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

Feng Xu[a], Yuan Yuan[a], Xiaoqian Shan[a], Changsheng Liu[a,*], Xinyi Tao[b], Yan Sheng[a], Huanjun Zhou[a]

[a] Key Laboratory for Ultrafine Materials of Ministry of Education, and Engineering Research Center for Biomedical Materials of Ministry of Education, East China University of Science and Technology, Shanghai 200237, PR China
[b] New World Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, PR China

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.

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.

* Corresponding author. Tel.: +86 21 64251358; fax: +86 21 64251358.
E-mail address: csliu@sh163.net (C. Liu).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
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$_{free}$

was the supernatant of HbPNPs suspension after centrifugation at 21,000 × *g* for 60 min. And finally, the specimen of $Hb_{measurement}$ was the prepared HbP suspension directly.

Therefore, the EE% and PCE% were calculated with the following equations:

$$EE\% = \frac{1 - OD_{free}}{OD_{total}} \times 100.$$

$$PCE\% = \frac{OD_{measurement} - OD_{free}}{OD_{total} - OD_{free}} \times 100.$$

2.3.4. Evaluation of the stability of the Hb and the suspension stability of the HbPNPs

2.3.4.1. The stability of the Hb. Fourier transform infrared (FTIR) and circular dichroism (CD) measurements were used to investigate the stability of Hb during encapsulation. For FTIR measurement, the Hb and HbPNPs were first washed with deionized water and then lyophilized. The lyophilized samples combined KBr were ground to fabricate KBr tablets and the mixture was compressed into a transparent tablet. The FTIR spectra were recorded from 4000 to 400 cm^{-1} by FTIR (AVATAR360, Nicolet, USA) (Zhang et al., 2005).

The secondary structures of the Hb and Hb loaded in HbPNPs were investigated by the far UV circular dichroism spectrum. Hb entrapped in HbPNPs was recovered by extraction (De and Girigoswami, 2006). Briefly, 10 mg of HbPNPs was dissolved in 2 ml solvents (1 ml dichloromethane and 1 ml ethyl acetate) and 10 ml phosphate buffer and kept vigorously shaking for 10 min. After clarifying, the aqueous Hb solution in supernatant was obtained by centrifugation under 5000 rpm for 10 min. To prevent autoxidation and denaturation of Hb, the extraction was under 4 °C. The CD spectra of the Hb and Hb extracted from HbPNPs were recorded on a JASCO spectropolarimeter (J-715, Japan) in the 190–250 nm wavelength region (0.2 nm resolution). The corresponding buffer baseline was subtracted from each spectrum. Each spectrum represented the average of eight scans. A quartz cuvette of 0.1 cm path length was used. The analysis temperature was under 25 °C.

2.3.4.2. Suspension stability of HbPNPs. The stability of the HbPNPs suspension was evaluated using a Turbiscan Lab Expert (Formulaction, France). The lyophilized samples were dissolved in PBS (pH 7.4, 0.01 mol/l) and transferred into a glass cylindrical cell. And then they were analyzed by a light beam emitted in near infrared (880 nm) wavelength which scanned the sample cell. Two synchronous optical sensors received, respectively, light transmitted through the sample and light backscattered by the sample. The backscattering (BS) was directly dependent on the particle mean diameter. These samples in the cell were scanned 120 h at 25 °C and the change in BS (ΔBS) in unit time was taken as a measure of the stability of the HbPNPs suspensions.

2.4. Uptake of the nanoparticles by macrophage cells in vitro

In vitro phagocytic uptake of HbPNPs was determined using marine peritoneal macrophages (MPM) harvested from SD mice (weight 250 ± 5 g). 20 ml cold PBS was injected into the abdominal cavity of sacrificed mice. 5 min later, the peritoneal cells were collected by flushing the peritoneal cavity with 10 ml of 0.9% NaCl. Then, the MPM were isolated by centrifuging at 2000 rpm for 10 min and re-suspended at a density of 1×10^5 cells/ml in Dulbecco's modified eagle medium (DMEM), supplemented with 10% fetal bovine serum, followed by being transferred to 24-well plate (1 ml for well) and incubated at 37 °C in atmosphere containing 5% CO_2 for 2 h. After incubation, the macrophages were washed with PBS to remove non-adherent cells. The adherent cells were further incubated in DMEM medium supplemented with 10% fetal bovine serum for 2 h. At the end of this incubation time, 300 μl aqueous suspensions of HbPNPs with different charges at a concentration 500 μg/ml were added. After 30 min incubation, the macrophage cells were washed and rinsed five times with PBS to ensure the removal of the HbPNPs adhered to the cells surface. Then, 300 μl/well of mixture of Triton-X 100 at 10% in acetonitrile were added to extract the coumarin 6 from the phagocytosed cells. Finally, the fluorescent intensity of the coumarin 6-containing extraction was analyzed by a fluoroskan ascent reader (Thermo Labsystems, Finland). And the percentage of uptake was calculated by division of the fluorescent intensity of total HbPNPs introduced into the cells by the fluorescent intensity of the nanoparticle-phagocytosed cells.

