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The effect of cationic starch on hemoglobin, and the primary attempt to encapsulate hemoglobin

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Abstract

Though starch has been a common material used for drug delivery, it has not been used as an encapsulation material for hemoglobin-based oxygen carriers. In this study, cationic amylose (CA) was synthesized by an etherification reaction. The interaction behaviors between CA and hemoglobin (Hb) were measured by zeta potential, size, and UV-Vis absorption spectra at different pH values. Cationic starch encapsulated Hb by electrostatic adhesion, reverse micelles, and cross-linking, and showed a core shell structure with a size of around 100 nm, when measured immediately after dispersing in PBS solution. However, we found that it was prone to swell, aggregate, and leak Hb with a longer duration of dispersal in PBS.

Keywords: artificial cells, blood, micelles, nanoparticle, starch

Introduction

Hemoglobin (Hb) has the ability to carry oxygen to tissue, but it cannot be injected into the body directly. Without the adjustment of 2,3-DPG, the oxygen affinity of Hb increases, and this induces insufficient delivery of oxygen to the tissue. Meanwhile, free Hb is apt to depolymerize into a dimer, which poisons the kidney. In addition, without the protection of the cell membrane and reductases, Hb tends to be oxidized into methemoglobin, which loses the ability to carry oxygen, and produces free radicals. Because of the above reasons, Hb has been transformed into several generations of Hb-based oxygen carriers (HBOCs). The first generation of HBOCs consisted of modified Hb, including polyhemoglobin, conjugated Hb, crosslinked tetrameric Hb, and recombinant human Hb (Squires 2002, Chang 2007). The second generation of HBOCs consisted of polySFHb-SOD-CAT, which crosslinked Hb, SOD, and CAT with glutaraldehyde (D'Agnillo and Chang 1997). Compared with the first generation of HBOCs, polySFHb-SOD-CAT not only prevented itself from oxidization, but also cleared free radicals and avoided ischemic reperfusion injury (Chang et al. 2004a, 2004b). The third generation of HBOCs were developed with the addition of carbonic anhydrase to form polySFHb-SOD-CAT-CA (Bian et al. 2012, Bian and Chang 2015). The nanoencapsulation of HBOCs was nanocapsulated Hb, the membrane protected the Hb from contact with surrounding tissue, and thus prevented the vasopressor effect and colloid osmotic pressure effect, had longer circulation time in vivo, eliminated the modification of Hb, and the nanosized particles delivered oxygen through the microvascular network to tissue (Chang 2007, Gao et al. 2013).

The materials encapsulated were mainly lipids and polymers. The lipids comprised phospholipids, cholesterol, fatty acids, and so on. Hb-liposomes, with a size of 250 nm, have received good evaluations in terms of safety and oxygen-carrying capacity in animals, and can even eliminate vasoconstriction (Sakai et al. 2004). However, it was found that glucose and reducing agents could not permeate the Hb-liposomal layer, hindering normal energy metabolism and increasing methemoglobin. Excessive methemoglobin weakened the oxygen-carrying capacity despite an increase in circulation time. Moreover, large amounts of encapsulation materials, such as lipids and cholesterol, become the resources for peroxidation, resulting in a decrease in the phagocytic function of the reticuloendothelial system. Polymers have similar amphiphilicity, without lipid inclusion and much larger molecular weights, compared with the lipids in liposomes. Polymersomes also have better permeability for larger molecules and effectively avoid peroxidation through lipids. Moreover, polymersomes may be designed with various functional properties, such as mechanical properties, permeability, biocompatibility, and stimuli-responsivity, by varying the polymers they contain. Most of the polymers in the preparation of encapsulated HBOCs are synthetic. However, the long-term effects of

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these polymers on the human body have not yet been confirmed. Thus, only a limited number of polymers have been approved by the Food and Drug Administration and other regulatory bodies (LoPresti et al. 2009). In contrast, many natural polymers and their derivatives have been widely used in drug delivery systems, namely, starch, collagen, chitosan, and so on (Degim and Celebi 2007). Although a limited number of reports have been found on the use of natural polymers for encapsulated HBOCs, these materials are potentially good candidates for encapsulated HBOCs because of their excellent biocompatibility and biodegradability (Gao et al. 2011).

