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Key parameters affecting the initial leaky effect of hemoglobin-loaded nanoparticles as blood substitutes

Xiaolan Zhang · Changsheng Liu · Yuan Yuan · Shiyu Zhang · Xiaoqian Shan · Yan Sheng · Feng Xu

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Abstract In order to realize long-term carrying/delivering oxygen and minimize the adverse effects of free hemoglobin (Hb) in vivo, Hb is desired to be confined in Hb-loaded nanoparticles (HbP), a novel blood substitute with potential clinical applications, and thus functions as the native red blood cells (RBCs). However, the initial burst release of Hb ("leaky effect") greatly underscores the significance of this work. The study described here wants to disclose the key preparative parameters, including polymer, excipients in the inner aqueous phase and solvent profile, affecting the Hb release behavior (the initial 24 h) from HbP fabricated by commonly used solvent diffusion/evaporation double emulsion technique. The results demonstrate that PEGlytated polymers, regardless of two- or tri-block copolymers show slower release compared with the corresponding non-PEGlytated ones. The higher polymer concentration yields lower initial release. PEG200, added as excipient facilitates Hb burst effect to about 38.4%, almost 17% increase compared to the control (~21%), whereas, PVA and Poloxamer188, due to amphiphilic nature, can effectively attenuate this leakage to about 13.0 and 5.1%, respectively. The diffusion/extraction rate from oil phase and the subsequent evaporation rate from the aqueous continuous phase of solvents impose different influences on Hb release. To reduce the burst effect, the initial diffusion/extraction rate should be slow, whereas, the concomitant evaporation rate should be as fast as possible. The results obtained here will be guidance's for the future tailored design of more desirable polymersome nanoparticle blood substitutes.

X. Zhang · C. Liu (✉) · Y. Yuan · S. Zhang · X. Shan · Y. Sheng · F. Xu
Key Laboratory for Ultrafine Materials of Ministry of Education, Engineering Research Center for Biomedical Materials of Ministry of Education, East China University of Science and Technology, Shanghai 200237, China
e-mail: liucs@ecust.edu.cn

X. Zhang
e-mail: zxlpsh@126.com

1 Introduction

The search for artificial blood substitutes has been carried out for many years. Increasing risks of infection, availability of adequate blood stores, and complex blood typing before transfusion, handling and storage have been problems for military and civilian institutions alike. The possibility that artificial blood substitutes, which eliminate many of these concerns will be of immense value to the medical community [1–3]. Stroma free Hb, when injected into body, however, is susceptible to dissociating from tetramer into dimmer structure and rapidly eliminates from the blood circulation, filters by the kidney and induces renal toxicity and other adverse effects [4]. To overcome the problems associated with free Hb, encapsulated Hb, simulating the structure of red blood cells (RBCs) and maintaining Hb in a protected, predominant inner environment [5], has been proposed and developed using a number of techniques [5–7]. In particular, the Hb-loaded nanoparticles (HbP) with about 200 nm size [7], which not only exhibit longer blood circulation compared to the larger-sized particles, but also can pass through blockages, with potential applications in thrombus and cancer therapy [8, 9], represents the new third generation of the blood substitutes.

It is well known that cell-free Hb from RBCs, very low in normal plasma, is not only subject to degradation and heme loss, but also readily scavenges NO, and may induce vessel construction or other adverse effects [10]. It has been turned out that the hypertension, increased systemic and pulmonary vascular resistance, morbidity, and mortality associated with administration of Hb-based blood substitutes have been widely attributed to the enhanced ability of cell-free Hb to scavenge NO [11–13]. So, to perform effective oxygen-carrying function to tissues and minimize the adverse effects in vivo bring forth the need to control the release of the free Hb from the blood substitutes. Unfortunately, up to date, there is little information available in this field, especially for the Hb-loaded polymeric nanoparticles.

In the previous study, our group has, for the first time, developed a novel porous HbP with high encapsulation efficiency (over 80%) and controlled particle size (about 200 nm) by a modified five-step solvent diffusion/evaporation double emulsion technique [7]. As an desired Hb-encapsulated blood substitute, it is anticipated that Hb can be restricted in HbP, just like the native RBCs. However, the high surface-to-volume ratio of nanosize and porous structure lead to above 20% of the entrapped Hb releases in the initial period 10 h (Fig. 1), a typical "leaky" phenomenon, which significantly offsets the oxygen-delivering capacity and have potential toxicity in vivo. Thus, controlling and attenuating this "leaky" effect become the major challenge in our next step.

The previous investigations have revealed that many factors, such as the polymer composition, molecular weight [14], polymer crystallinity [15], polymer and surfactant concentration [16], preparation temperature [17], additives in inner aqueous phase [18], and drug/protein loading [19] as well, have obvious effects on drug/protein release profile. Herein, the present study we want to extend the study and explore the effects of key preparative parameters on the Hb initial release (24 h), and thus find favorable strategies to attenuate Hb leakage for the future tailored design of desired polysome blood substitute.