2.5. Cytotoxicity of the HbPNPs

Cytotoxicity of the HbPNPs with different surface charges was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay in human umbilical vein endothelial cell-derived cell line, ECV304. ECV304 cell suspensions (100 μl; 2×10^4 cells) were dispensed (4 wells for each particle type) into 96-well plates (Costar New York, USA) and incubated overnight (12 h) to allow for cell adherence. Culture medium was replaced with 100 μl of nanoparticles/culture medium suspensions with different concentrations of 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml and incubated at 37 °C for 24 h. ECV304 cells without nanoparticles were used as the control. At the end of control, 30 μl of MTT (5 mg/ml) solution was added into each well and was allowed to react at 37 °C for 4 h. The solution was removed and 100 μl of dimethylsulphoxide (DMSO) was added into each well. Then the plate was incubated for 15 min at 37 °C. Absorption at 490 nm was measured with $SPECTRA_{max}$ PLUS 384 (Molecular Devices Corp., USA). The cell viability was calculated by the following formula:

$$\text{cell viability}\,(\%) = \frac{\text{optical density (OD) of the treated cells}}{\text{OD of the untreated cells}}.$$

2.6. In vivo blood clearance and biodistribution of HbPNPs

ICR mice, body weight between 20 and 25 g (provided by Sino-British Sippr/BK Lab Animal Ltd., Shanghai, China), were used for blood clearance and body distribution investigations. The mice were fasted overnight but had free access to water. The suspensions of HbPNPs were prepared in NaCl 0.9% and injected intravenously into the tail vein (10 ml of solution corresponding to 1 g HbPNPs/kg). After i.v. injection for predetermined time, the animals were sacrificed by cervical dislocation. Organ samples, consisting lung, liver, heart, spleen, kidney and brain were removed, washed with distilled water and accurately weighed. Blood samples were collected at predetermined time intervals after i.v. injection in mice.

Biodistribution and blood residence of particles were determined by measuring the fluorescent intensity of coumarin 6 after their extraction from the particles in blood or organs into acetonitrile. The extractions were carried out as follows. Blood samples were added into distilled water containing 0.1 mM ethylene diamine tetraacetic acid (EDTA) before being frozen. Organ samples were homogenized with equivalent volume of distilled water and then frozen, thawed and mechanically shaken. This procedure was repeated three times to completely destroy cells. After that, blood and organ samples were lyophilized over 36 h, and the dried samples were extracted by acetonitrile, and mechanically shaken for 18 h in a water bath at 25 °C. The concentration of coumarin 6 was assayed on a fluoroskan ascent reader (Thermo Labsystems, Finland) using λ_{ex} = 485 nm and λ_{em} = 530 nm. The cumulative percentage of coumarin 6 in organs and in blood can be calculated

Table 1
Physical characteristic of HbPNPs with different surfactants.

Formulation	Mean size (nm)	PDI	PCE (%)	ζ-Potential in water (mV)	ζ-Potential in PBS (mV)	EE (%)
Untreated HbPNPs	167.8 ± 5.4	0.200	78.8 ± 7.3	−19.5 ± 1.1	−12.3 ± 1.6	87.2
Anionized HbPNPs	155.2 ± 8.2	0.158	82.2 ± 3.2	−36.3 ± 2.3	−25.4 ± 2.1	86.3
Cationized HbPNPs	192.2 ± 9.3	0.164	79.3 ± 6.2	+5.46 ± 0.31	+3.28 ± 0.25	84.5

(Hans and Lowman, 2002). Three mice were i.v. injected for each sampling point.

2.7. Statistics analysis

All data were generated in three independent experiments and expressed as mean ± standard errors. Data were analyzed with Student's *t*-test. The difference between treatments was considered significant when $p < 0.05$ in a two-tailed analysis.

3. Results and discussions

3.1. Physicochemical properties of the prepared HbPNPs

In this research, surface charge the HbPNPs was modulated by introduction of CTAB and SDS surfactant into the second external aqueous solutions. CTAB and SDS, which have cationic and anionic character at physiological pH (7.4), respectively, were adsorption onto the surface of the HbPNPs. For comparison, HbPNPs without surfactant were also included as the control. Table 1 summarizes the physicochemical characteristics of the PLA–PEG HbPNPs modulated with different surfactants. As previously mentioned, the size of the colloidal carriers is a key for the biological fate of the nanoparticles, and nanoparticles within a size range between 70 and 200 nm are less susceptible to macrophage uptake. So, in this study, we first tailored the particle size and DLS results showed that the HbPNPs formulated in this study were all in the size range of 100–200 nm with unimodal distribution. No significant difference in particle size before and after surface modification could be detected.

The zeta potential of PLA was found to be −37.7 ± 3.1 mV (in deionized water, data not shown). After PEGylation, the zeta potential of mPEG–PLA–mPEG NPs was decreased to −19.5 mV in deionized water and −12.3 mV in PBS. It has been approved that ζ-potential of the untreated HbPNPs was negative due to the presence of ionized carboxylic groups of the mPEG–PLA–mPEG. Note that the zeta potentials of the cationized and anionized HbPNPs were totally different, having appreciably positive (+3.28 mV in PBS and +5.46 mV in deionized water) and negative (−25.4 mV in PBS and −36.3 mV in deionized water) values, respectively. It is clear that after modulated by CTAB and SDS, even in PBS, the zeta potentials of the HbPNPs exhibited an absolute 15 mV increase and 13 mV decrease, respectively. That is to say, the surface charge of the PEG–PLA HbPNPs can be successfully modulated by surfactant with charge properties. It is also noticed that as previously reported, the zeta potential of the HbPNPs measured in PBS was all obviously lower than that determined in deionized water, but the extent was obviously low than the report where the zeta potential in PBS was near zero (Roser et al., 1998). We think this maybe related to the existence of PEG on the nanoparticle surface, which might affect the interaction of nanoparticles with the ions in PBS. In view of the PBS is more near the physiological environment *in vivo*, so in the following section, we correlated the nanoparticle charges measured in PBS with the fate and behaviors of nanoparticles *in vitro* and *in vivo*.

