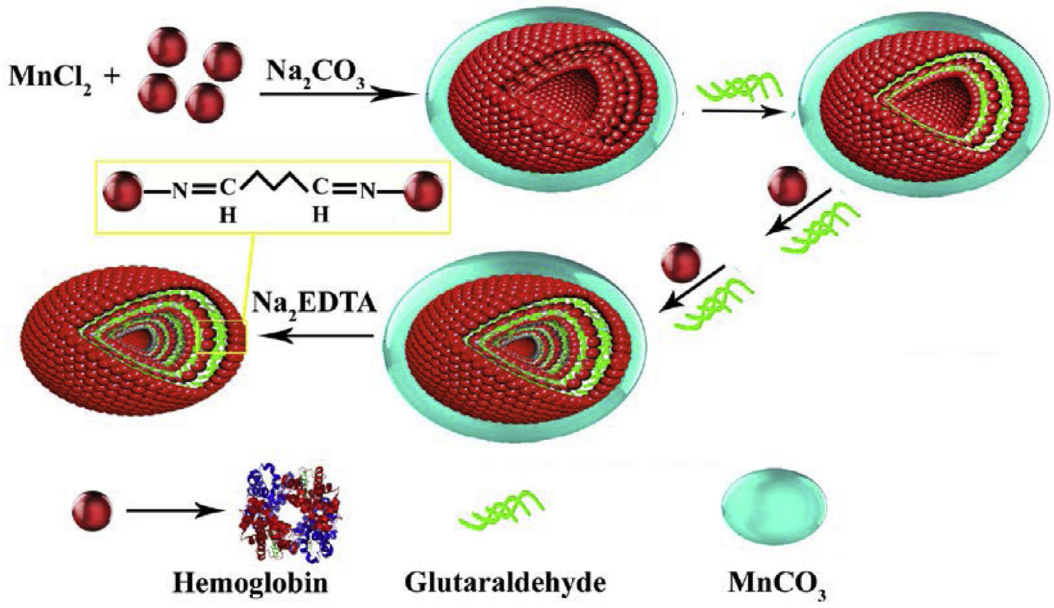

Scheme 1. Schematic illustration to show the assembly process of $\{Hb/GA\}_n$ nanospheres.

2. Experimental

2.1. Apparatus and reagents

Electrochemical measurements were carried out on a CHI 660D electrochemical workstation (CH Instruments Co., China). Glassy carbon electrodes (GCE, 4 mm in diameter) were as the working electrode, a platinum wire as the counter, and a saturated calomel electrode (SCE) as the reference electrode. Ultraviolet-visible (UV-vis) absorption spectrum was recorded with a UV-2450 spectrophotometer (shimadzu, Japan). Scanning electron microscopy (SEM) and energy-dispersive X-ray analysis spectroscope (EDS) analysis were performed using an electron microscope (JEOL JSM-6510LV, Japan). Fourier tansform infrared spectrum was measured with Nicolet iS50 spectrometer. Electrochemical impedance spectroscopy (EIS) was performed in 0.10 M KNO_3 solution including 5.0 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$(1:1) as the supporting electrolyte recorded with the AUTOLAB PGSTAT302N electrochemical working station (Metrohm Co. Ltd., Switzerland), accompanying with the frequency from 1.0 mHz to 100 kHz.

Hemoglobin from bovine blood (Hb, lyophilized powder protein) and ethylenediamine tetraacetic acid disodium salt (Na_2EDTA) were purchased from Sinopharm Chemical Reagent Co.,Ltd. Chitosan (92.5% deacetylation, CS) was brought from Nantong Shuanglin Company. Glutaraldehyde (GA, 25% water solution) was obtained from Shanghai Ling Feng Chemical Reagent Co., Ltd. Chitosan powder was dissolved in 0.05 M HCl to form 0.2 wt% solution and filtered using a 0.45 mm syringe filter.

2.2. Fabrication of Hb-coated spheres

The preparation process of the hemoglobin-loaded nanospheres encapsulating multilayer Hb with GA as the linking agent is illustrated in Scheme 1. Briefly, mixing 0.20 g Hb powder and 10 mL 0.25 M $MnCl_2$ solution, and sonicated at 4 °C with ice bath for 30 min, then the Hb-loaded nanoparticles are formed by rapidly adding 10 mL 0.25 M Na_2CO_3 solution into the above prepared mixture and washed three times with filtered double-distilled water based on 0.22 μm microfiltration membrane. Subsequently, the Hb-loaded $MnCO_3$ nanospheres were incubating in 0.025% GA prepared by pH 7.4 phosphate buffer solution for 2 h, followed by washing three times with buffers to obtain the first layer Hb-crosslinked $MnCO_3$ nanospheres, named as $\{Hb/GA\}_1$–$MnCO_3$. As time passes, increasingly more new Hb accumulated through imine linkage between aldehyde groups in GA and amino groups in Hb. Therefore, we followed on to immerse $\{Hb/GA\}_1$ in Hb solution for 12 h to form the second Hb layer after washing three times to remove the unabsorbed hemoglobin. Afterwards, incubating in GA solutions for another 2 h and washing three times with buffer solutions (pH 7.4). Repeatedly, the five-layer hemoglobin nanospheres ($\{Hb/GA\}_5$–$MnCO_3$) can be obtained. Finally, the hemoglobin loaded nanospheres are gained by immersing the $\{Hb/GA\}_5$–$MnCO_3$ nanoparticles in 0.20 M pH 7.0 Na_2EDTA solution to remove $MnCO_3$ and stored at 4 °C for future use.

2.3. Electrode modification

Firstly, the glassy carbon electrode (GCE) was successively polished with abrasive paper, alumina slurry and chamois leather, followed by ultrasonically cleaned in ethanol, water and then dried at room temperature. Secondly, the clean electrode was electrodeposited at potential of −2.0 V for 5 min in fresh prepared CS solution, then washing and dried in the air. Thirdly, 20 μl hemoglobin-coated nanospheres suspension was dropped onto the surface of the CS modified electrode. Finally, the obtained electrode was kept at room temperature and expressed as $\{Hb/GA\}_n$-CS-GCE.

3. Results and discussion

3.1. Characterization and size of $\{Hb/GA\}_n$nanospheres

We present the corresponding scanning electron microscopy (SEM) images of the obtained $MnCO_3$, $\{Hb/GA\}_n$–$MnCO_3$ and $\{Hb/GA\}_n$ nanospheres dispersions (n = 1 and 5) dropped on glass carbon electrodes using a JEOL JSM-6510LV filed emission scanning electron microscope with the voltage from 1 kV to 20 kV. As shown in Fig. 1C, pure $MnCO_3$ particles with a shape of peanut and a clear seam are clearly observed as early work reported [21]. However, when covered with Hb, the shape of the one-layer Hb (Fig. 1A) loaded and the five-layer Hb (Fig. 1D) loaded nanospheres became oval and the seams on surfaces of the nanospheres disappeared. Those results demonstrate that Hb have

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