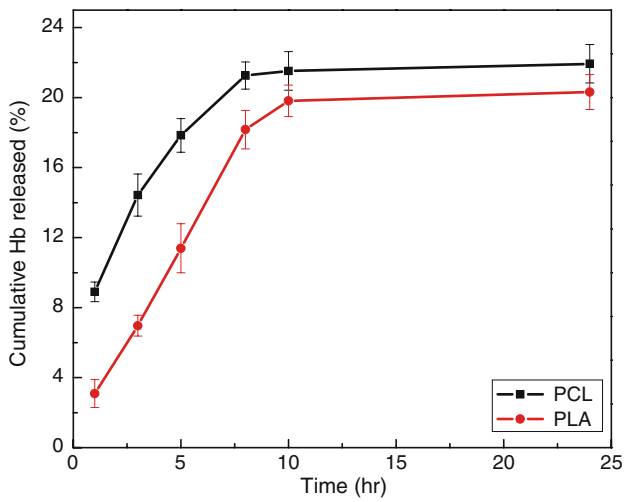

Fig. 1 The initial release (24 h) of hemoglobin-loaded PCL and PLA HbP

2 Materials and methods

2.1 Materials

Bovine Hb (64.5k MW) in lyophilized form is purchased from Yuanju Biotechnology Company, Shanghai. Polyvinyl alcohol (PVA) (1750 ± 50 DP, 87–89% hydrolyzed) is obtained from Sinopharm Chemical Reagent Co. Ltd., Shanghai. Poloxamer188 is purchased from BASF Corporation. Poly(ε-caprolactone) (PCL), poly(ε-caprolactone-ethylene glycol) (PCL-PEG), poly(DL-lactide) (PLA) and PEG-PLA-PEG are obtained from Chengdu Institute of Organic Chemistry, Chinese Academy of Science, China. The composition and molecular weight are characterized according to the specification of the manufacturer (Table 1). All other reagents are of analytical grade.

2.2 Preparation of hemoglobin-loaded nanoparticles (HbP) and characterization

HbP is prepared by a modified five-step solvent diffusion/evaporation double emulsion (w/o/w) technique. Briefly, 0.5 ml Hb solution (150 mg/ml) as inner aqueous phase is emulsified in 5 ml of organic solvent containing 10 mg polymer (oil phase) by ultrasonic (JYD-900, Zhixin Instrument Co. Ltd., Shanghai) for 15 s. Thereafter, the primary emulsion is poured into aqueous solution followed by two steps of re-emulsification by a high-speed homogenizer (WX500CY, Shanghai Weiyu company, China) for 15 s and 2 min, respectively. The double emulsion is subsequently added to 200 ml aqueous continuous phase

Table 1 The characterization data of the polymers used

Polymer	PEG MW (Dal)	PEG content (%)
PCL (45k)	0	0
PCL (45k)-PEG	6k	10
PCL (45k)-PEG	6k	30
PCL (45k)-PEG	20k	10
PLA (48k)	0	0
PEG-PLA (48k)-PEG	5k	5
PEG-PLA (48k)-PEG	5k	15
PEG-PLA (48k)-PEG	5k	30

solution to remove the solvents under stirring. The nanoparticles are finally collected by centrifugation, washed three times with deionized water, and lyophilized using a freeze-drier (FD-3, Beijing Boyikang instrument company, China). The detailed preparation procedures can be found in the previous report [7]. The morphology and size of the nanoparticles is observed by transmission electronic morphology (JEM-2010, Japan), size and size distribution is studied by dynamic light scattering analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd.).

2.3 In vitro release study

2.3.1 Hb concentration determination

Hb molecule has three conformational states, including oxyhemoglobin, deoxyhemoglobin and the oxidized state of methemoglobin. The stroma free Hb, in general, coexhibits the three states. The standard cyanomethemoglobin method to assess Hb concentration, however, shows limitations in repeatability, especially when Hb concentration is low. So a standard calibration curve of Hb concentration versus optical absorption is established at the wavelength of 523 nm, which is the isobestic point for the three conformational states of Hb independent of pH [20], to determine the amount of released Hb. The obtained curve of linearity range is very well ($R^2 > 0.9999$).

2.3.2 In vitro release study of HbP

The lyophilized nanoparticles (ca. 50 mg) are suspended in 5 ml of phosphate buffer (pH 7.4), and the suspensions are incubated at 37°C under continuous orbital rotation at 100 rpm. At predetermined intervals (1, 3, 5, 8, 10 and 24 h), the suspension is centrifuged and 4 ml of the supernatant is withdrawn, and subjected to a spectrophotometer to determine the Hb concentration at 523 nm using the established calibration curve. The same volume of fresh buffer is added back to the suspension. The in vitro release analyses for each sample are carried out in triplicates. The percentage of cumulative Hb released with respect to the amount of Hb encapsulated is investigated as a function of incubation time.