The encapsulation efficiency is 87.2%, 86.3% and 84.5% for the untreated HbPNPs, anionized HbPNPs and cationized HbPNPs, respectively and the PCE% of the untreated HbPNPs, anionized HbPNPs and cationized HbPNPs were 78.8%, 82.2%, 79.3%, implying that surfactant did not significantly affect the EE% and PCE%. Under TEM observation (Fig. 1), the untreated HbPNPs, all had a fine spherical shape with a relatively monodisperse size distribution. The particle size viewed in the TEM pictures was in agreement with that obtained by laser light scattering.

3.2. The biostability of the Hb and suspension stability of the HbPNPs

The maintenance of the protein structure is prerequisite for the fulfillment of its physiological function. So, a preliminary study of the structure change was necessary. Infrared spectroscopy is one of the well-recognized techniques in studies of the chemical structure of polypeptides and proteins. FTIR spectra of Hb and the entrapped Hb are shown in Fig. 2. The N–H bending vibration (amide II) was located at 1541 and 1542 cm^{-1} for Hb and the HbPNPs, respectively, while those for the C=O stretching vibration (amide I) at 1675 and 1677 cm^{-1}, respectively. The result showed that the shift in the frequencies of amide I band and amide II band is not obvious.

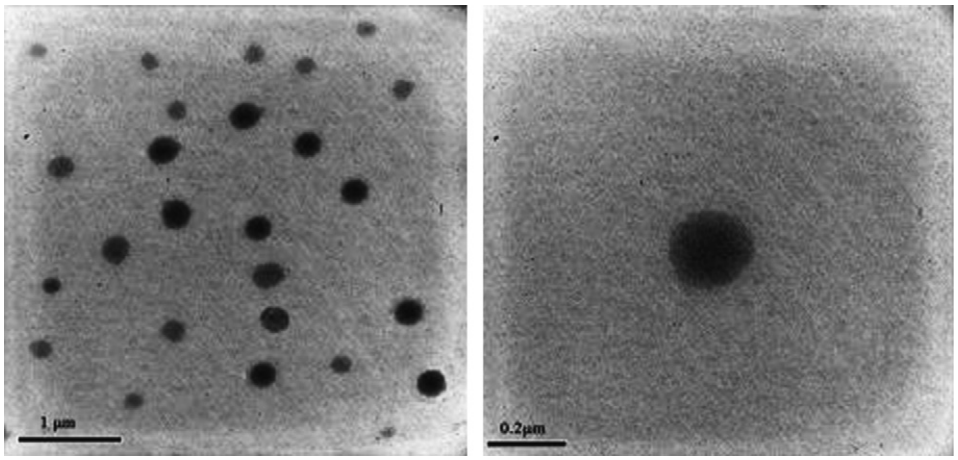

Fig. 1. Typical images of Hb-loaded polymeric nanoparticles taken by TEM.

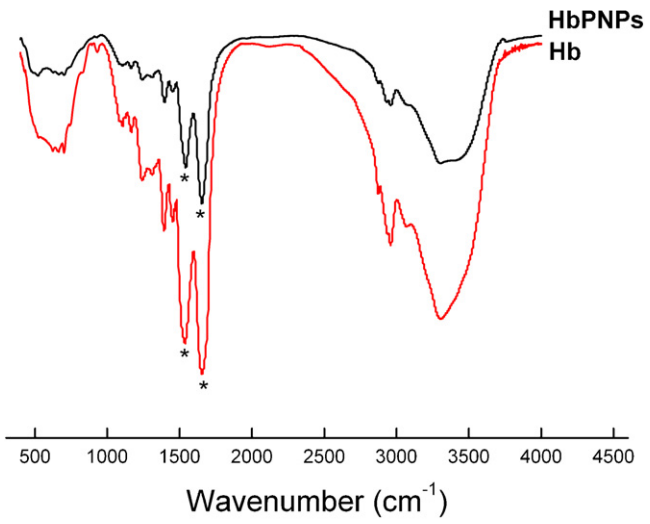

Fig. 2. FTIR spectra of hemoglobin and post-encapsulation (spectra marked with (*) correspond to the spectral features (amide I and amide II) of hemoglobin).

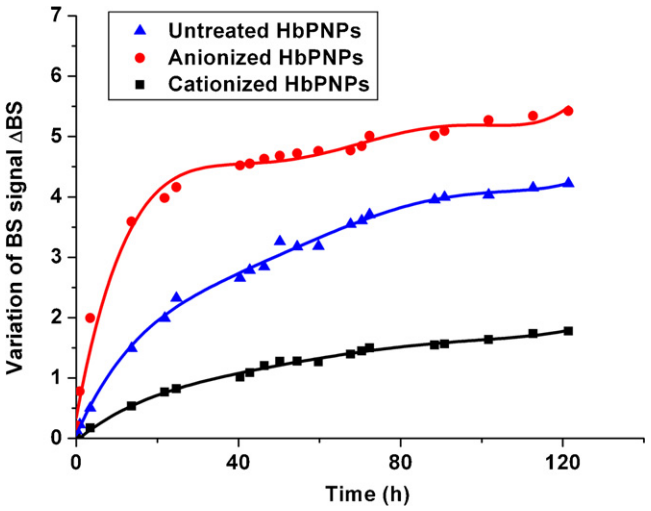

Fig. 4. Variation of backscattering signal (ΔBS) vs. time for the untreated HbPNPs cationized HbPNPs and anionized HbPNPs suspension. The mean ΔBS was measured over 120 h on sample portion comprised between 0.15 and 5.0 mm.

