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Insight into the fabrication of polymeric particle based oxygen carriers

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ABSTRACT

For the sake of protein stability and targeted application as blood substitutes, formulation customization of hemoglobin-loaded polymeric particles (HbP) was conducted via a double emulsion method. Screening of the emulsification parameters was firstly performed for the stability of Hb, and the structure and functions of recovered Hb could be well preserved via CD and UV–vis spectroscopy investigation. In the optimized conditions, Hb was loaded into the polymeric matrix formed of three material compositions. They were poly(ε-caprolactone)(PCL), poly(ethylene glycol)-block-poly(allyl glycidyl ether) (functionalized with mercaptopropionic acid)-block-poly(ε-caprolactone) (PEG-PAGE(MPA)-PCL), and the blend of the two polymers. The morphology, internal structure, in vitro leakage and hemocompatibility of the HbP products were characterized in detail, and the encapsulation mechanism was explored by the combined analysis of the encapsulation efficiency, non-specific protein adsorption and in vitro leakage studies. Results showed that the burst release effect found in homopolymers could be alleviated by use of block copolymers due to the reduced protein adsorption, and completely avoided by further cross-linking of particles through carbonyl-amino condensation reactions. The amphiphilic copolymers showed relatively high stability in blood and no interference with blood components compared with hydrophobic PCL. These results suggest that both the optimization of emulsion formation and material composition are prerequisite for stable formulations of Hb encapsulated in polymeric particles.

1. Introduction

The boom of protein engineering in recent years allows the production of functional proteins having large potential markets and evokes the enthusiasm of scientists to combat some of the most hard-to-treat diseases. Albeit with hundreds of protein drugs approved for clinical use by the US Food and Drug Administration, the development of protein therapeutics is still in its infancy, and the clinical utilization of therapeutical proteins is greatly limited by their fragility (Di Marco et al., 2010; Yadav et al., 2011).

Extended from the concept of microencapsulation, the internalization of biomolecules within confined artificial systems maintains the vitality and improves the stability of therapeutic agents against enzymatic degradation. And on the other hand, emulsions, involved of small droplets dispersed in immiscible solvent, have already been widely used in the cosmetic and food industry (Chappat, 1994). The introduction of emulsification into the pharmaceutical area offers new opportunities for the design of versatile hybrid systems to build biomimetic cellular structure and fight diseases and functional deficiency (Kim et al., 2006; Marguet et al., 2013).

Due to the biodegradable, non-antigenic nature and ease of scale up, aliphatic polyesters are promising materials for construction of sustained release system via double emulsion method (Benoit et al., 1999; Cleland and Jones, 1996; Wei et al., 2011). In recent years, hemoglobin-loaded polymeric particles (HbP) have emerged as a new type of cellular hemoglobin-based oxygen carriers (HBOCs) for the adjusted diameters and avoidance of hemoglobin (Hb) leakage into the plasma (Meng et al., 2004; Zhang et al., 2008; Zhao et al., 2007). However, due to the frangibility of active components and relatively harsh preparation procedures, each protein or peptides requires its own specific condition for stability, solubilization and encapsulation. Several issues account for the difference of Hb from traditional pharmaceutical proteins. First, as the paradigm for understanding the structure–function relationships of proteins, the structure of Hb is

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more complex than other encapsulated proteins, such as albumin and insulin, for the existence of prothetic group and the folding of multi-subunits. Second, the oxygen delivery capability of Hb is performed within the nanosystem circulating in the blood, different from the function of drugs after released from the vectors. The leaked free Hb is always toxic for increased autooxidation rate, higher oxygen affinity, tetramer instability and nitric oxide reactivity (Ronda et al., 2008; Tsuchida et al., 2009). And last, towards the targeted application to act as the biomimetic erythrocyte in blood, both the compatibility with blood components and the plasma stability of the polymeric particles are prerequisite to avoid the premature recognition of the immune system.

In this respect, towards the preparation of a safe and efficacious formulation of HbP via W/O/W emulsification, the harmful effect on Hb during emulsification was particularly analyzed in this work, specifically, the stability damage and uncontrollable leakage. The emulsification speed and organic solvents were screened for the maintenance of Hb bioactivity, and the encapsulation and controlled release of Hb was optimized by varied material components and cross-linking of the functional triblock copolymers. Furthermore, the blood cell compatibility of the particles was investigated by incubation with rat blood in vitro.

