Porosity and Semipermeability of Hemoglobin-Loaded Polymeric Nanoparticles as Potential Blood Substitutes

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Abstract: Porosity and semipermeability, allowing life-sustaining small molecules to penetrate, but hemoglobin (Hb) and other enzymes to cut off, predominantly affect the functionalities of the Hb-loaded polymeric nanoparticles (HbPNPs) as blood substitutes. In this article, HbPNPs formulated in the size range of 110–122 nm were prepared by a modified double-emulsion method with poly(lactic acid) (PLA)-based polymers. The influences of the main preparation conditions, including solvent composition, stirring speed, Hb concentration and polymer matrix, on the porosity were investigated in details. To evaluate the porosity of HbPNPs, a novel nondestructive testing method based on molecular weight cut-off (MWCO) was developed, and an effusion approach was applied to investigate the pore size in the particle shells with poly(ethylene glycol)s (PEGs) of different molecular weights (PEG200, PEG400, PEG600) as probes. Moreover, *in vitro* diffusion behaviors of ascorbic acid and reduced glutathione from HbPNPs fabricated with various polymer matrices were studied. The MWCO of HbPNPs by changing solvent composition, stirring speed, Hb concentration, and polymer composition varied from 200 to 600, especially the PEGylation of the polymer, which exhibited obvious influence on the MWCO of HbPNPs. Ascorbic acid with molecular weight 176.1 could diffuse into PEGylated nanoparticles with mPEG content of 5–30 wt % freely, while reduced glutathione with molecular weight 307.3 could not penetrate when mPEG content reached 30 wt %. These results suggest that the HbPNPs optimized with MWCO between 400 and 600 can facilitate the transport of all those life-sustaining small molecules.

Keywords: porosity; semipermeability; nanoparticles; molecular weight cut-off; probes

INTRODUCTION

The search for a clinically useful blood substitute is stimulated by the limitations of blood due to its insufficient supply, safety, and costs.[1–3] Although an excellent oxygen carrier, even highly purified stroma-free hemoglobin (Hb) cannot be used for infusion, because it is rapidly cleared by the kidney and causes renal toxicity and other adverse effects.[4] Encapsulation has been proposed as an advanced alternative to protect the enclosed Hb from the external environment, thereby helping to avoid renal toxicity, preventing vasoconstriction and escaping the rejection by immune system.[5] In the past few years, many researches have focused on encapsulating concentrated Hb in phospholipids[6,7] and polymeric[8,9] vesicles. Among them, liposomes have been shown to be a promising approach for the design of oxygen carrier, but plain liposomes are molecular assemblies and generally regarded as unstable capsules, such as fusion, aggregation, and shear-induced destruction.[10] Furthermore, lipid bilayer membrane is relatively dense to produce a close system. To overcome these drawbacks, biodegradable hemoglobin-loaded polymeric nanoparticles (HbPNPs), especially with about 100–200 nm size, has been developed. Previous investigations have revealed that HbPNPs, which not only possess a more stable and permeable system just like the natural red

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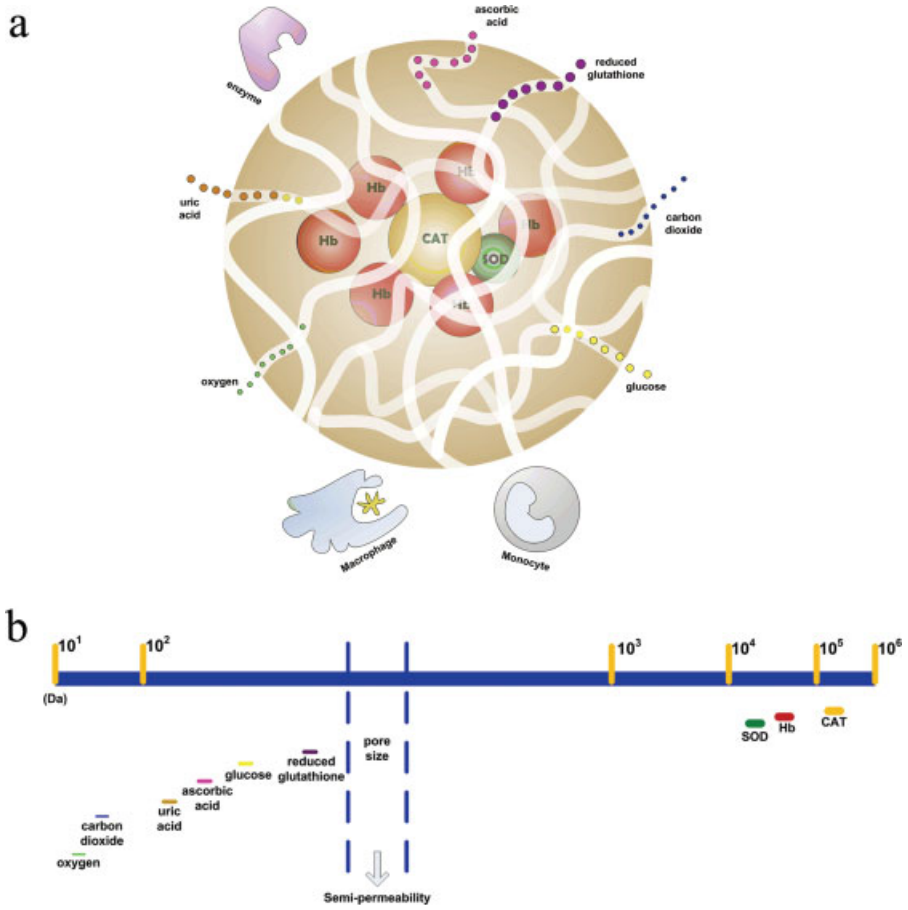

Figure 1. (a) Schematic representation of the porous structure and diffusion behavior of life-sustaining small molecules from artificial RBCs with a PLA membrane. (b) Schematic representation of the molecular size comparison of life-sustaining substances involved in artificial RBCs. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

blood cells (RBCs) but also exhibit longer blood circulation compared to the larger-sized particles and keep the same P_{50} as native Hb used in the preparation, represent a new generation of blood substitutes.[11,12]

An active in-and-out exchange system, allowing the life-sustaining small molecules such as oxygen, carbon dioxide, glucose in plasma, and metabolic products to diffuse into/out of the system, is the prerequisite of maintaining the normal functionalities of natural RBCs. So, from this viewpoint, the basic idea of designing artificial RBCs is, Hb and the enzyme system, including superoxide dismutase (SOD) and catalase (CAT), are coencapsulated inside the polymer membrane, which is permeable to small hydrophilic molecules, such as glucose and uric acid involved in Embden-Meyerh of pathway, oxygen and carbon dioxide participating in gas transport *in vivo*, and the large molecules, such as Hb, immune cells, cannot diffuse out/in [as shown in Figure 1(a)]. In addition, proper diffusion behavior of metahemoglobin (metHb) reducing agents present in human plasma, such as ascorbic acid and reduced glutathione, is vital for maintaining the oxygen-carrying capacity of Hb.[13,14] So, it is of great importance to understand and tailor the mass transport properties of these vesicles.