Fig. 3 presents the CD spectra of Hb and the Hb extracted from HbPNPs. It can be seen that the CD spectra exhibited an intensive positive peak about 195 nm and pronounced negative bands about 208 and 222 nm, which is a typical a-helix CD spectrum of Hb (Zhu et al., 2002). The very similar a-helix structure indicated the maintenance of the secondary structure of Hb during the suppression process (Palateh et al., 2006). Therefore, the combination of FTIR and CD measurements demonstrated the spatial structure of Hb did not undergo any significant change after loading process.

The suspension stability of the HbPNPs for clinic administration is a key factor to be considered. The backscattering, which is directly dependent on the mean particles size and volume fraction, has been widely used to detect the stability of nanoparticle suspension. So, in this study, backscattering variations (ΔBS) of different HbPNPs suspensions were studied over time using a Turbiscan optical analyzer, which has already been successfully used to investigate the stability and homogeneity of liquid mixtures on the basis of turbidity measurements (Lemarchand et al., 2003). The variation of BS signals as a function of time for HbPNPs suspensions with different surface charges are compared in Fig. 4. After 5 days of scanning, ΔBS variation was only 4.22%, 5.42% and 1.78% variation, respectively, for the untreated HbPNPs, cationized HbPNPs and anionized HbPNPs, indicating that there was no significant aggregation or sedimentation occurred in HbPNPs suspension with different surface charges.

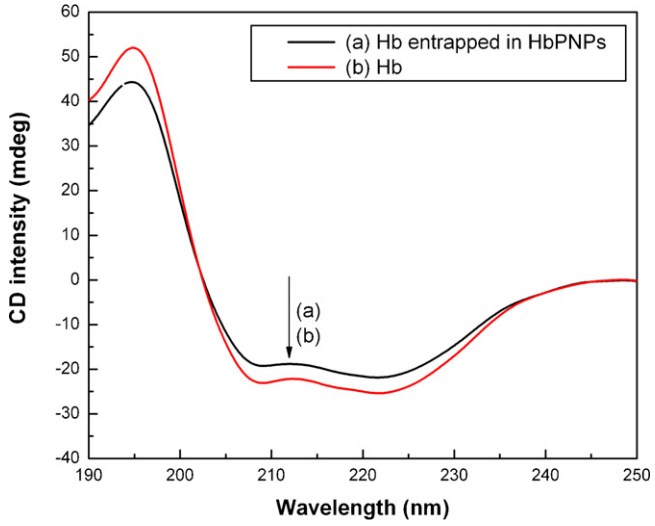

Fig. 3. CD spectra of hemoglobin and post-encapsulation.

It has been reported that, in general, the higher the ζ-potential absolute values of nanoparticles, including positive and negative, the more stable of the nanoparticle suspension can be obtained (Freitas and Müller, 1998; Gallardo et al., 2005). Thus, the lowest ΔBS variation was observed in the anionized HbPNPs suspension which possessed the highest absolute value of ζ-potential. Nevertheless, the cationized HbPNPs with ζ-potential only about +3.28 mV also showed the excellent stability. A potential explanation for this phenomenon might involve the inherent sterically hindered effect of PEG molecules. mPEG–PLA–mPEG, as shell polymer, formed a U-shape to concentrate the PLA units in the core and the PEG tails projected out into the water. The presence of a hydrophilic PEG steric barrier could provide effective steric stabilization. Thus, the cationized HbPNPs suspension, even with relative low absolute value of ζ-potential, also did not occur obvious aggregation or sedimentation.

3.3. Cytotoxicity of the HbPNPs

In vitro cellular activity of ECV304 cells after exposure to the untreated HbPNPs, cationized HbPNPs and anionized HbPNPs at the concentrations of 62.5, 125, 250, 500, 1000 μg/ml for 24 h was investigated and the results are shown in Fig. 5. It could be seen that compared with the control in which no HbPNPs was added, the untreated, anionized and cationized HbPNPs did not exert cytotoxic effects. Rather, to different extent, improved the cell viability in the range from 62.5 to 1000 μg/ml, implying that mPEG–PLA–mPEG nanoparticles are biocompatible in a large concentration range. Although cationized agents had been reported to cause serious cellular toxicity due to electrostatic interaction with negatively charged cellular membrane (Fischer et al., 2003), but the results here showed cationized HbPNPs exhibited no obvious cytotoxicity.

3.4. Macrophage uptake of HbPNPs in vitro

In this work, coumarin 6, a commonly used fluorescent marker (Davda and Labhasetwar, 2002), was employed as the fluorescent marker to evaluate the biological behaviors of the HbPNPs with different surface charges. In order to verify the reliability of the data obtained from the *in vitro* and *in vivo* experiments, *in vitro*

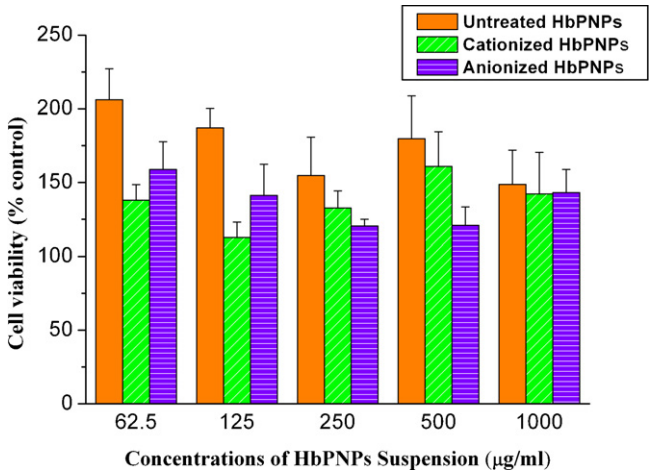

Fig. 5. *In vitro* viability of ECV304 cells treated with the untreated HbPNPs, cationized HbPNPs and anionized HbPNPs at the same concentration 62.5, 125, 250, 500, 1000 μg/ml after 24 h culture, respectively ($n = 4$). The cell viability (%) was calculated by the optical density (OD) of the treated cells/OD of the untreated cells.