3 Results and discussion

Generally speaking, for a micro-/nano-particle delivery system, drug/protein release may undergo the following three stages: (i) disassociation or desorption of drug/protein located on or near the particle surface; (ii) diffusion through pores or channels formed during the particle solidification; (iii) The release as consequence of erosion and degradation of polymer matrix. It can be deduced that the former two stages may account for the initial drug/protein release. Thus, to attenuate the initial burst effect, on the one hand, drug/protein should be restricted in the inner core instead of on the surface association or adsorption. On the other hand, the porosity and pore size should be controlled to cut off the entrapped drug/protein.

HbP, with uniform spherical shape and an average diameter about 200 nm characterized by TEM image and size distribution (Fig. 2) fabricated by the modified double emulsion technique in our experiment, is accompanied with built-in connecting porous structure, which is designed to function as semipermeable membrane and mimic an active in-and-out exchange with outer environment like RBCs, allowing the small molecules such as O_2 and CO_2, the life-sustaining glucose in plasma and metabolic products to diffuse into/out the system. The reducing agents like ascorbic acid and glutathione present in plasma can also successfully transfer into the system to suppress Hb oxidation. The schematic description is shown in Fig. 3. However, the availability of connecting porous channels and nanosize of HbP facilitate Hb leakage, which, in fact, set a great obstacle to the development of Hb-loaded

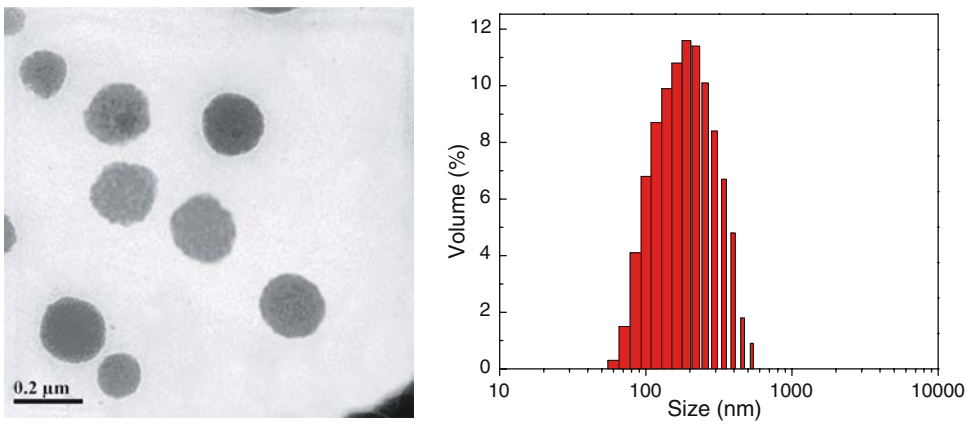

Fig. 2 TEM image and the size distribution of Hb-loaded nanoparticles (HbP)

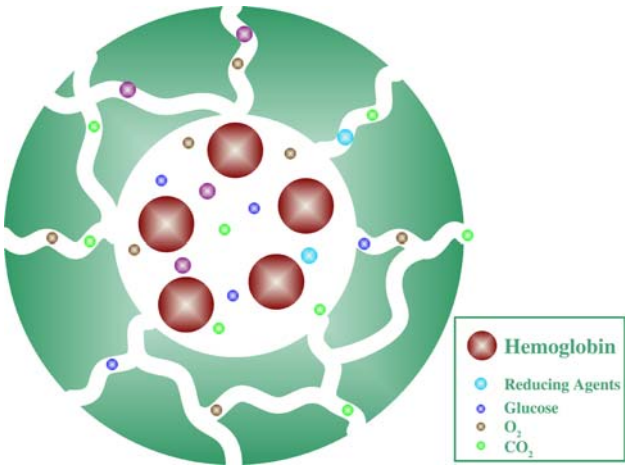

Fig. 3 Schematic representation of HbP structure

nanoparticle blood substitutes. The leaky behavior of Hb is mainly correlated with the preparation process. However, few studies are found focus on describing the process-related aspects affecting the release of Hb encapsulated. So, in this work, some key fabrication parameters including polymer, excipients added in inner aqueous phase and solvents are investigated on the basis of the preliminary work. In the previous investigations, 24 h have always been selected for the initial release of drug/protein [18], but from the Hb release patterns in our study (Figs. 4–10), it can be seen that the Hb, released from the HbP, monotonically increases with increasing release time up to 10 h, and slightly increases or remains constant afterwards, indicating that the leaky effect mainly takes place in the initial 10 h. So, the following leaky effect is investigated and compared in the initial 10 h.