To our great knowledge, most drug/protein-loaded particles, especially fabricated by double-emulsion solvent diffusion/evaporation technique, prefer to assume a multinucleate structure rather than a single core-shell and also display a three-dimensional structure (3D). In this case, the transport of small molecules in and out of particles is pore-dependent diffusion, including the porosity on the surface and within the internal channels.[15] It is well established that the 3D structure of particles is dominantly determined by the preparation process conditions.[16] So, mass transfer behavior, 3D structure of particles, and preparation conditions are intimately related and greatly affect each other. This is especially true for HbPNPs, in which the large molecular weight Hb is encapsulated, and this is expected to change the microstructure of nanoparticles, and thus affecting their physical properties, particularly the release kinetics of small molecules through particles. Therefore, there is a need to understand the relationship between preparation conditions and the 3D porous structure of particles. Up to date, there are a considerable number of researches on the release profile of protein or peptide through nanoparticles or microparticles[15,17]; however, relatively few investigations have focused on the effect of formulation

TABLE I. Characteristics of HbPNP With Different Polymer Matrices

Particle type	M_w (kDa)	Polydispersity	EG:LA (%weight)	Size (nm)	Zeta Potential (mV)	PEG EE[c] (%)	mPEG Coating Efficiency (%)
PLA	40	1.321		118 ± 7	−32.5 ± 1.3	90.22 ± 0.42	
PLA-mPEG5K[a]	150	1.282	5:95	122 ± 9	−22.9 ± 1.8	91.63 ± 0.45	81 ± 2
	110	1.347	10:90	116 ± 8	−18.9 ± 0.7	91.28 ± 0.55	85 ± 4
	40	1.573	30:70	110 ± 9	−11.8 ± 1.1	90.11 ± 0.61	93 ± 2
PLA-mPEG (10%)[b]	15	1.584	10:90	113 ± 10	−16.4 ± 0.3	90.56 ± 0.52	86 ± 1
	48	1.434	10:90	115 ± 8	−17.7 ± 0.5	91.07 ± 0.49	85 ± 2

Solvent composition DCM/ACE (1:1, v/v), stirring speed 700 rpm, Hb concentration 100 mg/mL.
All values indicate mean ± SD for $n = 3$ independent experiments.
[a] The mPEG molecular weight is constant, according to the provider, 5 kDa.
[b] The mPEG content is constant, according to the provider, 10 wt %.
[c] Nonsignificant (NS).

factors on the porosity and permeability of nanoparticles used as blood substitutes. Additionally, due to the nanoscaled diameter, the pore size of connecting porous channels within nanoparticles is too small to find an appropriate apparatus to observe it directly. Thus, the development of a novel strategy to detect the pore parameter of HbPNPs is also urgently needed.

Double-emulsion solvent diffusion/evaporation technique is one of the most popular methods to encapsulate hydrophilic drugs, especially protein and peptide drugs, into particles.[18] In the previous study, our group has successfully prepared biodegradable HbPNPs with higher encapsulation efficiency, desirable particle size, and available porous structure by an improved double emulsion method.[19] However, the porosity and diffusion behavior of various small molecules are yet unknown, which are essential for determining whether or not the fabricated HbPNPs would function as natural RBCs *in vivo*. In this study, we want to evaluate and optimize the porous structure and semipermeability of HbPNPs. For this purpose, the effects of solvent composition, stirring speed, Hb concentration, and polymer matrix on the porous parameter of HbPNPs were investigated in details. To investigate the porosity of HbPNPs, a novel method based on the molecular weight cut-off (MWCO) concept was established in this study. As we know, MWCO, a widely used parameter in membrane field, represents the boundary of molecular weight at which the smaller molecules can diffuse through the membrane freely, but the larger molecules cannot. So, this reminds us the possibility to use the MWCO to estimate the pore size of HbPNPs. To achieve this, poly(ethylene glycol)s (PEGs) of different molecular weights with Hb were encapsulated in the internal phase as probes, and the effusion rates of PEGs from HbPNPs were investigated to quantify the porosity. PEG is selected because it is hydrophilic, nonionic, easily available, and has a series of different molecular weights (such as PEG200, PEG400, PEG600). Also, PEGs have been used to study the passive transcellular permeability in immortalized human corneal epithelial cell.[20] Moreover, ascorbic acid and reduced glutathione, two important reducing agents for metHb present in human plasma, were used as small molecular models to further verify the experiment results. To our knowledge, this study is critical to investigate and optimize the mass diffusion behavior of HbPNPs as blood substitutes, and also this is the first time that a method using PEGs of different molecular weight as probes to study the porosity of nanoscaled particles has been proposed.

MATERIALS AND METHODS

Materials

PLA (M_w 40 kDa) and PLA–mPEG (M_w 150 kDa, M_{wPEG}:M_{wPLA} = 1:19; M_w 110 kDa, M_{wPEG}:M_{wPLA} = 1:9; M_w 40 kDa, M_{wPEG}:M_{wPLA} = 3:7; M_w 15 kDa, M_{wPEG}:M_{wPLA} = 1:9; M_w 48 kDa, M_{wPEG}:M_{wPLA} = 1:9) were supplied by DaiGang Biotechnology, Jinan, and characterized with regard to their compositions and molecular weights by GPC and ^{1}H NMR (Table I). The PLA–mPEG diblock copolymers used in this study are indicated as PLA–mPEG*a*(*b*), where *a* is the molecular weight of mPEG (Da) and *b* represents the content of mPEG (wt %). Purified bovine Hb in lyophilized form, ascorbic acid, and reduced glutathione were purchased from YuanJu Biotechnology Company, Shanghai. PEG200 (polydispersity index = 1.02), PEG400 (polydispersity index = 1.01), and PEG600 (polydispersity index = 1.04) were obtained from Sigma. All other regents were of analytical grade.