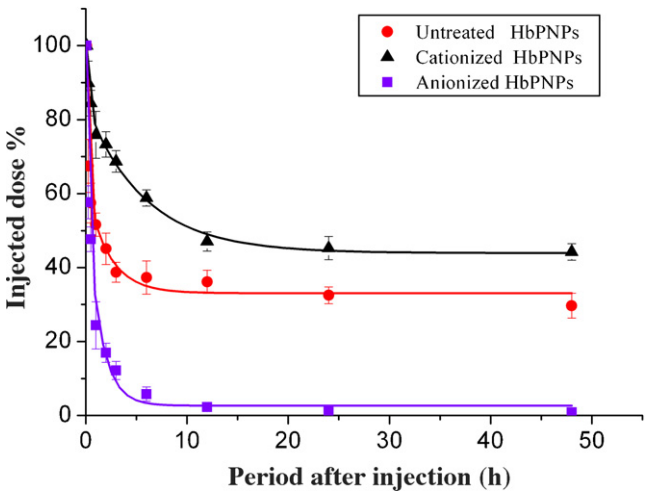

Fig. 7. Blood clearance of the untreated, cationized and anionized HbPNPs in mice intravenous injection.

release study of coumarin 6 and the cytotoxicity assay from/of the labeled HbPNPs were first carried out in this study (data not shown). The results revealed that less than 0.8% of the dye is release from the HbPNPs during the 48 h period tested. Additionally, cytotoxic assays illustrated that the coumarin 6-labeled HbPNPs were non-toxic. Therefore, the coumarin 6 incorporated in the HbPNPs as a marker could not interfere with their inherent biological behaviors *in vitro* and *in vivo*.

Fig. 6 shows the cellular uptake percentage of HbPNPs with different surface charges by marine peritoneal macrophages after 30 min incubation. Media used in all incubations contained 10% FBS to mimic the opsonization events *in vivo*. The percentage of the fluorescent intensity of the coumarin 6 extracted from cells in the total fluorescent intensity of the HbPNPs added into cells was defined as the percentage of macrophage uptake. It could be seen that after 30 min incubation, the uptake percentage of the untreated HbPNPs was about 24%. After cationized by CTAB, only 12% of the HbPNPs were uptaked by MPM. But the cellular uptake percentage of anionized HbPNPs attained 52%. It is clear that the surface charges of HbPNPs could greatly affect the uptake of nanoparticles by MPM. It is also clear that the HbPNPs with low positive charge could mask the phagocytosis of macrophage.

It has been well-established that for both negatively and positively charged particles, the extent of phagocytosis was increased with the increasing absolute ζ-potential values, and there was no significant difference in the phagocytosis between the cationized and anionized surfaces when compared at a ζ-potential of the same absolute value (Roser et al., 1998). When ζ-potential was zero, the uptake was the lowest. Consistent with the above phenomena observed by Roser et al. (1998), our results here also showed that the cationized HbPNPs with the lowest absolute ζ-potential value could effectively avoid the uptake by the MPS cells, and the anionized HbPNPs with the highest absolute ζ-potential value promote the uptake by the MPS cell.

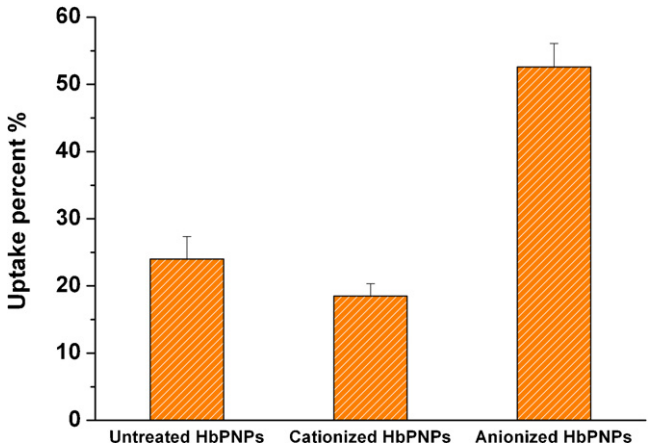

Fig. 6. Uptake of nanoparticles by MPM.

3.5. Biodistribution of HbPNPs in vivo

To fully understand how the body handles the HbPNPs, the pharmacokinetics and biodistribution of HbPNPs were monitored and compared. Fig. 7 shows the blood clearance curves of HbPNPs with different surface charges after intravenous injection. It could be seen that the anionized HbPNPs were quickly removed from the blood circulation after administration. In contrast, the cationized HbPNPs exhibited a markedly delayed blood clearance and 40% of the injected dose still remained in blood circulation even after 48 h. The half-lives ($T_{1/2}$) of the neutral, cationized and anionized HbPNPs in blood circulation was 1.198, 10.991 and 0.354 h, respectively (shown in Fig. 8). Apparently, the half-lives of the HbPNPs in the blood circulation strongly depend on the surface electric charges. And compared with the untreated HbPNPs, the half-life of the cationized HbPNPs was prolonged approximately 9-fold. It should be noted that the blood clearance curves of the HbPNPs can be divided into two stages: an initial rapid disappearance and a subsequent slower one. This might cause by that, HbPNPs after i.v. injection, distributed immediately into the organs through blood circulation. As a result, the concentration of HbPNPs in blood was decreased rapidly and a dynamic equilibrium would reach between HbPNPs concentrations in organs and blood.