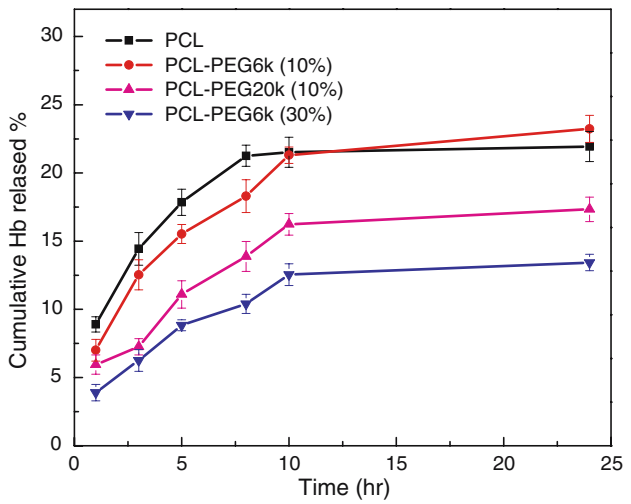

Fig. 4 Effects of PCL and PEGylated PCL copolymers on the Hb initial release

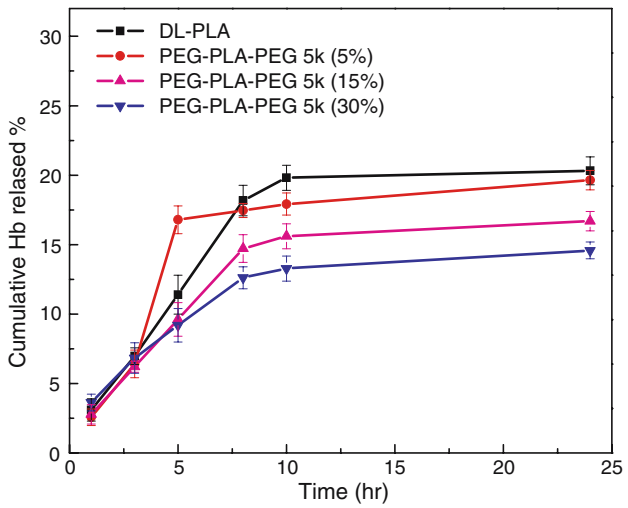

Fig. 5 Effects of PLA and PEGylated PLA copolymers on the Hb initial release

3.1 Effect of the composition of polymer on the HbP release

The polymer properties in large part determine the particle surface structure, which in turn determines the drug release. In this part, the effects of the PEGylation on the release profile are investigated with the PCL and PEGylated PCL two-block copolymer, PLA and PEGylated PLA tri-block copolymer. The results are shown in Figs. 4 and 5, respectively. From the Fig. 4, it can be seen that hydrophilic PEG segments induce a dramatic reduction of the protein release, but the extent of reduction varies depending on the molecular weight and content of PEG. In the case of PCL, the cumulative released Hb during 10 h is about 21.52%. But after PEGylation with PEG 6k (10%), PEG 20k (10%) and PEG 6k (30%), the cumulative

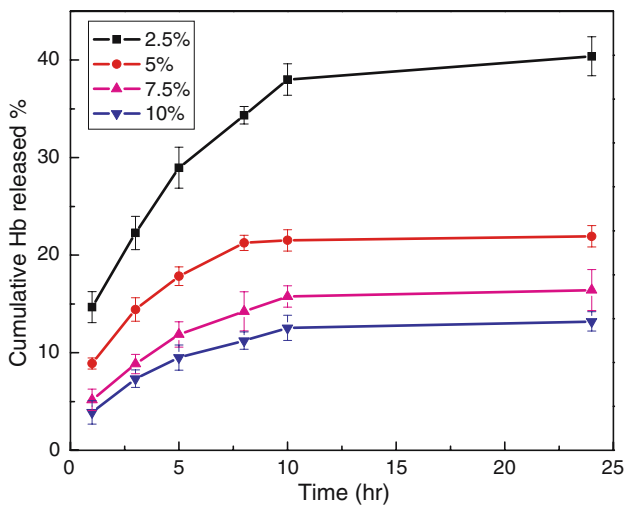

Fig. 6 Effects of polymer concentration on the Hb initial release

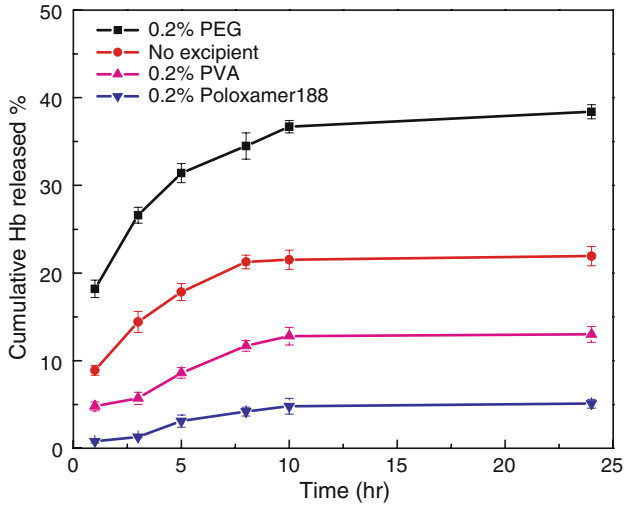

Fig. 7 Effects of excipients added in inner aqueous phase on the Hb initial release