2. Material and methods

2.1. Materials

$PEG_{5\,K}$-$PAGE_5$-$PCL_{27\,K}$ was synthesized by sequential living ring-opening polymerization of allyl glycidyl ether (AGE) and ε-caprolactone (CL) with $mPEG_{5\,K}$ as the macroinitiator (Wang et al., 2013). The subscripts 5 K and 27 K referred to the molecular weight of each block and 5 denoted the degree of polymerization of PAGE block, all determined by ^1H NMR analysis. Afterward, the PAGE block was functionalized by 3-mercaptopropionic acid (MPA) via thiol-ene chemistry to endow carboxyl groups on the side chains of the copolymers. Poly(ε-caprolactone)($PCL_{29\,K}$) was kindly supplied by Prof. Xuesi Chen from Changchun Institute of Applied Chemistry. The two different polymer structures PEG-PAGE (MPA)-PCL and PCL were abbreviated as PECC and PCL, respectively. Bovine Hb was purchased from Shanghai Kayon Biological Technology Co. Ltd. and stabilized under CO atmosphere to afford COHb. Bovine serum albumin (BSA) was obtained from sigma, fluorescein isothiocyanate-labeled BSA(FITC-BSA) and Hb(FITC-Hb) were prepared according to reported literatures (Wischke and Borchert, 2006). Other solvents were analytical grade and used without further purification.

2.2. Preparation of W/O/W particles

Particles loaded with Hb were prepared by a two-step procedure. Typically, 1 mL of Hb solution (~50 g L^{-1}) was emulsified for 1 min in 10 mL of organic solvent containing the polymers (5 g L^{-1}) to obtain the primary W1/O emulsion. Then the pre-emulsion was emulsified for another 2 min in 50 mL of phosphate buffer solution (20 mM PB, pH 7.4) to get the final W1/O/W2 emulsion. Both of the emulsification steps were carried out with a homogenizer (BME 100LX, Weiyu Corp. Shanghai, China) in an ice bath. After the evaporation of organic solvent, the Hb-loaded particles were collected by centrifugation (12,000 rpm × 10 min). Three material compositions were used, including PCL, PECC, and the blend of the two polymers (1:1, w/w), PECC/PCL.

For the screening of optimal emulsifying speed for maintenance of Hb structure, high shearing was applied to Hb solutions for 5 min with varied rates. And for the investigation of solvent type (dichloromethane or ethyl acetate) on protein bioactivity, Hb was emulsified according to the above procedure without addition of polymers in the organic solvent. Circular dichroism (CD) spectra of Hb were obtained on a JASCO J-600 spectropolarimeter at room temperature, 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 300–190 nm, using quartz cuvettes. And UV–vis spectra were recorded on a UV-2450 UV–vis spectrophotometer (Shimadzu Scientific Instruments) at room temperature.

2.3. Characterization of the Hb-loaded particles

The encapsulation efficiency, gas-binding capacity, oxygen affinity and methemoglobin (metHb) level were determined according to the procedures as described in our previous paper (Li et al., 2012).

Samples for transmission electron microscopy (TEM) measurements were prepared by drying an aqueous droplet of the HbP dispersion onto a carbon-coated grid, and analysed on a JEOL JEM-1011 electron microscope operating at an acceleration voltage of 100 kV.

The surface morphology of HbPs was analyzed by environmental scanning electron microscopy (ESEM). The ESEM images of HbPs deposited on a silicon wafer were recorded with a model XL 30 ESEM FEG from Micro FEI Philips after coating with gold film.

For the confocal laser scanning microscopy (CLSM) observation of encapsulated Hb, dual-fluorescence system was established by fluorescence labeling of polymer matrix with Nile red and Hb with FITC. The particle-forming course was similar except for the use of Hb solution containing 30% FITC-Hb as the internal water phase and polymer solution containing 0.02% Nile red as the oil phase. The collected fluorescence-labeled particles were rinsed three times by PB for the absence of fluorescence in the supernatant. CLSM images of fluorescence-labeled HbP were collected with a Leica TCS SP2 CLSM system (Leica Microsystems Heidelberg GmbH, Germany) equipped with a 100× oil immersion objective. A droplet of the HbP suspension was dripped on a glass slide, covered with a coverslip and visualized directly.

2.4. Protein adsorption studies

The protein adsorption capability of the polymers was measured by using FITC-BSA as a model protein. Typically, 20 mg of the polymers was dissolved in 5 mL of $CHCl_3$, and thin film was formed by evaporation of the solvent in a petri dish. Then the film was incubated with 5 mL of phosphate-buffered saline solution (PBS) containing FITC-BSA (0.2 mg/mL) at 37 °C for 2 h. The non-adsorbed FITC-BSA on the film was washed with PBS, and collected with the supernatant. After centrifugation to remove the suspended polymer film, the UV–vis absorption of the non-adsorbed protein solution was measured via UV–vis spectrophotometer. PCL, PECC and the blend of both polymers (1:1) were processed according to the same testing protocol.

2.5. Cross-linking of HbP

The cross-linking of the particles was realized by condensation reactions of the carbonyl groups on the copolymers with diamine. Typically, the carbonyl groups were firstly activated by EDC·HCl and sulfo-NHS (10 fold molar of carbonyl groups) for 1 h at pH 6.0. After adjustment of the pH to 7.4, 2, 2′-(ethylenedioxy) bis (ethylamine) (6 fold molar of carbonyl groups) was added, and then the mixture was kept stirring overnight. The cross-linked HbP was collected by centrifugation.

The diameters of cross-linked particles were determined by dynamic light scattering (DLS) experiments at a 90° scatter angle performed on a Brookheaven ZetaPlus Analyzer (Brookheaven