In this study, the cationized HbPNPs exhibiting the most prolonged blood circulating property was selected to investigate the

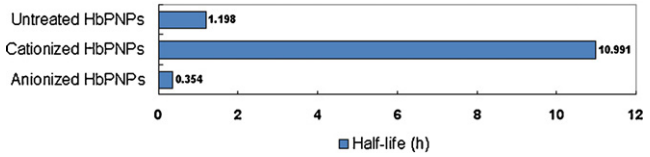

Fig. 8. Half-life ($T_{1/2}$) in blood circulation of the untreated, cationized and anionized HbPNPs.

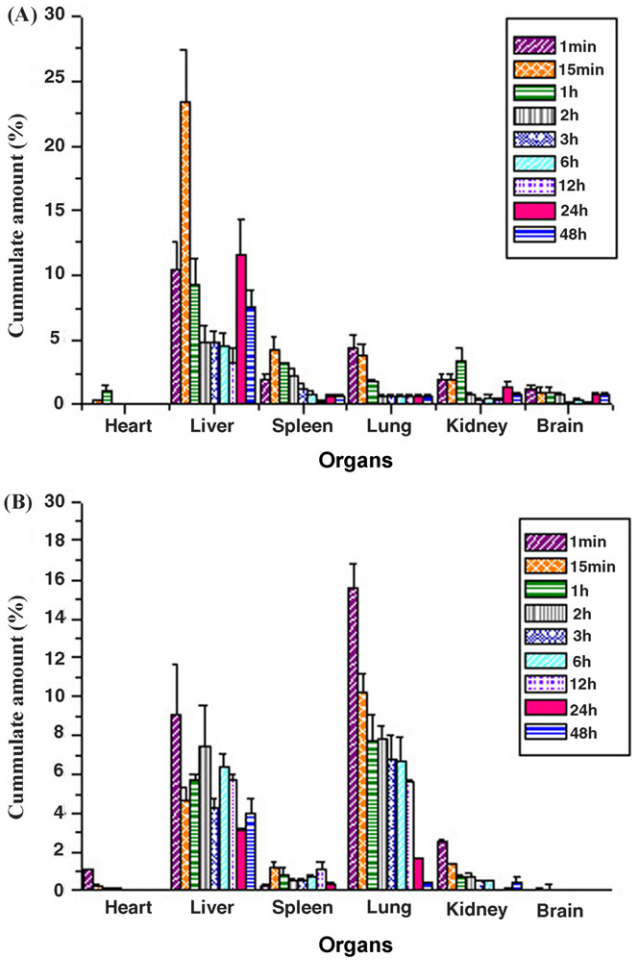

Fig. 9. The cumulated amount of the untreated HbPNPs (A) and cationized HbPNPs (B) in main organs at different time within 48 h after i.v. injection.

biological fate of HbPNPs *in vivo* after i.v. administration. The neutral HbPNPs was also selected for comparison. The biodistribution profiles of the neutral HbPNPs and cationized HbPNPs in various organs at 48 h after intravenous administration are shown in Fig. 9. After i.v. injection, both untreated HbPNPs and cationized HbPNPs were mainly distributed to the liver, spleen, lung and kidney. This corresponded well to the trend of HbPNPs concentration in blood. Meanwhile, the HbPNPs accumulation in heart and brain were kept at a quite low level of less than 1.2 injected dose%. These results indicated the uptake of these HbPNPs to heart and brain could be negligible.

The major pathway for the removal of nanoparticles from blood appeared to be the nanoparticles capture in MPS tissues, and especially in the liver. As liver is the main MPS organ, there seems logical to exist a correlation between the level of increased blood retention and their reduced sequestration by liver. It was noted that after 15 min intravenous injection, approx. 5-fold untreated HbPNPs accumulated in liver than the cationized HbPNPs. A similar trend was observed for spleen accumulation although the absolute percentage was much less than that of the liver. So, it can be inferred that cationization of the HbPNPs could contribute substantially to reduce the uptake of reticuloendothelial system (RES) and eventually to achieve the long circulation property of HbPNPs. These results are in agreement with the previous results obtained *in vitro* macrophage uptake. Interestingly, the cationized HbPNPs accumulated in lung to a greater extent than the untreated HbPNPs. It was possible that cationized HbPNPs were more easily to be absorbed to the beds of blood capillary, while a large of blood capillary enriched in lung. The results indicated that cationized nanoparticles had greater potential to be used as blood substitute than the neutral and anionized HbPNPs.