Methods

Nanoparticles Preparation. HbPNPs were fabricated by an improved double-emulsion solvent diffusion/evaporation method as previously described by Zhao et al.[19] with some modifications. Briefly, 0.5 mL Hb solution was emulsified in 5 mL organic solvent containing PLA or PLA–mPEG (100 mg) by an ultrasonic generator (JYD-900, ZhiXin Instrument, Shanghai) for 12 s. Thereafter, the primary emulsion was poured into 50 mL 0.5% polyvinyl alcohol (PVA) aqueous solution followed by two steps of re-emulsification by a high-pressure homogenizer (AH110D, ATS Engineering, Canada) at 200 bar for 15 s and 3 min, respectively. The double emulsion was subsequently added to 150 mL 0.5% PVA solution, and then it was vacuumed to completely remove the solvents. In this study, to measure the porosity, a series of PEGs (PEG200, PEG400,

PEG600) with the concentration of 300 mg/mL was entrapped in the internal phase with Hb. The nanoparticles were collected by centrifugation (GL-21M, Shanghai Centrifuge Institute, Shanghai) at 21,000*g* for 60 min, followed by washing with distilled water for three times to remove the free Hb or PEGs on the particle surface, and then the nanoparticles were vacuumed to completely remove residual solvents before lyophilization. The entire process was maintained at 4°C by thermostatted water bath.

Several batches of Hb- and PEG-loaded nanoparticles were fabricated with four varying process conditions such as solvent composition, stirring speed, Hb concentration, and polymer matrix to reveal the influence of process variables on porous parameters of HbPNPs.

Characterization of HbPNPs. Physicochemical Characterization of HbPNPs. The particle size and zeta potential of prepared HbPNPs were determined by photon correlation spectroscopy (PCS) and laser Doppler anemometry (LDA) at 25°C under an angle of 90° in a Zetasizer Nano ZS (Malvern Instrument, Malvern, UK), respectively.

The morphology of the HbPNPs was observed under transmission electronic microscope (TEM) (JEM-2010, Japan) and atomic force microscope (AFM) (SPA-400, Japan). The samples for TEM analysis were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar films for viewing. Samples for AFM analysis were prepared by deposition of particles on freshly cleaved mica, followed by air-drying overnight at 30°C.

Measurement of PEG Encapsulation Efficiency. Encapsulation efficiency (EE) percentage (%EE) was calculated as the ratio of amount of PEG entrapped to the total amount of PEG added initially. Hb- and PEG-loaded nanoparticles were suspended in 1*N* NaOH for 1 h to which distilled water was added followed by acidification with 1*N* HCl. Amount of PEG entrapped was then measured at 200 nm using an UV–vis spectrophotometer (U-2001, Hitachi). Hb-loaded nanoparticles (prepared by the same volume of Hb solution as inner aqueous phase) were used as a blank to correct for interference.

Determination of Surface Composition of HbPNPs by ^{1}H NMR. To study the influence of different polymers on the surface composition of the HbPNPs, two types of ^{1}H NMR analysis were employed.[21] Firstly, to determine the real composition, the HbPNPs were freeze-dried, dissolved in $CDCl_3$, and analyzed by ^{1}H NMR (Avance 500, Bruker, Germany). Secondly, to determine how much of the mPEG was either inside or around the particles, the isolated nanoparticles were suspended in D_2O and analyzed then by ^{1}H NMR. Under this condition, it was expected that only the mPEG chains that migrated to surface and projected toward the external aqueous phase will be seen by ^{1}H NMR, while PLA will remain in a solid core phase and thus invisible. The results are presented as "mPEG coating efficiency," which is the percentage of soluble mPEG coating around the nanoparticles with respect to the total amount of mPEG in the nanoparticles.

Porosity Measurements. The prepared Hb- and PEG-loaded nanoparticles (0.2 g) were suspended in phosphate buffer (10 m*M*, pH 7.4, 1 mL) and enclosed in a dialysis bag, which was then placed into phosphate buffer (10 mL) at 37°C under shaking. At defined time intervals, 10 mL of release medium was collected and replaced with fresh medium. The amount of PEG released was measured by spectrophotometry at a wavelength of 200 nm. The effusion profiles of PEGs from HbPNPs were determined to quantify the porosity.

In Vitro Diffusion Behavior of Ascorbic Acid and Reduced Glutathione. HbPNP (0.2 g) was incubated with 1 m*M* ascorbic acid and 1 m*M* reduced glutathione, respectively, suspending in Tris buffer (0.1*M*, pH 7.4). The suspensions were kept at 37°C under shaking. At predetermined times, 3 mL suspension was drawn and the metHb level in HbPNP was determined by the method described in the following section.

Measurement of metHb in Nanoparticles. MetHb in nanoparticles was periodically measured using a standard cyanomethemoglobin method at 630 nm as described by Zhang et al.[22] Briefly, the absorbance of 3 mL sample was measured at 630 nm against a blank reference of the nonloaded nanoparticles (prepared by the same volume of phosphate buffer as inner aqueous phase) to correct for interference, which is taken as OD_1. Thus, OD_1 is the absorbance of the mixture of deoxyhemoglobin (deoxyHb), oxyhemoglobin (oxyHb), and metHb. Thereafter, 10 μL of KCN stock solution (one part 20% KCN and one part 10 m*M* phosphate buffer, pH 7.4) was added to 3 mL of sample and mixed. Then, the absorbance was read at 630 nm against 10 μL of KCN stock solution and 3 mL of nonloaded nanoparticles suspension, which is taken as OD_2. MetHb reacts with cyanide to form cyanomethemoglobin, which has little or no absorbance at 630 nm. Thus, OD_2 is the absorbance of oxyHb and deoxyHb. Then the metHb concentration can be calculated by Eq. (1). The millimolar extinction coefficient for metHb at 630 nm is 3.7 L mM^{-1} cm^{-1}. The amount of Hb in HbPNP was determined according to Eq. (2). Hb_{total} and EE% refers to the total amount of the Hb added during the preparation and EE, respectively. EE% was determined according to the previous method.[19] Then the metHb percent can be calculated by the Eq. (3).

$$\mathrm{MetHb_{free}}\ (\mathrm{m}M) = (\mathrm{OD_1} - \mathrm{OD_2})/3.7 \quad (1)$$

$$\mathrm{Hb_{encapsulated}} = \mathrm{Hb_{total}} \times \mathrm{EE}\% \quad (2)$$

$$\mathrm{MetHb_{free}}\ (\%) = \mathrm{MetHb}/\mathrm{Hb_{encapsulated}} \times 100\% \quad (3)$$

Statistics Analysis. Data were generated in three independent experiments and expressed as mean ± standard errors. Statistical data analysis was conducted using SPSS software. Predictive value (p) less than 0.05 was considered statistically significant.