Starch, which is a natural biopolymer, is abundant, cheap, and biocompatible. As a drug delivery material, starch has been easily fabricated into various forms of carriers for controlled drug delivery. The isoelectric point of Hb (pI) is in the range of 6.8–7.0, so Hb shows a negative charge. To encapsulate the Hb, cationic starch could be tried. The interaction behaviors between chitosan and Hb have been reported, and chitosan can obviously associate with Hb to form a protein–chitosan complex, which affects the microstructure of Hb (Chen and Liu 2008). In this study, we will illustrate the effect of cationic starch on Hb, and primarily attempt to encapsulate bovine Hb with cationic starch (CSBH).

Materials and methods

Materials

Glycidyltrimethylammonium chloride (GTAC), bovine Hb, and AOT were purchased from the Sigma-Aldrich Trading Co. Ltd. (Shanghai, China). Other chemicals and reagents were purchased from Sinopharm Chemical Reagent Co. Ltd (Xi'an, China). Carbon support film on copper was purchased from Beijing Zhongjingkeyi Technology Co. Ltd. All were used without further purification.

Preparation of cationic starch

To prepare cationic starch, 5 g of starch was suspended in 10 mL of water, and 0.45 g of NaOH was dissolved in 10 mL of water. The two were mixed together, and the mixture was stirred for 10 min. Next, 5.6 g of GTAC was added into the mixture and thoroughly stirred in a water bath, at 60°C for 3 h. At the end of the synthesis reaction, 1% of glacial acetic acid was used to neutralize the pH value, and the mixture turned from yellow to white. The cationic starch obtained was separated and washed with 95% ethanol several times. Finally, the cationic starch was dried under 0.1 MPa at 50°C and ground to a powder prior to use.

Measurement of the zeta potential, main peak size and UV-Vis absorption spectra of cationic starch and Hb at different pH values

The zeta potential of 0.25% cationic starch was measured and calculated by intensity using a Malvern Nano-ZS 90, which is based on non-invasive back scatter. The zeta potential and main peak size of the mixture (0.25% cationic starch + 1 mg/mL Hb) were measured and calculated by intensity using a Malvern Nano-ZS 90. The pH value of the mixture was adjusted with 0.5 mol/L of NaOH. The UV-Vis absorption spectra of the mixture at different pH values were determined by UV-Vis Spectrum Spectrometer (Pgeneral, China).

Preparation of CSBH

1. Oil phase: 2.22 g of AOT was dissolved in 10 mL isooctane.
2. Water phase: 1.8 mL of 1% cationic starch was mixed with 0.4 g of Hb, and 0.5 mol/L of NaOH was added until the pH value was 7.6.
3. W/O reversed-phase microemulsion: The water phase was dropped into the oil phase, and stirred for15 min at 37°C.
4. Crosslinking: For each gram of starch, 0.3 ml of epichlorohydrin was added and stirred for 24 h at 37°C.
5. Separation: The reacted solution was centrifuged at 20,000 r/min for 20 min, and the sediment obtained.

The morphology and size of CSBH

CSBH were dispersed in a PBS solution, and dropped immediately and 1 h later, respectively, on carbon support film, and air dried. Then, the morphology was measured using transmission electron microscopy (TEM) (Hitachi, Japan). The size distribution of the sample was measured and calculated by intensity using a Malvern Nano-ZS 90 at each 10 min interval.

Statistical analysis

Test data have been shown as mean ± SD of three independent experiments, except when otherwise indicated. One-way ANOVA was used to analyze the statistically significant differences, followed by the Newman-Keuls post-hoc test wherever appropriate. P values less than 0.05 ($P < 0.05$) were considered statistically significant.

Results

The zeta potential, main peak size, and UV–Vis absorption spectra of the mixture of cationic starch and Hb at different pH values

As shown in Figure 1, the zeta potential of 0.25% cationic starch was 46.50 ± 3.84 mv at 25°C. The zeta potential of the mixture (0.25% cationic starch and 1 mg/mL Hb) was 34.20 ± 3.10 mv. As shown in Table I, the initial pH value of the mixture (0.25% cationic starch and 1 mg/mL Hb) was 4.35, and the zeta potential was 34.20 ± 3.10 mv, the size of the main peak was 351.2 nm. The pH value of the mixture increased with the addition of 0.5 mol/L of NaOH. The zeta potential of the mixture decreased from 34.20 ± 3.10 mv to 16.6 ± 1.30, as the pH increased from 4.35 to 6.82, and it decreased from 25.80 ± 2.02 mv to 16.30 ± 1.27 mv as pH increased from 7.22 to 8.18. The size at main peak of the mixture decreased from 351.2 to 164.6 nm as pH increased from 4.35 to 6.82, and it increased from 138.7 to 186.2 nm as pH increased from 7.22 to 8.18.