According to the accepted mechanism, the adsorption of plasma proteins onto the surface of colloidal carriers influences the distribution in the body by affecting the uptake into the mononuclear phagocytic systems (MPS). Avoidance of the uptake by MPS is a prerequisite for targeting colloidal carriers to other organs and tissues than liver and spleen. A widely used method of camouflaging or masking NPs is the use of surface adsorbed or grafted shielding groups which can hinder the hydrophobic and electrostatic interactions that help plasma proteins bind to particle surface. Some examples of polymer systems have been tried in the literature as shielding groups, including polyethylene glycol (PEG), polyvinyl alcohol (PVA), polysaccharides, etc. Among them, PEG appeared as an ideal candidate, and was extensively used to coat the surface of NPs, since it has been shown to successfully weaken the MPS uptake and lead to prolonged blood circulation time. Based on these premises, in the previous study, we substituted mPEG–PLA–mPEG for PLA with the primary objective to repel the adsorption of plasma protein by the steric hindrance and modulation of the physicochemical of the nanoparticles. But the results indicated that even with 30% PEG content, the zeta potential of the nanoparticles was as high as −19.5 mV (in water) and −12.3 mV (in PBS) and the half-life in blood circulation was still only 70 min, which cannot meet the requirement of the clinic application. Therefore, in this study, we want to further modify the surface charge of HbPNP by different surfactants, thus influencing the phagocytic uptake into macrophages.

The results obtained here showed that the surface charges of the HbPNPs can be easily positively and negatively modulated by CTAB and SDS.

No significant difference in particle size with the different modulation methods could be detected. Since all preparations had comparable average diameters and polydispersity coefficients, the influence of size on *in vitro* phagocytosis and *in vivo* biodistribution could be neglected. Compared with the untreated HbPNPs prepared without surfactant, the cationized HbPNPs with +3.28 mV can avoid the macrophage uptake and prolong the blood circulation time. But in the case of the anionized HbPNPs with −25.4 mV, the blood longevity was greatly shortened. As previous report, the process of opsonization plays an important role in the recognition and phagocytosis of nanoparticles by the macrophages of the RES. To our great knowledge, there are many kinds of opsonins (such as immunoglobulins G and M, complementary protein, platelet thrombin, fibronectin and so on) in the blood stream. Once administrated into the blood stream, the nanoparticles surface will rapidly be occupied by these opsonins via passive or electrostatic adsorption. Consequently, these opsonin-bound nanoparticles can easily be recognized by the specific receptor on the surface of macrophages plasma membrane. And the nanoparticles will be endocytosed by the mononuclear phagocyte system. From this viewpoint, the cationized HbPNPs with low absolute positive charge may inhibit the absorption of opsonins and the endocytosis of macrophages, and thus prolong the blood circulation time. But the anionized HbPNPs with high absolute negative charge might promote, to some extent, the protein absorption. As a result, the endocytosis of macrophages was enhanced and the blood resident time was shortened.

Another potential explanation for the effect of the surface charges might relate to the configuration of the PEG chain on the nanoparticle surface in water medium, which has been established to play a large role in the steric repulsion efficacy *in vitro* and *in vivo*. A “brush” configuration vs. a “mushroom”, or flat, regime can provide a conformational mobility of PEG molecules that result in higher cloud density and uniformity that may enhance the shielding effect (Zahr et al., 2006). The cationized HbPNPs had a lowest

uptake percentage and the longest blood circulation compared to the untreated and anionized HbPNPs with the same nanoshell composition. One could hypothesize that the cationized HbPNPs assumes brush-like conformation in the presence of cationized CTAB. Therefore, if every PEG molecule attached to the surface is in a brush regime, the hydrated shell is developed and the shielding effect is maximal.

The blood circulation time has great effect on the efficacy of protein/drug nanoparticle, in particular for the intravenous delivery system. The commonly used method to evaluate the biological fate of nanoparticles is *in vivo* animal experiment, which is very expensive and time-consuming. In this study, prior to the *in vivo* animal study, *in vitro* cellular uptake experiment was carried out to evaluate the phagocytosis of nanoparticles by the macrophages of RES. Correlated the results obtained from the *in vitro* cellular uptake and the *in vivo* animal experiment, it can be found that the less the nanoparticles endocytosed by macrophages *in vitro*, the longer of the blood circulation *in vivo*. Therefore, it can be inferred that the *in vitro* preliminary phagocytic uptake experiment could be used to forecast about the blood circulation longevity of nanoparticles *in vivo*.

4. Conclusion

In this study, the effect of the surface charge of Hb-loaded polymeric nanoparticles (HbPNPs) on the blood clearance and biodistribution was investigated. The HbPNPs prepared by double emulsion (W/O/W) technique, whether untreated, cationized or anionized, all had small particle size, monodisperse, excellent stability and low cytotoxicity. This study showed that the biological fate of HbPNPs was greatly influenced by the surface charge of nanoparticles. The anionized HbPNPs were rapidly eliminated from the blood circulation, while the cationized HbPNPs with low absolute values of ζ-potential could help to prolong the blood circulation time. The half-life ($T_{1/2}$) in blood circulation for them was about 11 h due to the cationized HbPNPs with low absolute value of ζ-potential could reduce uptake by MPS efficiently. Thus HbPNPs surface modified by CTAB could be an effective oxygen carrier with long circulation time.

Acknowledgements

The authors acknowledge the financial support from the National High Technology Research and Development Program of China (863 program) (No. 2004AA-302050) and from Shanghai Nanotechnology Special Foundation (No. 0452nm022).

References

Avgoustakis, K., Beletsi, A., Panagi, Z., Klepetsanis, P., Livaniou, E., Evangelatos, G., Ithakissios, D.S., 2003. Effect of copolymer composition on the physicochemical characteristics, in vitro stability, and biodistribution of PLGA–mPEG nanoparticles. Int. J. Pharm. 259, 115–127.

Awasthi, V.D., Garcia, D., Goins, B.A., Phillips, W.T., 2003. Circulation and biodistribution profiles of long-circulating PEG-liposomes of various sizes in rabbits. Int. J. Pharm. 253, 121–132.