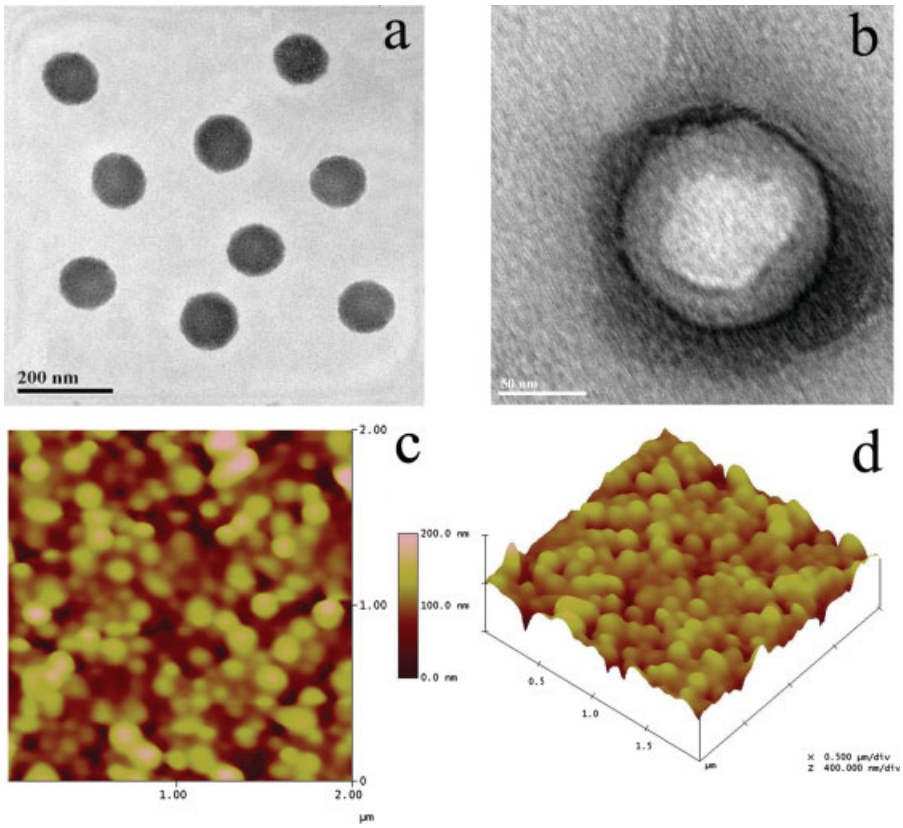

Figure 2. Images of HbPNP taken by TEM and AFM [solvent composition DCM/ACE (1:1, v/v), stirring speed 700 rpm, Hb concentration 100 mg/mL, polymer matrix PLA]. (a, b) TEM; (c, d) AFM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

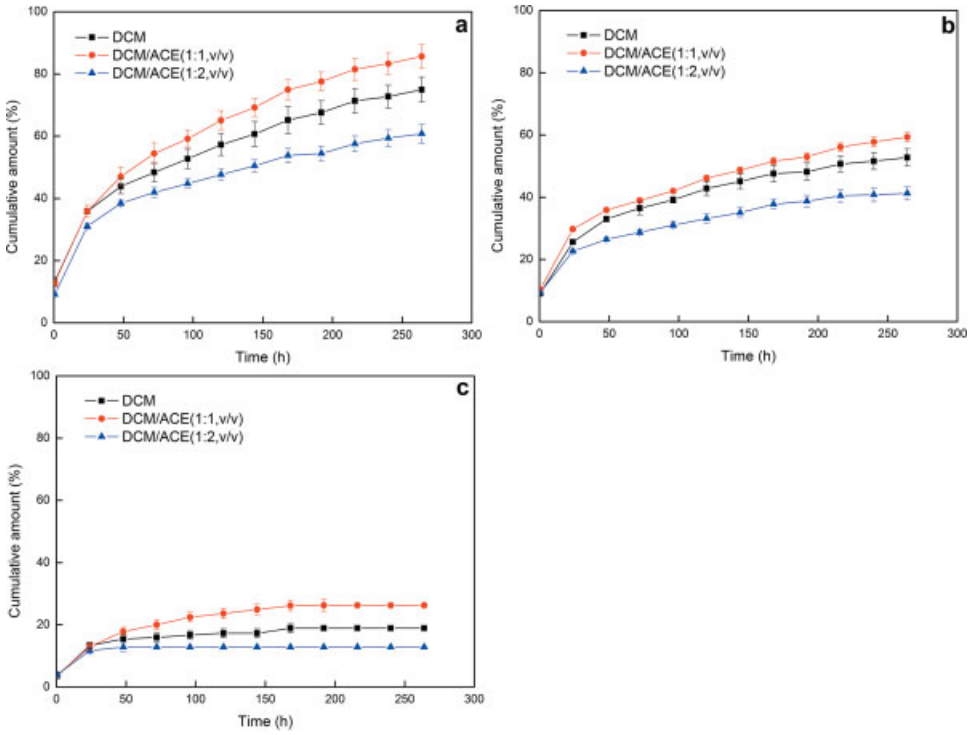

Figure 3. Effect of solvent composition on PEG release rate of HbPNP (stirring speed 700 rpm, Hb concentration 100 mg/mL, polymer matrix PLA). (a) PEG 200; (b) PEG 400; (c) PEG 600. The values plotted are mean ± SD of three experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS

Characterization of HbPNPs: Morphology, Particle Size and Zeta Potential

As shown in Figure 2, the morphologies of HbPNPs imaged by TEM and AFM appeared to be spherical and uniform in size with smooth round surface.

Table I summarizes the characteristics of the HbPNPs prepared with different polymer matrices. Particle size distribution determined by DLS showed unimodal distribution. Different formulations showed similar particle size in the range of 110–122 nm. Additionally, the size of the nanoparticles (data not shown) did not vary significantly with the variation of the solvent composition, stirring speed, and Hb concentration, except for the emulsification strength, as shown in the prior work by Zhao et al.[19]

PLA HbPNPs presented the largest negative zeta potential values of −32.5 mV. The results in Table I also showed that the zeta potential was affected by the mPEG content in polymer matrix rather than mPEG chain length. HbPNPs made of high mPEG content (30%) presented smaller negative zeta potential (−11.8 mV) than those made of low mPEG content (5, 10%). But the zeta potential value (−16.4, −17.7, −18.9 mV) was hardly changed by varying the mPEG chain length (500, 2000, 5000).

PEG Encapsulation Efficiency

The EEs for PEG loading are presented in Table I. EEs were about 90% and there was no significant difference ($p > 0.05$) between the different polymer matrices. Also, the variation of formulations in each batch, such as solvent composition, stirring speed, and Hb concentration, had no apparent effect on PEG EE (data not shown). These results preclude the variation of the release profiles of PEGs resulting from the different PEG content in the HbPNP.

