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Preparation, characterization and in vivo investigation of blood-compatible hemoglobin-loaded nanoparticles as oxygen carriers

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ABSTRACT

Although many attempts have been made to design advanced hemoglobin-based oxygen carriers (HBOCs), no clinically viable product has been widely approved, because they do not perform normal blood functions, such as coagulation, hematologic reactions and stability. Additionally, the in vivo oxygenation of hemoglobin-loaded nanoparticles (HbPs) encapsulated with polymers has seldom been proved. Herein, HbPs of approximately 200 nm with good stability were successfully fabricated and exhibited oxygen-carrying capacity. The HbPs preserve the biological and structure features of hemoglobin according to UV–vis spectrophotometry, Fourier transform infrared (FTIR) spectroscopy and circular dichroism (CD) spectral analysis. In vitro, the HbPs showed a viscosity comparable to that of blood with no obvious effects on red blood cell aggregation. At the same time, blood compatibility was characterized in terms of platelet function, clot strength, speed of clot formation, degree of fibrin cross-linking and hemolysis rate. After intravenous administration of HbPs to mice with controlled hemorrhages, blood flow recovery and maintenance of systemic oxygenation were observed.

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1. Introduction

Medical demands for blood are high, but resources for transfusion are lacking, especially in developing countries, and therefore, the development of a suitable blood substitute is a persistent and urgent need in biomedicine [1]. However, cell-free hemoglobin (Hb) cannot be used as a blood substitute due to its short circulation time, nephrotoxicity, and high oncotic pressure [2]. Where red blood cell transfusion is not an option, hemoglobin-based oxygen carriers (HBOCs) can enable survival in acutely anemic patients, with the potential benefits including long-term storage, availability, and lack of infectious risk [3]. However, several side effects of current HBOCs hindered their utility for short-term application in acute blood loss, including scavenging of endothelial nitric oxide (NO), hemoglobin (Hb) extravasation across the blood vessel wall, and heme-mediated oxidative side reactions [4,5].

Notably, Hb encapsulation can protect the surrounding tissues and blood components from direct contact with tetrameric Hb, thereby solving the problems associated with molecular Hb, preventing hypertension caused by NO depletion, and prolonging the Hb circulation half-life [6]. Various types of these cellular HBOCs have been investigated, i.e., lipidic nanoparticles (lipidic NPs) and polymeric nanoparticles (polymeric NPs) [7–9]. Hemoglobin-loaded nanoparticles (HbPs) designed with polymers have attracted significant interest due to the possibility of developing polymers with tailored characteristic. We fabricated HbPs using PEG-PLGA in view of three reasons. First, the mechanical flexibility of poly(lactide-*co*-glycolide) (PLGA) with high elastic modulus play an important role in red blood cells-mimicking and prolonging circulation time [10]. Second, approved by the US FDA, the biodegradable and biocompatible (PLGA) material is a safe-to-administer polymer employed for in vivo applications, including anticancer drugs and antioxidants [11,12]. Third, surface engineering with poly(ethylene glycol) (PEG) might create NPs capable of evading the reticulo-endothelial system uptake and exhibiting prolonged residence in blood systems [13,14]. However, due to the

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frangibility of quaternary structure–function relationships of Hb, the difference of oxygen delivery from the drugs delivery, and the application of HbPs in blood, it has remained challenging to make HbPs that exhibit a homogeneous diameter, good stability, blood compatibility, and good O_2 delivery capacity [15]. In order to minimize the oxidation of Hb and homogenize the HbPs size, the effects on maintenance of the desirable diameter and Hb bioactivity for encapsulation by double emulsion method were studied through investigation of the composition of the organic solvent, the emulsifier and the copolymer/drug mass ratio. Herein, the HbPs, with sufficient and effective capacity for oxygen supply, showed little negligible leakage, blood compatibility and a viscosity comparable to that of whole blood in our study.

Evaluation of in vivo oxygenation is the goal and foundation of application of polymeric HbPs. In vitro measuring oxygen equilibrium curves were used in many researches [6,16–18] to reflect the oxygen-binding affinity of HbPs. However, evaluation of in vivo oxygenation after polymeric HbPs infusion remains a challenge and no animal data are available that can support the effects of in vivo oxygenation [4]. Thus, we used a controlled hemorrhage model to demonstrate the efficiency of in vivo oxygenation of the HbPs. The HbPs showed blood flow recovery and systemic oxygenation for resuscitation.

2. Materials and methods

2.1. Materials

Blood was extracted from healthy male cattle (experimental animals from JinXiu DaDi Agricultural Park, Beijing, China). The Hb solutions were prepared by osmotic rupture of the RBC membrane followed by centrifugation. The sample was used either undiluted or diluted with normal saline [19]. The Hb concentration used for HbP preparation was 24 g/dL, and the MetHb concentration was less than 3%. The MetHb and Hb concentrations of the sample were measured using a blood gas analyzer (Radiometer ABL90COOX, Denmark). Electrophoresis with SDS-PAGE was conducted to determine whether the prepared Hb sample was of good purity.