As shown in Figure 2, the mixture of cationic starch and Hb was yellow at pH levels > 7 (pH 8.18, 7.92 and 7.22), while it was red at pH levels < 7 (pH 6.82, and 6.32). The UV-Vis

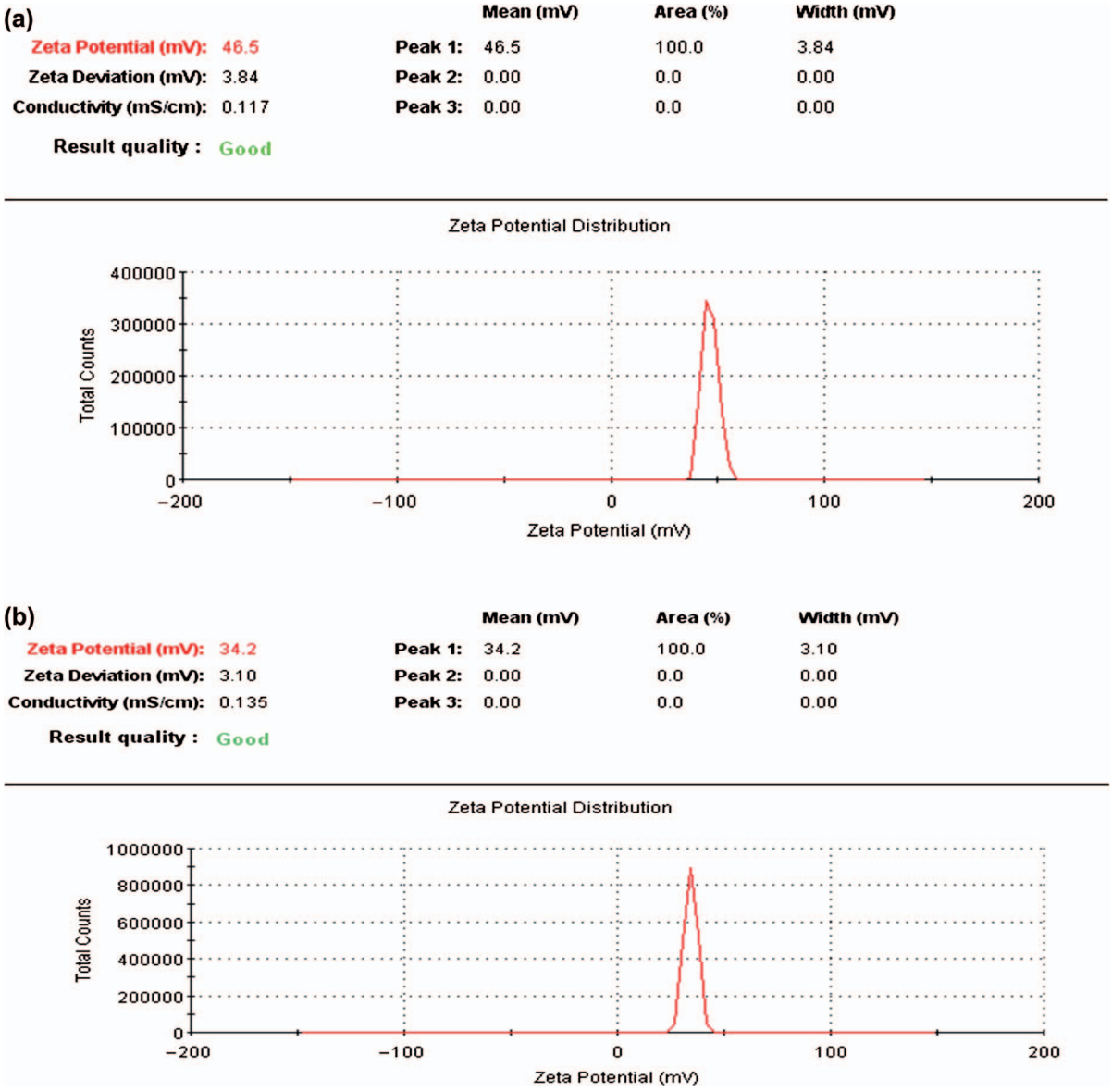

Figure 1. (a) The zeta potential of 0.25% cationic starch, and (b) the zeta potential of the mixture (0.25% cationic starch and 1 mg/mL hemoglobin).