^{1}H NMR: mPEG Coating Density

The NMR spectrum was used to quantify the mPEG coating efficiency of the HbPNP prepared by the different polymers. As shown in Table I, most of the mPEG was located on the surface of the HbPNP (more than 80%). It could also be seen that the mPEG coating efficiency was obviously affected by the mPEG content in the copolymer rather than mPEG chain length, which was generally consistent with the results of zeta potential, as will be latter discussed.

Effect of the Preparation Parameters on the Porosity of HbPNPs

Effect of Solvent Composition. Figure 3 shows the effects of the composition of organic solvent on the pore microstructure of HbPNPs, where the HbPNPs were fabricated with varying volume ratio of dichloromethane (DCM) and acetone (ACE) at DCM/ACE (1:0), DCM/ACE (1:1, v/v), and DCM/ACE (1:2, v/v). At first glance, the *in vitro* release of PEG markers from HbPNPs showed a typical biphasic profile, a rapid release in the initial hours followed by a slow and sustained release. And the release rate of PEG markers from HbPNPs fabricated by the specific solvent was in the order of PEG200 > PEG400 > PEG600, in a typical molecular weight-dependent manner. Compared with the pure DCM solvent, when binary solvent DCM/ACE was adopted, some small but significant differences in release rate kinetics were observed at the long release period. The release rates of the PEGs from HbPNPs fabricated with DCM/ACE (1:1, v/v) were obviously higher than that fabricated with DCM. With further increasing the ratio of ACE/DCM to 2:1, the release of PEG200, PEG400, and PEG600 decreased. However, the MWCOs of the HbPNPs, prepared from the binary or single solvent, were between 400 and 600, and no obvious changes were observed.

Effect of Stirring Speed. Stirring speed during solvent evaporation had a significant influence on MWCO of particles and the diffusion behaviors of the small molecules. We prepared HbPNPs from PLA at the DCM/ACE solvent composition of 1:1 (v/v) and Hb concentration of 100 mg/mL and investigated the effect of the stirring speed at 500 and 700 rpm. The results (data not shown) revealed that the release profile was strongly affected by the stirring speed. When stirring speed was 700 rpm, the MWCO of HbPNPs was between 400 and 600. While the stirring speed decreased to 500 rpm, the release rate of PEG400 dropped and then the MWCO was 200–400. It can be hypothesized that larger pores or altered surface shielding could be achieved by increasing the stirring speed from 500 to 700 rpm.

Effect of Hb Concentration. The effects of Hb concentration in initial internal phase on the resulting PEGs release kinetics are illustrated in Figure 4, where the Hb concentrations were 100, 200, 300, and 400 mg/mL, respectively. From Figure 4, it can be seen that for all the formulations, the MWCO of HbPNPs ranged from 400 to 600. And further observation could find that with the increasing of Hb concentration from 100 to 300 mg/mL, the release rate of PEGs appeared to drop step by step. But when the Hb concentration increased to 400 mg/mL, the release rate of PEGs quickened.

Effect of Polymer Matrix. To investigate the effects of polymer matrix on MWCO of particles, two sets of experiments were carried out. In the first set of experiment, four batches of particles made of PLA (M_w 40 kDa), PLA–mPEG500 (10%) (M_w 15 kDa), PLA–mPEG2000 (10%) (M_w 48 kDa) and PLA–mPEG5000 (10%) (M_w 110 kDa), which concerned the same mPEG content (10%) and different mPEG chain lengths (500, 2000, 5000), were studied. In the second set of experiment, PLA (M_w 40 kDa), PLA–mPEG5000 (5%) (M_w 150 kDa), PLA–mPEG5000 (10%) (M_w 110 kDa) and PLA–mPEG5000 (30%) (M_w 40 kDa)

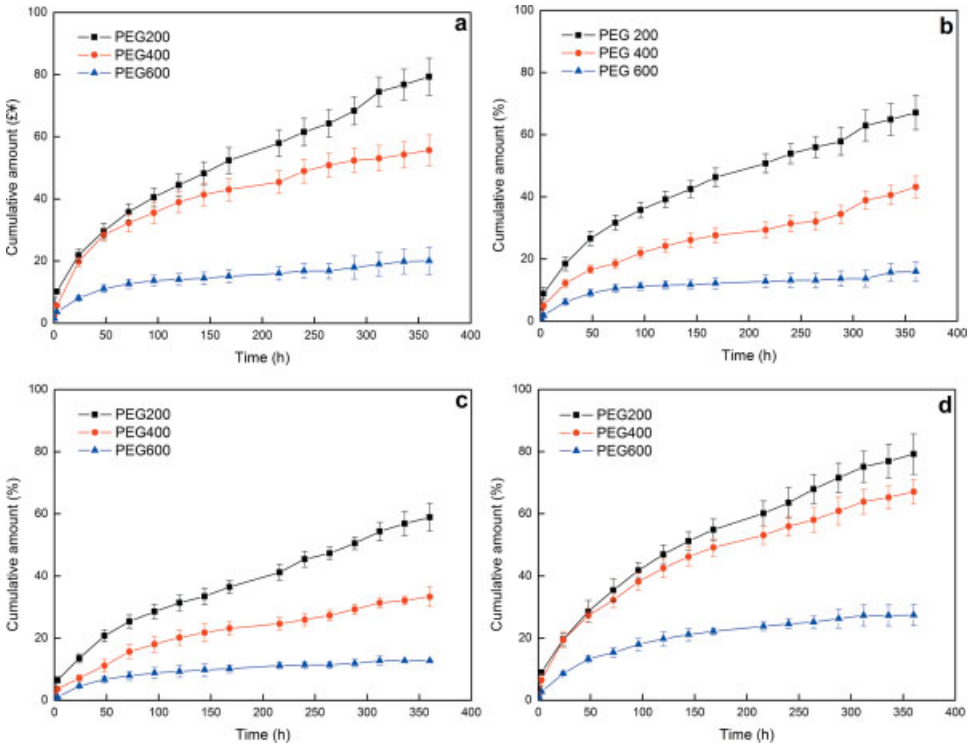

Figure 4. Effect of Hb concentration on PEG release rate of HbPNP [solvent composition DCM/ACE (1:1, v/v), stirring speed 700 rpm, polymer matrix PLA]. (a) 100 mgHb/mL; (b) 200 mgHb/mL; (c) 300 mgHb/mL; (d) 400 mgHb/mL. The values plotted are mean ± SD of three experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