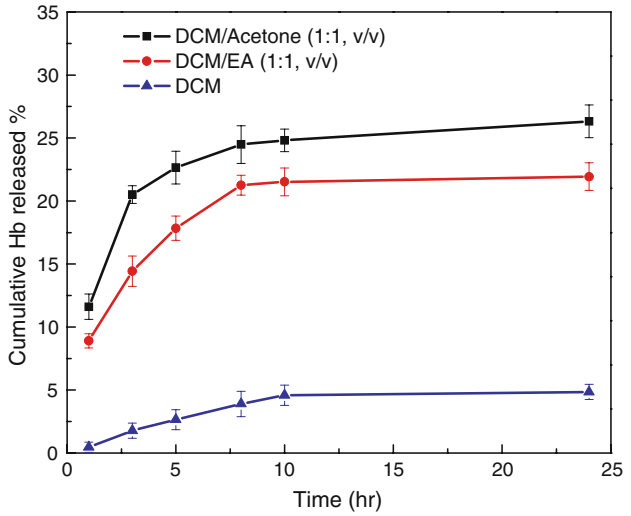

Fig. 8 Influence of solvent composition on Hb initial release

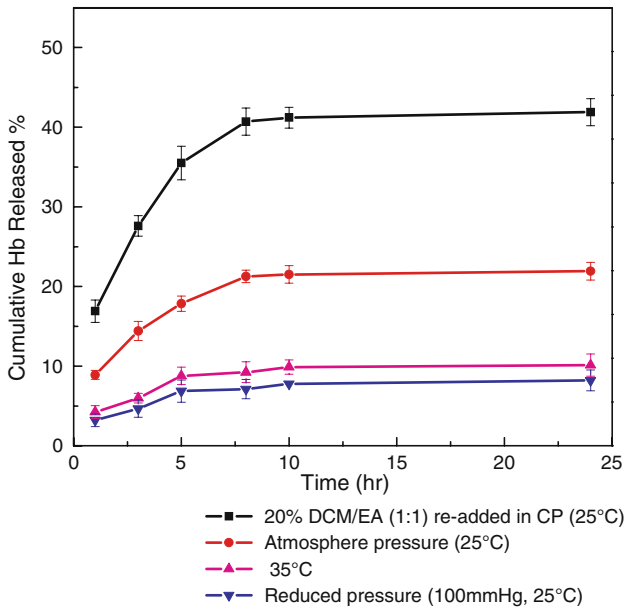

Fig. 9 Influence of solvent evaporation profile on Hb initial release

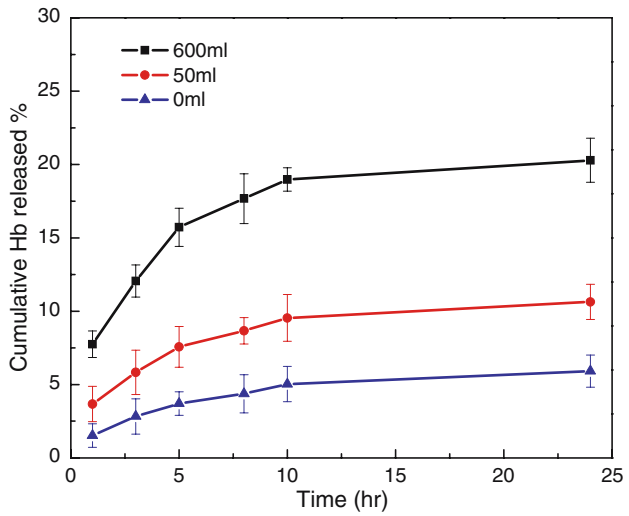

Fig. 10 Influence of aqueous continuous phase volume on Hb initial release

released Hb is 21.30, 16.23 and 12.54%, respectively. This phenomenon is further confirmed by the results in Fig. 5. For example, PLA as polymer matrix leads to 19.81% Hb release during 10 h, while 13.28% for PEG-PLA-PEG (30%). The results above indicate that PEGylated copolymers can effectively suppress the Hb release, irrespective of two- or tri-block copolymers. This is somewhat contradictory to other previous researchers that drug release rate enhanced with the increase of PEG length [21] or other hydrophilic segment contents [22]. The sharply opposite results achieved may arise from the different drug nature and preparation methods. They encapsulated hydrophobic drugs by nanoprecipitation [21], with comparison to our hydrophilic protein and double emulsion fabrication. It is believed that hydrophilic PEG chains would orient not only towards the external aqueous medium but also towards the inner aqueous phase. Thus, the protein reservoirs would be theoretically surrounded by a PEG barrier [23], which would restrict Hb inside to form core-loading, and thereby increase the difficulty for Hb to diffuse out.