absorption spectra of the mixture were similar at pH levels of 6.82, 6.32, 4.35, and 8.18, which is the characteristic peak of methemoglobin at 500 nm and 632 nm. The UV-Vis absorption spectra of the mixture were similar at pH values of 7.92 and 7.22, which is the characteristic peak of oxyhemoglobin at 541 and 577 nm. Compared with the mixture at other values of pH, the mixture of cationic starch and Hb at pH 7.92 and 7.22 had smaller size at main peak and Hb in mixture was at the state of oxyhemoglobin with the ability of oxyen carrying.

The morphology and size of CSBH

As shown in Figure 3, CSBH showed a core shell structure, with a size around 100 nm, when it was measured immediately after dispersal in PBS solution. After being dispersed in PBS solution for 1 h, the nanoparticles aggregated, with an increase in size, and the core shell structure also disappeared, which illustrated that the CSBH nanoparticles were prone to swell and aggregate, and the Hb leaked from the nanoparticles.

Table I. The zeta potential and the size at main peak of the mixture (0.25% cationic starch and 1 mg/mL hemoglobin) at different pH values.

Initial pH	0.5 mol/L NaOH(μl)	Instant pH	Balanced pH	Zeta potential(mv)	Size at main peak(nm)
4.29	12	8.33	8.18	16.30 ± 1.27	186.2
4.28	10	8.34	7.92	20.70 ± 1.62	155.6
4.27	8	7.24	7.22	25.80 ± 2.02	138.7
4.30	7	6.68	6.82	16.60 ± 1.30	164.6
4.36	5	5.92	6.32	26.00 ± 2.04	201.1
4.35	0	4.35	4.35	34.20 ± 3.10	351.2