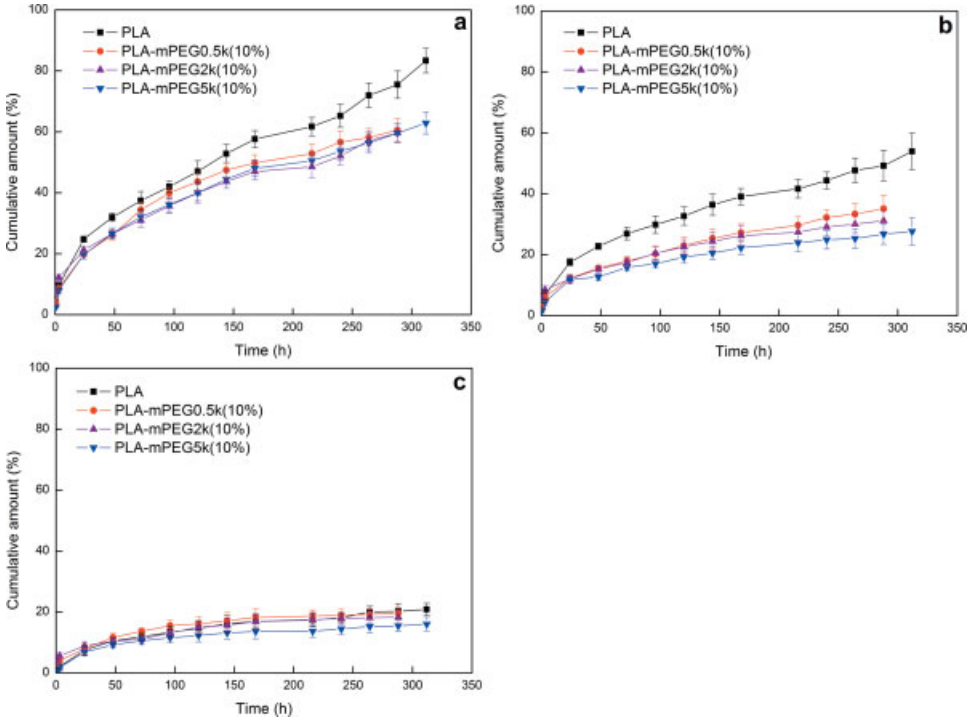

Figure 5. Effect of mPEG chain length in polymer matrix on PEG release rate of HbPNP [solvent composition DCM/ACE (1:1, v/v), stirring speed 700 rpm, Hb concentration 100 mg/mL]. (a) PEG200; (b) PEG400; (c) PEG600. The values plotted are mean ± SD of three experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

were applied to investigate the effect of the mPEG content (5, 10, 30%).

As shown in Figure 5, the release of PEG markers from the PEGylated nanoparticles was much slower than that from the corresponding PLA nanoparticles. The mPEG chain length (500, 2000, and 5000) had no apparent effect on the release of PEG markers, and the MWCOs of HbPNP with different mPEG chain lengths were all around 400–600. Figure 6 showed some distinct differences in the release profiles of PEGs from the HbPNPs with different mPEG contents. It can be seen from Figure 6(b,c) that, when 5 and 10 wt % of PEG block were introduced into the PLA chains, the release rate of PEG400 decreased compared with Figure 6(a), and the MWCO of HbPNP dropped from 400–600 to 200–400. Increasing the mPEG content from 10 to 30 wt %, as shown in Figure 6(d), almost PEG200 hardly diffused through the particle wall and hence MWCO was around 200. It is clear that polymer matrix, especially the mPEG content in diblock copolymer, has great influence on the diffusion behaviors of small molecules.

In Vitro Diffusion Behavior of Ascorbic Acid and Reduced Glutathione. To further verify the small molecular diffusion behavior of HbPNP and also to demonstrate the permeability of the metHb reducing agents present in human plasma, the changes of metHb level in HbPNP before and after cultivation with ascorbic acid (1 m*M*) or reduced glutathione (1 m*M*) were investigated. Four formulations, including PLA nanoparticles and PLA–mPEG nanoparticles with the same mPEG chain length (5000) and different mPEG contents (5, 10, and 30%), were employed. The metHb level as the function of incubation time was shown in Figure 7, taken PLA–mPEG5000 (30%), absence of ascorbic acid or reduced glutathione, as a control group. Figure 7(a) showed that the release rate of ascorbic acid decreased step by step with an increment of the mPEG content in polymer matrix. In Figure 7(b), it can be noticed that reduced glutathione hardly diffused through the nanoparticles by increasing the mPEG content to 30%. These are largely in accordance with the previous results in Figure 6.

DISCUSSION

The natural red blood cell is an active open system; nutritious and metabolic substances could pass in and out freely and also the vital function of binding/delivering oxygen and carbon dioxide is guaranteed, whereas larger molecules such as antibodies and proteins cannot enter. In general, these substances needed for cellular processes enter/leave RBCs mainly by simple diffusion and facilitated diffusion. Simple diffusion occurs when small molecules (like oxygen) pass through the lipid bilayer of the cell membrane, whereas facilitated diffusion depends on carrier proteins imbedded in the membrane to allow specific substances (like glucose) to pass through. But for artificial blood substitutes, protein-mediated diffusion is hard to come true; the porous structure is the exclusive access to accomplish it. Thus, for these artificial RBCs, porous structure and semipermeability are very important.

To establish the promising long-circulating oxygen carriers, the cellular Hb-based artificial blood substitutes should be typically designed to be uniform in size, hydrophilic on surface to prolong circulation time, ideal oxygen-carrying capacity,[11,12,23] and high Hb EE to minimize the dosage. In the past few years, our group have succeeded in preparing a novel HbPNP with high EE (87.35%) and oxygen-carrying capacity (27 mmHg and 1.946) near to that of native BHb.[19] In this study, we used the same method with little modification to fabricate HbPNP with a narrow size range of 110–122 nm and high mPEG coating density reaching 93%. The profile of HbPNP surface [Figure 2(b)] appeared discontinuous in silhouette after stained with dye, which was supposed to be the pores. However, it is difficult to observe the size of pores directly from TEM and AFM. So, in this study, a novel nondestructive method based on the MWCO concept was established to investigate the porosity of HbPNP.

Preparation of nanoscaled particles by double-emulsion solvent diffusion/evaporation method is a complex process in which the organic solvent generates pores in the structure during its diffusion/evaporation.[24] In general, solvent elimination profile includes two stages. The first step is the initial diffusion/extraction of solvent from the dispersed oil phase to external aqueous continuous phase (CP) and the subsequent step is the evaporation from the CP/air interface to environment. It is believed that the porous structure directly derived from both the solvent diffusion and the solvent evaporation followed.[19] Process parameters, which affect the solvent diffusion and solvent evaporation, were all supposed to have influence on the porosity of HbPNP. So, in this study, the effects of organic solvent and stirring speed were firstly investigated.

