International Journal of Pharmaceutics 377 (2009) 199–206

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Long-circulation of hemoglobin-loaded polymeric nanoparticles as oxygen carriers with modulated surface charges

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ARTICLE INFO

Article history:
Received 7 December 2008
Received in revised form 7 May 2009
Accepted 8 May 2009
Available online 18 May 2009

Keywords:
Hb-loaded polymeric nanoparticles
Blood long-circulation
ζ-Potential
Biodistribution

ABSTRACT

The aim of this study was to investigate the effects of the surface charges on the *in vitro* macrophage cellular uptake and *in vivo* blood clearance and biodistribution of the hemoglobin-loaded polymeric nanoparticles (HbPNPs). The surface charges of the HbPNPs fabricated from mPEG–PLA–mPEG were modulated with cationized cetyltrimethylammonium bromide (CTAB) and anionized sodium dodecyl sulphate (SDS), respectively. *In vitro* macrophage cellular uptake and *in vivo* biodistribution of the coumarin 6-labeled HbPNPs with different electric charges were investigated, and the half-lives in the circulation were pharmacokinetically analyzed. The particle sizes of the HbPNPs were all below 200 nm with a narrow size distribution and high encapsulation efficiency (>84%). And the ζ-potentials of the untreated, cationized and anionized HbPNPs in phosphate buffered sodium chloride solution (PBS) were −12.3, +3.28 and −25.4 mV, respectively. The HbPNPs did not occur significant aggregation or sedimentation, even after 5 days. Compared with the untreated HbPNPs, 1-fold decrease/increase of the uptake percentage associated with the cationized/anionized HbPNPs was observed. *In vivo* experiment demonstrated that the calculated half-life of the cationized HbPNPs was 10.991 h, 8-fold longer than that of the untreated HbPNPs (1.198 h). But the anionized HbPNPs displayed opposite effect. Furthermore, the cationized HbPNPs mainly accumulated in the liver, lung and spleen after 48 h injection. MTT results showed that the HbPNPs with different surface charges all exhibited slight toxicity. These results demonstrated that the CTAB-modulated HbPNPs with low positive charge and suitable size have a promising potential as a long-circulating oxygen carrier system with desirable biocompatibility and biofunctionality.

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1. Introduction

As a result of severe shortage of donor blood, the infection and spread of various diseases caused by traditional blood transfusion and the short storing time of crude blood, seeking for safe and efficient blood substitutes have received a lot of attention throughout the world (Stephen, 2000; Moore, 2003; Greenburg and Kim, 2004). Biodegradable hemoglobin-loaded polymeric nanoparticles (HbPNPs), which mimic the structure of the native red blood cells (RBCs), have attracted great interest in recent years (Chang, 1999, 2006; Piras et al., 2008). As a blood substitute, HbPNPs are desired to retain in blood circulation and function as RBCs with long-term carrying/delivering oxygen capacity. However, just as the other nanoparticles, a major limitation facing the intravenous delivery of HbPNPs is their rapid elimination from the systemic circulation by blood monocytes and cells of the mononuclear phagocyte system (MPS), thus greatly underscores their oxygen-carrying capacity. It is well-established that phagocytosis is a cellular phenomena and initiated by the attachment of the foreign particles to the surface receptors of the phagocytic cells (Gaur et al., 2000; Avgoustakis et al., 2003). And this attachment can be facilitated by the absorption of plasma proteins (opsonins) to the particle surface. Thus, fabrication of HbPNPs with a surface that can evade opsonin adsorption and the subsequent clearance from the blood by phagocytic cells is one of the key fundamental issues necessary to engineer long-circulating HbPNPs.

It has been accepted that the physical and chemical properties, including particle size, surface charge and surface hydrophilicity, are important to determine the biological fate of the nanoparticles after i.v. administration (Tabata and Ikada, 1988; Awasthi et

Abbreviations: CD, circular dichroism; CTAB, cationized cetyltrimethylammonium bromide; DLS, dynamic light scattering; DMEM, Dulbecco's modified eagle medium; DMSO, dimethylsulphoxide; EDTA, ethylene diamine tetraacetic acid; EE%, encapsulation efficiency; FTIR, Fourier transform infrared; HbPNPs, hemoglobin-loaded polymeric nanoparticles; ICR, Institute of Cancer Researcch; MPM, marine peritoneal macrophages; MPS, mononuclear phagocyte system; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide dye; PBS, phosphate buffer solution; PDI, polydispersity index; PEG, poly(ethylene glycol); RBCs, red blood cells; SD, Sprague–Dawley; SDS, sodium dodecyl sulphate; TEM, transmission electronic morphology.

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doi:10.1016/j.ijpharm.2009.05.015

al., 2003). The nanoparticles above approximately 200 nm will be immediately removed by the spleen as a result of mechanical filtration (Kissel and Roser, 1991). On the other hand, nanoparticles with diameters below approximately 70 nm will increase accumulation in the liver due to penetration of such small particles through fenestrate in the endothelial lining of the liver (Litzinger et al., 1994). According to these investigations, the "ideal" size requirements for nanoparticles developed for intravenous drug delivery are between 70 and 200 nm. Surface hydrophilicity, to some extent, can evade MPS uptake and prolong the residence of nanoparticles in blood. Poly(ethylene glycol) (PEG), either through covalent attachment of PEG to surface functional groups or through physical adsorption of PEG to the surface, has illustrated a decreased uptake by cells of the MPS and an increased circulation time in the blood stream (Tobío et al., 1998; Li et al., 2001). About the effect of the surface charges on the body distribution, many attempts have been made to investigate, but the results have been confused. It turns out that compared with the neutral or positive charged nanoparticles, the negative surface charge facilitates the clearance of nanoparticles from the blood circulation (Stolnik et al., 1995). But Gbadamosi et al. demonstrated that a lower negative charge decreased uptake; Ikada et al. reported that the introduction of negative charges into the dextran molecule prolonged its half-life in the circulation and this trend became more marked with increasing molecular weight of the dextran (Yamaoka et al., 1995; Gbadamosi et al., 2002). These previous results suggest that the surface charge of colloid carriers is indeed important in determining the circulation time in blood. But which surface charge best suits each individual target is a pending question up to now. A search of the previous literature, there is little information available about the effect of the surface charge on polymeric nanoparticles used for oxygen carriers.