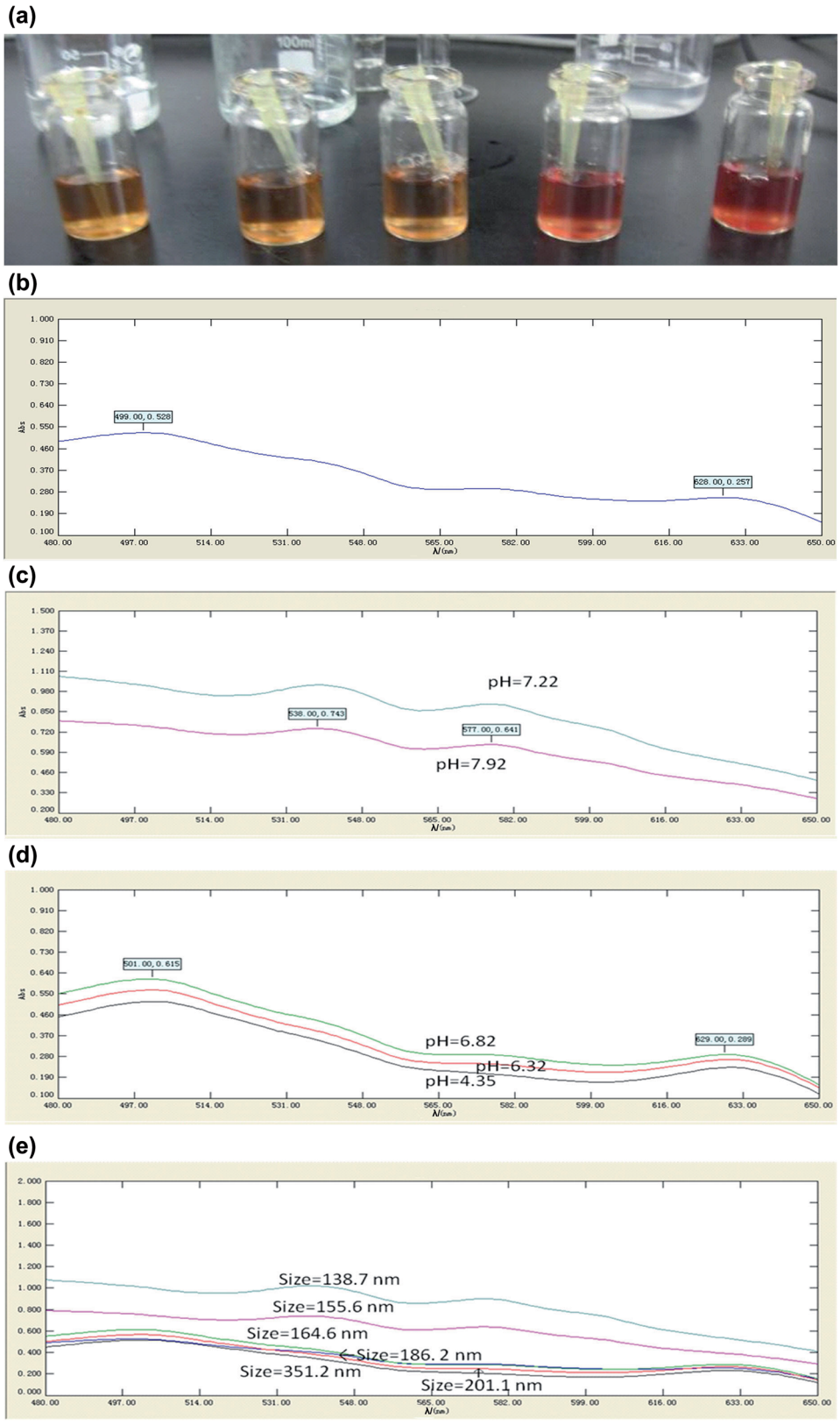

Figure 2. (a) The mixture (0.25% cationic starch and 1 mg/mL hemoglobin) at different pH value (8.18, 7.92, 7.22, 6.82 and 6.32 from left to right), (b) UV-Vis absorption spectrum of cationic starch and hemoglobin at pH 8.18, (c) UV-Vis absorption spectrum of cationic starch and hemoglobin at pH 7.92 and 7.22, (d) UV-Vis absorption spectrum of cationic starch and hemoglobin at pH 6.82, 6.32 and 4.35, (e) UV-Vis absorption spectrum of cationic starch and hemoglobin at above all pH value.

As shown in Figure 4, the size of CSBH was 168.00 ± 0.16 nm (PDI = 0.111), with only the main peak size of 182.70 nm, when CSBH was dispersed in PBS solution immediately. The size of the CSBH became larger with increasing time of immersion. The size was 257.50 ± 0.42 nm at 10 min, which was larger than 200 nm. When CSBH was immersed

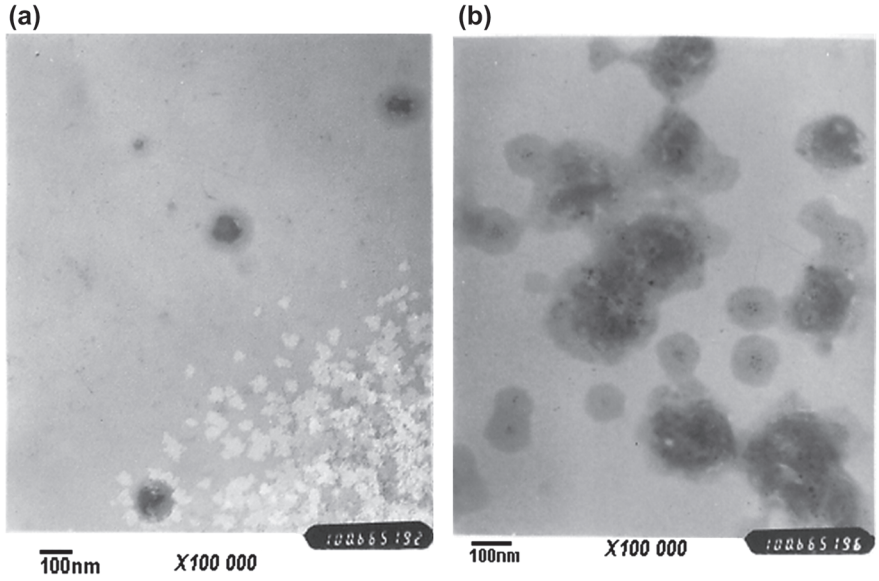

Figure 3. TEM pictures of cationic starch encapsulated hemoglobin without metal spraying under the multiple of 10×105. Cationic starch encapsulated bovine hemoglobin were dispersed in PBS solution and respectively dropped (a) immediately and (b) 1 h later on carbon support film.

in PBS solution for 1 h, the size was 342.80 ± 14.93 nm (PDI = 0.389), with two peaks at 873.70 nm (63.6%) and 214.80 nm (46.4%).