The organic solvent is a very important parameter, as it can influence the 3D structure of the particles, especially the porous surface and internal channels, which small molecules diffuse in and out.[25] DCM is a popular organic solvent due to its excellent solvency, which can dissolve large amount of polymers, and easier removal as a consequence of low boiling point (39.8°C). However, poor solubility in water (2.0%, w/v) leads to a slow exchange rate of solvent (DCM) and water, a relatively slow diffusion/extraction rate, thus setting a relatively "undisturbed" environment for polymer precipitation and the embryonic particle formation, which tends to form smooth and compact structure.[26] When the half volume of DCM was replaced by ACE, which is miscible in water, quick diffusion into external water phase was responsible for a relatively fast precipitation rate of the polymer, resulting in more porous surface and internal channels and hence the high release of PEGs. But when the ratio of ACE/DCM is up to 2:1, the diffusion rate of solvent was too fast and the surface/interface of the primary nanoparticles formed was turbulent and irregular.

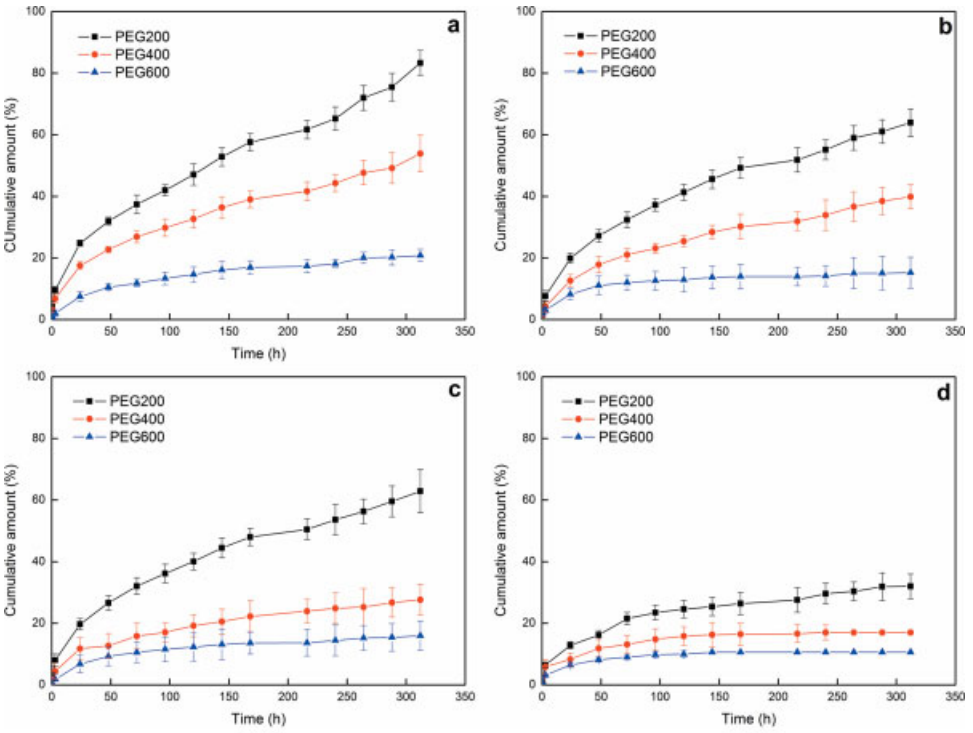

Figure 6. Effect of mPEG content in polymer matrix on PEG release rate of HbPNP [solvent composition DCM/ACE (1:1, v/v), stirring speed 700 rpm, Hb concentration 100 mg/mL]. (a) PLA; (b) PLA–mPEG5000 (5%); (c) PLA–mPEG5000 (10%); (d) PLA–mPEG5000 (30%). The values plotted are mean ± SD of three experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

After solvent evaporated completely, the final nanoparticles with small-sized porous surface and internal channels were produced and the release rate of PEGs was consequently reduced.

Stirring devices, such as propeller and magnetic stirrer, had obvious influence on the drug release profile of microcapsules.[27] The results obtained in this study showed that accelerating the stirring speed significantly increased the release of PEGs. This may be due to the fact that stirring process provides the energy to disperse the oil phase into the external water phase. Because of the higher mass transfer, a high stirring speed produced a high energy leading to

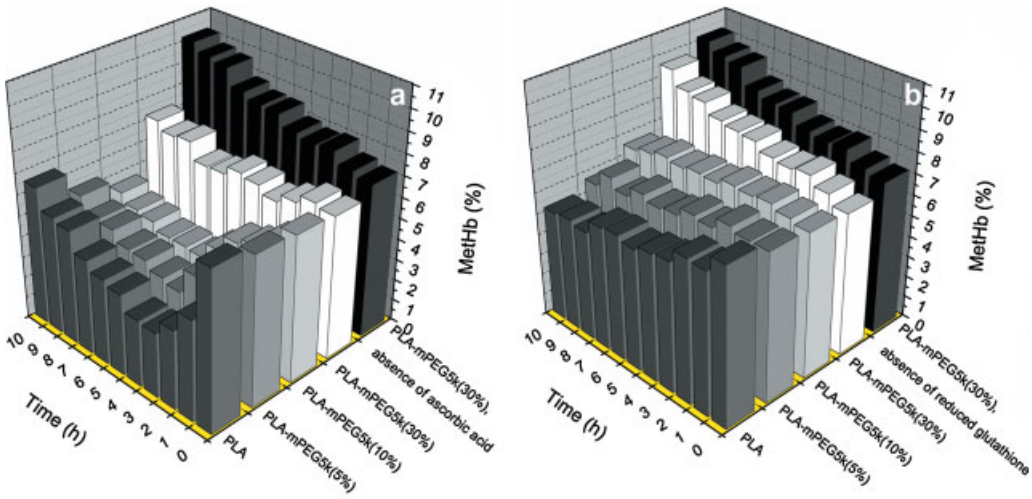

Figure 7. Diffusion behavior of reducing agents from HbPNP based on various polymer matrices with different mPEG contents [solvent composition DCM/ACE (1:1, v/v), stirring speed 700 rpm, Hb concentration 100 mg/mL]. (a) Ascorbic acid; (b) reduced glutathione. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

a fast removal of organic solvent into the external water phase and subsequently a quick polymer precipitation, resulting in a more porous matrix and defective skin surface.

With the exception of solvent-diffusion-controlled morphology, there existed differences between the formulations with various Hb loadings. Consistent with the viewpoints of Gorner et al.[28] and Takada et al,[29] when a relatively high content of Hb was initially available to be accommodated in the same size of nanoparticles, it would prone to forming a heterogeneous matrix. So, from this viewpoint, with the increasing of the Hb concentration from 100 to 300 mg/mL, the internal structure became more compact and dense. Subsequently, the release rate of PEGs from the nanoparticles decreased. But, further increasing the Hb concentration to 400 mg/mL, the release rate of PEGs contrarily rose up. This may be explained as follows: excessive Hb in the internal aqueous phase led to a high osmotic pressure, which drove the Hb into the external aqueous phase and thus came into being as large pores in the matrix.