In earlier work, our group focused on the development of a novel porous HbPNPs with high encapsulation efficiency (over 80%) and controlled particle size (about 70–200 nm) using the improved double emulsion and solvent diffusion/evaporation technique. Compared with the PCL matrix, PEGylation led to a 7.2-fold increase of the longevity of HbPNPs. But the half-life of the HbPNPs is still low as 160 min (Zhao et al., 2007). Therefore, in this study, we attempt to further prolong the blood residence by modulating the surface charge of the PEGylated HbPNPs. For this purpose, the blood circulation time and body distribution of HbPNPs with different surface charges were investigated. These HbPNPs were prepared using mPEG–PLA–mPEG as shell polymer by double emulsion (W/O/W) and solvent diffusion/evaporation technique, and cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) ionic surfactants were used to modulate the surface charge of HbPNPs. The blood clearance characteristic and biodistribution of these HbPNPs following intravenous administration were determined over 48 h in mice.

2. Materials and methods

2.1. Materials

mPEG–PLA–mPEG (Mw 16 kDa, Mw PEG:Mw PLA = 30:70) was purchased from DaiGang Biotechnology Co., Ltd., Jinan. Bovine Hb in a lyophilized form CTAB and SDS were supplied by YuanJu Biotechnology Company, Shanghai. Coumarin 6 was obtained from Sigma Chemical Company. Methylene chloride, acetic ether, acetone, span80 and other chemicals used were of analytical grade.

2.2. Preparation of Hb-loaded polymeric nanoparticles (HbPNPs)

The HbPNPs were prepared by a modified double emulsion (W/O/W) and solvent diffusion/evaporation technique based on the method proposed by Freytag et al. (2000). Briefly, 100 mg mPEG–PLA–mPEG and 0.15 g span80 were dissolved in 5 ml mixture of methylene chloride, acetone and acetic ether in the volume ratio of 3:1:1 as organic phase. Then 0.5 ml Hb solution with the concentration of 150 mg/ml was emulsified into organic phase by ultrasonic (JYD-900, ZhiXin Instrument Co., Ltd., Shanghai) for 15 s to form a primary W/O emulsion. This emulsion was then added to 50 ml aqueous solution of CTAB or SDS (0.1%, w/v) and homogenized by high pressure homogenizer at 200 bar for 3 min (AH110D, ATS Engineering Inc., Italy), leading to the secondary W/O/W emulsion. The double emulsion was subsequently added to 150 ml aqueous solution and then the resultant dispersion was stirred using a magnetic stirrer under atmospheric pressure. Finally, 200 ml nanoparticles suspension was obtained. The HbPNPs were collected by Labscale™ TFF System (Millipore Bioprocess Division, American), and then were lyophilized for 48 h to remove residual solvents. Fluorescence-labeled HbPNPs were prepared in the same way by incorporating 0.25% (w/w) coumarin 6 in the initial organic solution.

2.3. Characterization of HbPNPs

2.3.1. Particle size and size distribution

The size of the HbPNPs was determined by dynamic light scattering, using a Malvern Nano-ZS instrument (Malvern Instruments, UK). All experiments were performed at 25 °C with a measuring angle of 90° to the incident beam. The correlation decay functions were analyzed by the cumulants method to determine the average particle diameter and polydispersity (defined as the variance of the log normal distribution of particle sizes). The values presented were the mean of three replicate samples together with the standard deviation.

2.3.2. Surface charge and morphology

The surface potential of the HbPNPs was analyzed using Malvern Nano-ZS instrument (Malvern Instruments, UK). The measurements were done in phosphate buffered sodium chloride solution (PBS) (pH 7.4, 0.01 mol/l) and deionized water using disposable zeta cells (DTS 1060) at 25.0 °C, respectively. A manual duration of about 25 sub-runs was used for each measurement. The mean ζ-potential was determined using phase analysis light scattering technique.

The morphology of the nanoparticles was examined by transmission electronic morphology (TEM) (JEM-2010, Japan). The nanoparticle samples for TEM analysis were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar® films for viewing.

2.3.3. Encapsulation efficiency (EE%) and pore-connecting efficiency (PCE%) measurement

The sulphocyanate potassium method was chosen to determine the EE% and PCE%, which represents the percentage of the partial Hb from the total encapsulated Hb within HbPNPs that connects with the exchange materials, as described by Zhao et al. (2007) in detail. Briefly, 2 ml 2 M hydrochloric acid was added into 2 ml specimen under shaking conditions, respectively. In the presence of 20% trichloroacetic acid solution, the specimens were stored at 37 °C for a period of 5 min. Then, 0.2 ml saturated potassium persulphate solution was added in 4 ml of supernatant after centrifugation again and then 0.8 ml of sulphocyanate potassium solution was added to each specimen. Finally, the values of OD at 520 nm were determined via a UV spectrophotometer (SPECTRA$_{max}$ PLUS 384, Molecular Devices Corporation, USA) before being calibrated by the specimen of control. The specimen of control was prepared from the external aqueous solution directly. The Hb$_{total}$ specimen was prepared from dissolving Hb, which was equal to the initial fed Hb into 200 ml external aqueous solution. The specimen of Hb$_{free}$

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