Discussion

The nanoencapsulation of HBOCs confronts us with a doubt on the safety of the encapsulating material. Starch might be a good candidate as HBOC-encapsulating material, for its non-toxicity, biodegradability, biocompatibility, low cost, and long shelf life. In this study, starch was etherified into cationic starch, and the interrelationship between cationic starch and Hb was illustrated by the zeta potential, size, and UV-Vis absorption. The pI of Hb is in the range of 6.8–7.0. When pH>pI, the negative charge of Hb turned stronger with the increase of pH value, the positive charge of mixture turned weaker, and the size of mixture turned larger for electrostatic adherence. On the other hand, when pH<pI, the positive charge of Hb turned stronger with the decrease of pH value, the positive charge of mixture turned stronger, and the size of mixture turned larger for the increase of hydrogen bond between -COOH of Hb and -OH of starch.. The UV-Vis absorption spectra of the mixture showed the characteristic peak of oxyhemoglobin at pH levels of 7.92 and 7.22, and showed the characteristic peak of methemoglobin at pH levels of 6.82, 6.32, 4.35, and 8.18. This illustrated that the unphysiological pH value influenced the hydrophobic microenvironment of Hb, and Fe^{2+} was oxidized into Fe^{3+} by water. The intensity of the spectrum was inverse with the size of mixture, which might be because the interaction turned stronger with the decrease of size, and then the electronic energy became stronger, which induced the absorption intensity of the spectrum to grow stronger. Therefore, when $7.22 < \text{pH} < 7.92$ and zeta potential of the mixture $>20.70 \pm 1.62$ mv, it not only guaranteed the activity of Hb, but also formed the electrostatic adsorption between Hb and cationic starch. Chen and Liu had reported that chitosan can greatly associate with Hb to form chitosan/Hb complexes by hydrogen bonding, electrostatic, and hydrophobic interactions. The association between chitosan and Hb obviously changed the behaviors and microstructure of Hb. The intrinsic UV-Vis absorption and fluorescence intensities of Hb increased with an increase of chitosan concentration. The α-helix in Hb was drawn and changed into β-sheet (Chen and Liu 2008).

Cationic starch-encapsulated bovine Hb was prepared in a water/AOT/isooctane reverse micelle system that contained nanosized water droplets which supplied a moderate environment, and controlled the size of the nanocapsule. The Hb was located in the center of the water droplet, whereas the cationic starch was in the outer layer. The Hb and cationic starch were arranged according to the different levels of hydrophilicity and the charges of Hb, cationic starch, and AOT, which further guaranteed the isolation of Hb from the organic solvent, thereby maintaining the activity of Hb. Additionally, cross-linked cationic starch also formed a strongly linked network to firmly encapsulate Hb and maintain the size of the nanocapsule. Cationic starch-encapsulated Hb showed a core shell structure with a size of around 100 nm, when it was measured immediately after dispersal in PBS solution. This illustrated that the preparation method agreed with our design. While the nanocapsule was apt to swell, it also aggregated and leaked Hb with the increasing time of immersion. When the size exceeds 200 nm, the nanocapsules would be eliminated by the reticuloendothelial system (Nan 2007). In the meanwhile, the Hb molecules that are free from the nanocapsule are directly filtered by the kidney, which will induce renal toxicity. Thus, the