From the above results, we can find that the most remarkable factor affecting the diffusion behavior of some polymer particles is polymer matrix, especially PEGylated polymer, which has been turned out to have great influence on the surface morphology.[30,31] Considerable researches have previously investigated the effect of PEG chain on the drug/protein release rate. For examples, Park et al.[30] and Matsumoto et al.[32] reported that the mass transfer of the PLA particles was significantly increased with the introduction of PEG chains due to the increase of polymer degradation. Somewhat contradictory to our results is that the diffusion of small molecules was not obviously affected by the mPEG chain length (500, 2000, 5000) (Figure 5), but a high surface density of mPEG effectively reduced the diffusion of small molecules through the particle surface (Figure 6). A potential explanation for this phenomenon involves a diffusion-based transfer model of PEGs rather than a polymer erosion-based model.

As mentioned above, the formation of nanoparticles is probably due to an interfacial instability arising from rapid diffusion of the solvent across the interface and then the polymer precipitation. Since PLA–mPEG copolymers could form nanoparticles with a well-defined multiple core-shell structure in the case of emulsion technique, most mPEG chains are, logically, oriented toward the external aqueous phase.[31] As there was no obvious difference in the particle size of various PLA–mPEG nanoparticles (Table I), changes in the release of PEG markers from the HbPNPs with different polymer matrices maybe related to the density of the mPEG chains on the surface. A larger number of mPEG blocks were incorporated into the PLA polymer; a higher surface coverage of mPEG chain was obtained, with 30 wt % content of mPEG reaching 93% mPEG coating efficiency (Table I). This would provide a "cloud" of hydrophilic steric barrier surrounding the nanoparticle and a shielded particle surface with reduced surface porosity was formed. Thus, the presence of mPEG coating played an important role on the diffusion behaviors of HbPNP. However, it can be seen in Figure 5 that the diffusion of small molecules was not affected by the changed mPEG chain length (500, 2000, 5000). This phenomenon might result from the nearly equal mPEG coating efficiency (84, 85, 85%) (Table I). These results indicated that coating PLA particles with different contents of mPEG was very efficacious in modulating diffusion behavior of small molecules through the particles, rather than those with different mPEG chain lengths. As an oxygen carrier, one potential issue associated with the observed high mPEG coating density on the treated nanoparticle surface was whether this hydrophilic shell thus created might entrap water, provide a diffusion barrier, and thus inhibit oxygen transport from the pulmonary capillary wall to the Hb core and conversely from Hb to the systemic capillary wall. We think this might be negligible, given the nanoscale shell dimensions. Further experiment is now going on in our group and we will report the results in the near future.

Just like other blood substitutes, to maintain a lower metHb level for a sustained time, the fabricated HbPNP had better be protected by the nonenzymatic reduction system consisting of small molecular reducing agents in the plasma.[13,14] Also, when used as probes, PEGs, due to their inherent hydrophilicity and flexibility, may swell in the aqueous solution, thus leading to some deviation from the real value. So, in the experiment, ascorbic acid and reduced glutathione, two important reducing agents in the plasma, were chosen as the models to address the potential of the reducing agents in the plasma to suppress the metHb in HbPNP and also to verify the results obtained by the above PEG probes. The results in Figure 7 demonstrated that reduced glutathione could reduce metHb in HbPNP slowly for a sustained time, while ascorbic acid was rapid and effective during the initial several hours. These results were largely identical to the phenomenon obtained for free metHb reduced by ascorbic acid and reduced glutathione.[13] The molecular weight of ascorbic acid is 176.1 g/mol, smaller than that of PEG200, while molecular weight of reduced glutathione is 307.3 g/mol, between those of PEG200 and PEG400. As a result, ascorbic acid could diffuse freely into PLA–mPEG nanoparticles with mPEG content of 5–30 wt %, while reduced glutathione could not pass through the nanoparticles when mPEG content in polymer matrix reached 30 wt %. In other words, mPEG content between 10 and 30 wt % was determined as a threshold value for optimal small molecules mass transfer. Also, the most optimum PEG surface coverage on liposomes surface was 10% of 5 kDa PEG, as reported by Phillips et al.[33] These results were in good agreement with the prior results in Figure 6, confirming that PEGs could conceivably be used as probes to detect the pore parameter of nanoparticles, also implying that the metHb content in the

prepared HbPNP could be effectively controlled by the reducing compounds in the plasma.

Taken together, the pore size of the HbPNP created by double emulsion solvent diffusion/evaporation method from PLA-based polymers could be successfully modulated from 200 to 600 MWCO. As we know, the substances needed for the functionality of natural RBCs include oxygen, carbon dioxide, the life-sustaining glucose, reducing agents in plasma and metabolic products. Among them, the largest is reduced glutathione with molecular weight of 307 g/mol. That is to say, in view of the practical clinic, the desirable pore size of the HbPNP should be bigger than 307 MWCO, allowing all those life-sustaining substances to diffuse freely. The results described above indicated, using the solvent (DCM or DCM/ACE), stirring speed (700 rpm), Hb concentration (100~400 mg/mL) and PLA-mPEG polymer (mPEG content no more than 10 wt%), the pores between 400 and 600 MWCO were created, which could function as semi-permeability: allowing overpass of all those life-sustaining small molecules, but acting as a barrier to the macromolecules. The comparison of molecular size involved in HbPNP was illustrated in Figure 1(b).

CONCLUSIONS

This study describes an efficacious, nondestructive detecting method, which is based on the definition of MWCO and an effusion approach with a series of low molecular weight PEG as probes, to quantify the porosity and semi-permeability of nanosized polymeric particles as blood substitutes. The MWCO of the HbPNP could be modulated in the range from 200 to 600 by changing the main preparation conditions. PEGylated polymer exhibited the most noteworthy influence on MWCO of HbPNP, which was further demonstrated by the diffusion behaviors of ascorbic acid and reduced glutathione. With the solvent of DCM or DCM/ACE, stirring speed of 700 rpm, Hb concentration from 100 to 400 mg/mL, and PLA–mPEG polymer with mPEG content no more than 10 wt %, the pores prepared were between 400 and 600 MWCO, facilitating the in-and-out exchange of the life-sustaining small molecules. The HbPNPs prepared are considered to have good potential to use as blood substitutes. Further study should be conducted to evaluate the *in vivo* oxygen carrier capability.

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