3.2 Effect of the polymer concentration on the HbP release

Varying PCL concentration in oil phase, the 10 h release is about 38.0% in the case of 2.5%, while 12.54% Hb releases when 10% polymer is employed (Fig. 6). Increasing the polymer concentration obviously yields more denser wall

structure, which can account for the slow release. On the other hand, the higher polymer concentration, the more viscous oil phase, and thus more difficult for water molecules to diffuse into the polymer matrix to lessen the formation of interconnecting channels or paths, which may also contribute to the lower release [16, 24], However, when the polymer concentration is over 10%, it is difficult to disperse the oil phase. To better understand the underlying release mechanism, PCL was used and fixed 5% in oil phase in the following study.

3.3 Effect of excipients added in inner aqueous phase on the HbP release

It was reported that excipients in inner phase would affect the stability of multiple emulsion, encapsulation and subsequently drug release [25, 26]. In this part, PEG200, PVA and Poloxamer188 are selected as the excipients in inner phase and coencapsulated with Hb (fixed with 0.2% in inner aqueous phase). As shown in Fig. 7, after 10 h incubation of the nanoparticles, about 36.7% of total Hb entrapped releases when PEG200 is used as excipient, with comparison to the marked decrease release about 12.8 and 4.8% when PVA and Poloxamer188 added, respectively. Addition of PEG200 facilitates Hb leakage compared to the control group (no excipient), while PVA and Poloxamer188 significantly attenuate this burst effect. It is well known that PVA tends to adsorp onto the microsphere surface despite of repeatedly washing procedures when PVA used in the continuous phase of the secondary emulsion [27]. Thus, it is reasonable to deduce that PVA also coats the inner pores and/or exits within the polymer matrix [28, 29]. Poloxamer188 and PVA are both amphipathic surfactants with high molecular weight, and tend to align themselves at the water/oil interface [30], stabilizing and restricting the entrapped Hb to form core-loading [16, 31], which may account for the lower release extent during the in vitro experiments. Whereas PEG200 shows limitation in stabilization the protein due to its more soluble nature in water and small molecular weight, and is susceptible to drain out from the inner phase during the multiple emulsion formation, which may leave more soluble channels and facilitate Hb migration to the particle surface, thus enhance the Hb leakage. The similar accelerating drug release was reported for PEG 400 added in inner aqueous phase [32].

3.4 Effect of the elimination rate of organic solvents on the HbP release

Solvent removal leads to polymer precipitation and then particle solidification. Thus the elimination manner directly determines the physiochemical properties of the particle structure, which then influences the drug release profile. Sato et al. [33] and Yang et al. [34], for example, have reported that high evaporation rate of solvent caused rapid drug release. Luan et al. [35] also suggested that fast exchange rate of solvent–nonsolvent (water) resulted in porous particles, and higher drug release. In contrast, Chung et al. [36] showed that higher solvent evaporation rate led to a slower drug release. This surprising finding warrants to further examination of solvent effects.

It is believed that, during the double emulsion process, 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). The subsequent step is the evaporation from the CP/air interface to environment. Then, in order to separatively identify the influence of the above two different steps, two sets of experiments are carried out. In the first set of experiment, various solvent compositions, including dichloromethane (DCM), DCM/ethyl acetate (EA) (1:1, v/v) and DCM/acetone (1:1, v/v) are employed to obtain various solvent diffusion/extraction rates. In the second set of experiment, the solvent diffusion/extraction is kept at the same condition and solvent evaporation rate is modulated with different temperatures and different pressures as following: atmosphere pressure at 25°C as control group, atmosphere pressure at 35°C, reduced pressure (100 mmHg) at 25°C.

As shown in Fig. 8, when the DCM, DCM/EA and DCM/acetone are applied, the 10 h cumulative Hb release is 4.59, 21.52 and 24.81%, respectively. In contrast with DCM used only, an obvious burst release occurs along with addition of EA or acetone into DCM. DCM is a popular organic solvent due to its excellent solvency, which can dissolve large amount of polymer, 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 nonsolvent (water), a relative slow diffusion/extraction rate. We believe that slow diffusion/extraction rate of DCM sets a relatively "undisturbed" environment for polymer precipitation and the embryonic particle formation, which tends to form smooth and compact structure [35]. Due to the miscible nature of EA (8.7%, w/v) and acetone with water, the addition of EA or acetone accelerates the exchange rate of solvent–nonsolvent, especially in the case of acetone, where the "disturbed" condition may lead to coarse and porous structure. On the other hand, more soluble cosolvents such as EA or acetone favor more water uptake and leave more aqueous channels or paths. The more water entrapped inside the particles, the more porous the particles will be. The addition of miscible solvents is the porosity precursors. Furthermore, the fast diffusion/extraction rate

of solvent may facilitate the inner Hb drain or migrate to the particle surface. These factors are interrelated in generating higher Hb release.