Chang, T.M.S., 1999. Future prospects for artificial blood. Trends Biotechnol. 17, 61–67.

Chang, T.M.S., 2006. Blood substitutes based on nanobiotechnology. Trends Biotechnol. 24, 372–377.

Davda, J., Labhasetwar, V., 2002. Characterization of nanoparticle uptake by endothelial cells. Int. J. Pharm. 233, 51–59.

De, S., Girigoswami, A., 2006. A fluorimetric and circular dichroism study of hemoglobin—effect of pH and anionizedamphiphiles. J. Colloids Interface Sci. 296, 324–331.

Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., Kissel, T., 2003. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 24, 1121–1131.

Freitas, C., Müller, R.H., 1998. Spray-drying of solid lipid nanoparticles (SLN™). Euro. J. Pharm. Biopharm. 46, 145–151.

Freytag, T., Dashevsky, A., Tillman, L., Hardee, G.E., Bodmeier, R., 2000. Improvement of the encapsulation efficiency of oligonucleotide containing biodegradable microspheres. J. Control. Release 69, 197–207.

Gallardo, V., Morales, M.E., Ruiz, M.A., Delgado, A.V., 2005. An experimental investigation of the stability of ethylcellulose latex: correlation between zeta potential and sedimentation. Eur. J. Pharm. Sci. 26, 170–175.

Gaur, U., Sahoo, S.K., De, T.K., et al., 2000. Biodistribution of fluoresceinated dextran using novel nanoparticles evading reticuloendothelial system. Int. J. Pharm. 202, 1–10.

Gbadamosi, J.K., Hunter, A.C., Moghimi, S.M., 2002. PEGylation of microspheres generates a heterogeneous population of particles with differential surface characteristics and biological performance. FEBS Lett. 532, 338–344.

Greenburg, G., Kim, H.W., 2004. Civilian uses of hemoglobin-based oxygen carriers. Artif. Organs 28, 795–799.

Hans, M.L., Lowman, A.M., 2002. Biodegradable nanoparticles for drug delivery and targeting. Curr. Opin. Solid State Mater. Sci. 6, 319–327.

Kissel, T., Roser, M., 1991. Influence of chemical surface-modification on the phagocytic properties of albumin nanoparticles. Proc. Int. Symp. Control. Release Bioact. Mater. 18, 275–276.

Lemarchand, C., Couvreur, P., Vauthier, C., Costantini, D., Gref, R., 2003. Study of emulsion stabilization by graft copolymers using the optical analyzer Turbiscan. Int. J. Pharm. 254, 77–82.

Litzinger, D.C., Buiting, A.M.J., Van Rooijen, N., Huang, L., 1994. Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene glycol)-containing liposomes. Biochim. Biophys. Acta 1190, 99–107.

Li, Y.P., Pei, Y.Y., Zhang, X.Y., Gu, Z.H., Zhou, Z.H., Yuan, W.F., Zhou, J.J., et al., 2001. PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. J. Control. Release 71, 203–211.

Moore, E.E., 2003. Blood substitutes: the future is now. J. Am. Coll. Surg. 196, 1–17.

Palateh, N., Bhad, S., Montazeri, R., Guidry, C.A., Haynie, D., 2006. Polypeptide multiplayer nanofilm artificial red blood cells. J. Biomed. Mater. Res. 81, 261–268.

Piras, A.M., Dessy, A., Chiellini, F., Chiellini, E., Farina, C., Ramelli, M., Valle, E.D., 2008. Polymeric nanoparticles for hemoglobin-based oxygen carriers. Biochim. Biophys. Acta 1784, 1454–1461.

Roser, M., Fischer, D., Kissel, T., 1998. Surface-modified biodegradable albumin nano- and microspheres: II. Effect of surface charges on in vitro phagocytosis and biodistribution in rats. Eur. J. Pharm. Biopharm. 4, 255–263.

Stephen, M.C., 2000. Blood substitutes in surgery. Surgery 127, 599–602.

Stolnik, S., Illum, L., Davis, S.S., 1995. Long circulating microparticulate drug carriers. Adv. Drug Deliv. Rev. 16, 195–214.

Tabata, Y., Ikada, Y., 1988. Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophage. Biomaterials 9, 356–362.

Tobío, M., Gref, R., Sànchez, A., Langer, R., Alonso, M.J., 1998. Stealth PLA–PEG nanoparticles as protein carriers for nasal administration. Pharm. Res. 15, 270–275.

Yamaoka, T., Kuroda, M., Tabata, Y., Ikada, Y., 1995. Body distribution of dextran derivatives with electric charges after intravenous administration. Int. J. Pharm. 113, 149–157.

Zahr, A.S., Davis, C.A., Pishko, M.V., 2006. Macrophage uptake of core–shell nanoparticles surface modified with poly(ethylene glycol). Langmuir 22, 8178–8185.

Zhang, Y., Zhu, W., Wang, B.B., Ding, J.D., 2005. A novel microgel and associated post-fabrication encapsulation technique of proteins. J. Control. Release 105, 260–268.

Zhao, J., Liu, C.S., Yuan, Y., Tao, X.Y., Shan, X.Q., Sheng, Y., Wu, F., 2007. Preparation of hemoglobin-loaded nano-sized particles with porous structure as oxygen carriers. Biomaterials 28, 1414–1422.

Zhu, Y.C., Cheng, G.J., Dong, S.J., 2002. Structural electrochemical study of hemoglobin by in situ circular dichroism thin layer spectroelectrochemistry. Biophys. Chem. 97, 129–138.