Normal saline was purchased from Shijiazhuang SiYao Limited Company (Hebei, China). Sodium citrate was purchased from Jiangsu YuLi Medical Equipment Limited Company (Jiangsu, China). The mPEG-PLGA (5000-45000) was purchased from Jinan Daigang Biotechnology Co. (Shandong, China). The PVA was purchased from Sinopharm Chemical Reagent Co. (China). Pluronic F68 was purchased from Adamas Reagent Co. (China). The EA and DCM were purchased from Guangdong Xilong Chemical Co. (China). 4% modified fluid gelatin were purchased from Gelofusine® (GEL: molecular mass 16000–26600 Da, PI 4.5 ± 0.3)

2.2. HbP preparation

Particles loaded with Hb were prepared via the double emulsion (w/o/w) technique. In brief, 40 mg of mPEG-PLGA was dissolved in 2 mL of EA, and 0.4 mL of Hb solution (12 g/dL) was added. The mixture was transferred to a centrifuge tube and emulsified via sonication for 3 min at 80 w. Next, the obtained emulsion was slowly added to 7 mL of 2% (m/v) PVA and 3 mL of 2% (m/v) pluronic F68, followed by vigorous stirring at room temperature for 10 min. The mixture was emulsified via sonication for 5 min at 250 w. Both emulsification steps were carried out in an ice bath. After vacuum evaporation of the solvent, the Hb-loaded particles were collected by centrifugation at 10,000 rpm for 10 min at room temperature and washed twice using distilled water. For screening of the optimal emulsification speed for maintenance of the Hb structure, high shearing was applied to Hb solutions for 5 min with varied rates. For investigation of the solvent type (DCM or EA) on protein bioactivity, Hb was emulsified according to the above procedure without the addition of polymers to the organic solvent.

2.3. Characterization

2.3.1. Size and morphology

The average size and PDI index were determined by DLS using a ZetaSizer Nano series Nano-ZS (Malvern Instruments Ltd., Malvern, UK). Determinations were performed at 633 nm with a constant angle of 90° at 25 °C after samples were appropriately diluted in distilled water.

The morphology of HbPs was confirmed using a TEM (EM-200CX; JEOL Ltd., Tokyo, Japan) following negative staining with uranyl acetate.

2.3.2. Determination encapsulation efficiency and stability

The EE% of HbPs were calculated as follows:

$$EE\% = \frac{(Hb_{total} - Hb_{free})}{Hb_{free}} \times 100\%$$

The amount of entrapped Hb was determined by measuring the difference in the amount of Hb between the initial amount of Hb (Hb_{total}) and the amount of free Hb in the supernatant (Hb_{free}). The cyanomethemoglobin method was chosen to determine the Hb_{total} and Hb_{free} as detailed elsewhere [20].

Stability experiments (repeated three times) were carried out by regularly detection of the sizes of HbPs in PBS solution at room temperature within a week. The concentration of the HbPs for stability experiments is consistent with the concentration of HbPs for the in vivo study on the controlled hemorrhage model. In vitro release study, the HbPs (incubated at 37 °C during 7 days) were suspended in 1 mL of saline. At determined intervals, the suspension was centrifuged, and the supernatant was withdrawn and subjected to a spectrophotometer to determine the Hb concentration. The same volume of fresh saline was added back into the suspension. Moreover, the in vitro release of the HbPs (incubated at 4 °C during 42 days), after being washed by fresh normal saline for three times, was also studied. The in vitro release analyses for each sample were carried out in triplicate.

2.3.3. Chemical structure and oxygen-carrying capacity

The FTIR spectra were recorded using an FTIR spectrometer (Spectrum One, PerkinElmer). The CD spectra of samples was obtained on a Jasco J-800 spectropolarimeter (Japan) with a path length of 1 cm, a bandwidth of 1.0 nm, a time constant of 2.0 s, and a wavelength range of 260–190 nm. The corresponding buffer baseline was subtracted from each spectrum, and smoothing was applied for further analysis. The absorption of Hb, MetHb and HbP suspensions was scanned on a UV–vis absorption spectrometer at room temperature (Helios β, Thermo). The MetHb solutions were prepared by making the Hb reacting with potassium ferricyanide.

The oxygen affinity (P_{50}) and Hill plots were determined by measuring the oxygen dissociation curve using a Hemox analyzer (TCS Scientific Corp., USA), which is based on dual-wavelength spectrophotometry for the measurement of Hb and a Clark electrode for measuring the partial pressure of oxygen. Measurements were collected at 37 °C using the temperature controller on the instrument. The P_{50} and Hill plots of HbPs were regressed by fitting the oxygen binding curves to the Adair equation. The P_{50} was defined as the oxygen partial pressure when oxygen saturation reached 50% on the oxygen dissociation curve. The Hill coefficient was determined from the cooperative extent of the four subunits in the Hb molecule and was taken from the slope of the Hill plot.

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