Figure 9 illustrates the control group leads to about 21.52% Hb release during 10 h. While, increase of evaporation temperature decreases Hb release (about 9.89%). The lowest release is observed for the group under the reduced evaporation pressure, where about 7.78% of the entrapped Hb is released during 10 h. Higher temperature or lower pressure both accelerate solvent evaporation rate [37]. As can be seen, Hb leakage is greatly suppressed other than facilitated when the solvent evaporation rate is accelerated. The fast solvent evaporation rate results in attenuated Hb leakage, which is found to be opposite to the effect of solvent diffusion/extraction rate on Hb release.

To our great knowledge, the initial diffusion/extraction rate of solvent from the oil phase determines the embryonic particle structure, wherein slow exchange rate tends to form denser structure because the polymer deposits under a relatively "static" condition, which may lead to fewer water molecules penetrating the polymer matrix, and thus less soluble channels available. The extracted solvent in CP, however, should be eliminated quickly as possible, because the presence of solvents can delay polymer precipitation and hinder the embryonic particle to proceed in solidification. In the mean time, nascent particles may be further eroded by the co-existed solvent. Furthermore, the delayed solidification of particles may facilitate Hb to drain to the particle surface. These factors work together to facilitate the Hb leakage. The adverse effect of delayed solvents can be further confirmed by re-addition of 20% solvents into the multiple emulsion suspension during the solvent evaporation process. The obvious Hb leakage about 41.2%, much higher than that in the control group (about 21.52%) occurs when the prolonged residence of solvents present (Fig. 9).

To further verify the above hypothesis, aqueous continuous phase (CP) volumes are varied, because CP volume can simultaneously modulate the solvent diffusion/extraction rate and evaporation rate. The aqueous continuous phase (CP) volumes are varied with 600, 50 and 0 ml. As shown in Fig. 10, 600, 50 and 0 ml CP result in the 10 h release about 18.98, 9.54 and 5.04%, respectively. It can be seen that the more CP used, the higher extent of Hb release occurs. Under the extreme condition (no CP present), the solvent diffusion/extraction rate greatly decreases, while the evaporation rate increases due to the shorter path for solvent expulsion. Therefore, the burst effect is reduced. Whereas, an extremely low concentration of solvent in CP when large amount of CP (600 ml) employed, provides a good concentration driving force for solvent diffusion/extraction, which tends to form more porous particle structure, and thereby higher protein release.

Taken together, solvent elimination rate plays an important role on the Hb-loaded nanoparticle release profile. The literatures available mostly emphasize the effect of solvent evaporation rate on the characterization of particles or drug release [33, 34, 36]. Here, it is found that the diffusion/extraction rate and the subsequent evaporation rate impose different influences on the protein release. From the experimental data, it is inferred that, to attenuate the burst leakage, the initial diffusion/extraction rate of solvent from the oil phase should be slow, which tends to form dense embryonic particles, while the concomitant evaporation in CP should be fast as possible to minimize the adverse influence of the solvent.

4 Conclusions

In the present study, the preparative parameters affecting the initial burst release of Hb-loaded nanoparticles are investigated. The polymer grafted PEG segment and higher concentration contribute to reduced Hb release. Excipients added in inner aqueous phase demonstrate different effects on release. Addition of PEG200 facilitates Hb burst release, whereas, PVA and Poloxamer188 show obvious attenuation of this leakage. Solvent elimination rate imposes two aspects on the protein release: diffusion/extraction rate from the oil phase to aqueous phase and the subsequent evaporation rate. The results illustrate, to attenuate the Hb leaky effect, the initial diffusion/extraction of solvent should be slow, while the concomitant evaporation should be fast as possible.

Although the lowest initial release is still over the limit requirement for cell-free Hb in normal human plasma in this work, from the Hb release pattern, the leaky effect mainly takes place in the initial 10 h. How to exactly modulate the pore size of nanoparticles and the protein/drug distribution, indeed, is some difficult, but unveiling the preparative variables governing the release behavior, and fundamental understanding of the relationship amongst these key parameters and the protein drug release mechanisms may be guidance's in the future design of more desirable polymersome nanoparticle blood substitutes or other drug delivery systems.

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References

1. R.M. Winslow, Adv. Drug. Deliv. Rev. **40**, 131 (2000)
2. C. Chauvierre, M.C. Marden, C. Vauthier, D. Labarre, P. Couvreur, L. Lecler, Biomaterials **25**, 3081 (2004)

3. S.L. Li, J. Nickels, A.F. Palmer, Biomaterials **26**, 3759 (2005)
4. V. Budhiraja, J.D. Hellums, Microvasc. Res. **64**, 220 (2002)
5. T.M.S. Chang, J. Intern. Med. **253**, 527 (2003)
6. F.T. Meng, G.H. Ma, Y.D. Liu, W. Qiu, Z.G. Su, Colloid Surf. B: Biointerf. **33**, 177 (2004)
7. J. Zhao, C.S. Liu, Y. Yuan, X.Y. Tao, X.Q. Shan, Y. Sheng, F. Wu, Biomaterials **28**, 1414 (2007)
8. A. Gabizon, D. Papahadjopoulos, Proc. Natl. Acad. Sci. **85**, 6949 (1988)
9. N. Iodoshima, C. Udagawa, T. Ando, H. Fukuyasu, H. Watanabe, S. Nakabayashi, Int. J. Pharm. **146**, 81 (1997)
10. C.D. Reiter, X.D. Wang, J.E. Tanus-Santos, N. Hogg, R.O. Cannon, A.N. Schechter, M.T. Gladwin, Nat. Med. **8**, 1383 (2002)
11. R. Lee, K. Neya, T.A. Svizzero, G.J. Vlahakes, J. Appl. Physiol. **79**, 236 (1995)
12. J.S. Olson, E.W. Foley, C. Rogge, A.L. Tsai, M.P. Doyle, D.D. Lemon, Free Radic. Biol. Med. **36**, 685 (2004)
13. K. Sampei, J.A. Ulatowski, Y. Asano, H. Kwansa, E. Bucci, R.C. Koehler, Am. J. Physiol.: Heart Circ. Physiol. **289**, H1191 (2005)
14. F.D. Cui, K. Shi, L.Q. Zhang, A.J. Tao, Y. Kawashima, J. Control Release **114**, 242 (2006)
15. S. Ghosh, J. Chem. Res. **4**, 241 (2004)
16. V. Coccoli, A. Luciani, S. Orsi, V. Guarino, F. Causa, P.A. Netti, J. Mater. Sci.: Mater. Med. doi:10.1007/S10856-007-3253-9
17. Y.Y. Yang, H.H. Chia, T.S. Chung, J. Control Release **69**, 81 (2000)
18. J. Wang, B.M. Wang, S.P. Schwendeman, Biomaterials **25**, 1919 (2004)
19. A.K. Bajpai, S. Bhanu, J. Mater. Sci.: Mater. Med. **18**, 1613 (2007)
20. W.G. Zijlstra, A. Buursma, Comp. Biochem. Physiol. **118b**, 743 (1997)
21. Y. Zhang, R.X. Zhuo, Biomaterials **26**, 6736 (2005)
22. Z.P. Zhang, S.S. Feng, Biomaterials **27**, 4025 (2006)
23. Y.P. Li, Y.Y. Pei, Z.H. Zhou, X.Y. Zhang, Z.H. Gu , J. Ding, J.J. Zhou, X.J. Gao, J. Control Release **71**, 287 (2001)
24. D. Klose, F. Siepmann, K. Elkharraz, S. Krenzlin, J. Siepmann, Int. J. Pharm. **314**, 198 (2006)
25. P. Johansen, Y. Men, R. Audran, G. Corradin, H.P. Merkle, B. Gander, Pharm. Res. **15**, 1103 (1998)
26. Y.K. Katare, A.K. Pand, Eur. J. Pharm. Sci. **28**, 179 (2006)
27. K.M. Shakesheff, C. Evora, I. Soriano, R. Langer, J. Colloid Interf. Sci. **185**, 538 (1997)
28. C. Bouissou, J.J. Rouse, R. Price, C.F.V. Walle, Pharm. Res. **23**, 1295 (2006)
29. F. Boury, T. Ivanova, I. Panaiotov, J.E. Proust, A. Bois, J. Richou, J. Colloid Interf. Sci. **169**, 380 (1995)
30. L. Mu, S.S. Feng, J. Control Release **80**, 129 (2002)
31. M.D. Blanco, M.J. Alonso, Eur. J. Pharm. Biopharm. **43**, 287 (1997)
32. J.M. Péan, F. Boury, M.C. Venier-Julienne, P. Menei, J.E. Proust, J.P. Benoit, Pharm. Res. **16**, 1294 (1999)
33. T. Sato, M. Kanke, H.G. Schroeder, P.P. Deluca, Pharm. Res. **5**, 21 (1988)
34. Y.Y. Yang, T.S. Chung, X.L. Bai, W.K. Chan, Chem. Eng. Sci. **55**, 2223 (2000)
35. X.S. Luan, M. Skupin, J. Siepmann, Int. J. Pharm. **324**, 168 (2006)
36. T.W. Chung, Y.Y. Huang, Y.Z. Liu, Int. J. Pharm. **212**, 161 (2001)
37. Y.Y. Yang, H.H. Chia, T.S. Chung, J. Control Release **69**, 81 (2000)