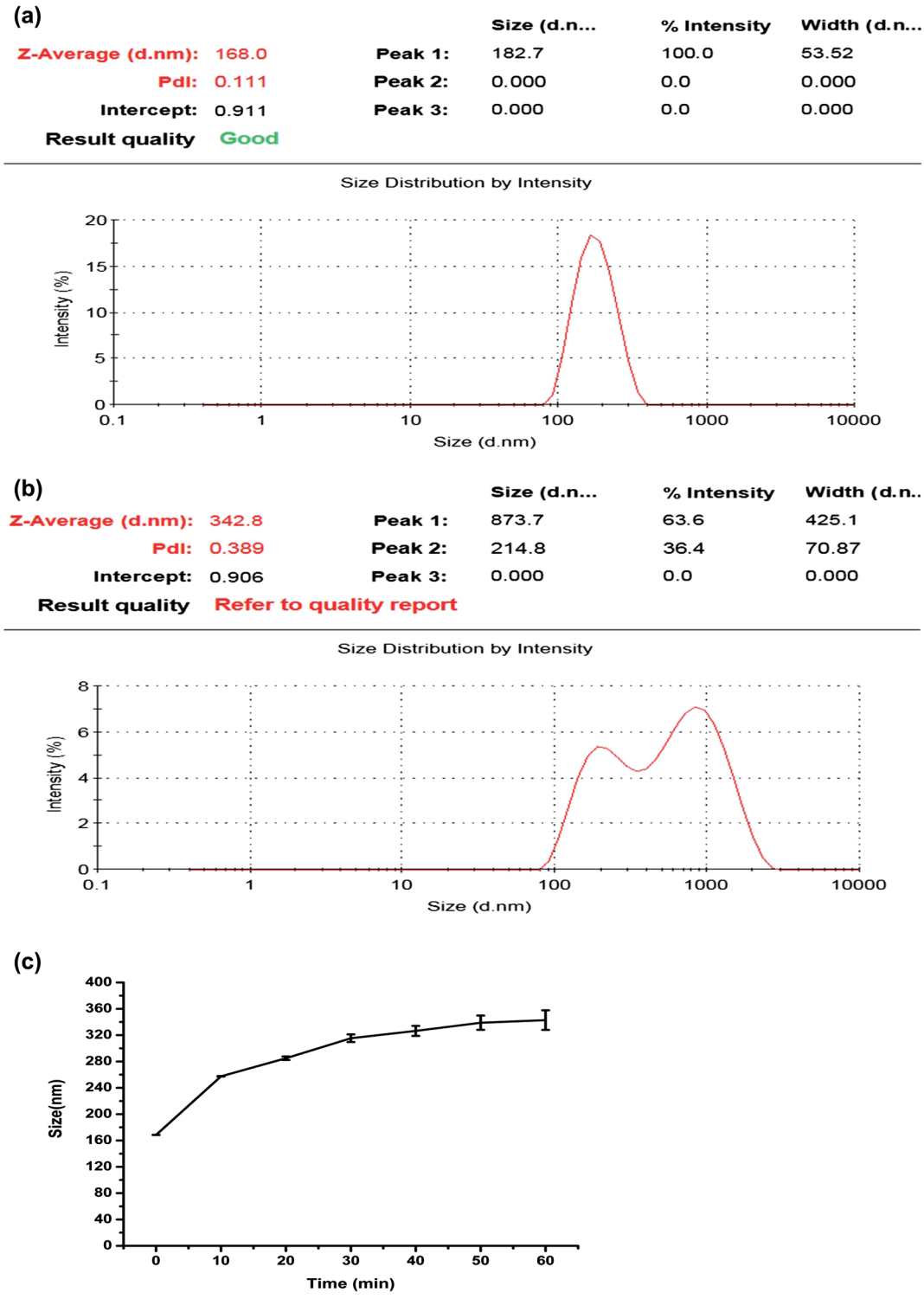

Figure 4. Cationic starch encapsulated bovine hemoglobin were dispersed in a PBS solution. Its size was measured by non-invasive back scatter in Malvern Nano-zs 90 (a) immediately, (b) 1 h later and (c) each 10 min.

anti-swelling starch with smaller steric hindrance should be considered in further research.

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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

References

Bian Y, Chang TM. 2015. A novel nanobiotherapeutic poly-[hemoglobin-superoxide dismutase-catalase-carbonic anhydrase] with no cardiac toxicity for the resuscitation of a rat model with

90 minutes of sustained severe hemorrhagic shock with loss of 2/3 blood volume. Artif Cells Nanomed Biotechnol. 43:1–9.
Bian Y, Rong Z, Chang TM. 2012. Polyhemoglobin-superoxide dismutase-catalase-carbonic anhydrase: a novel biotechnology-based blood substitute that transports both oxygen and carbon dioxide and also acts as an antioxidant. Artif Cells Blood Substit Immobil Biotechnol. 40:28–37.
Chang EJ, Lee SH, Mun KC, Suh SI, Bae JH, Kim SP, et al. 2004a. Effect of artificial cells on hepatic function after ischemia-reperfusion injury in liver. Transplant Proc. 36:1959–1961.
Chang EJ, Lee TH, Mun KC, Kim HC, Suh SI, Bae JH, et al. 2004b. Effects of polyhemoglobin-antioxidant enzyme complex on ischemia-reperfusion in kidney. Transplant Proc. 36: 1952–1954.
Chang TM. 2007. Artificial cells: biotechnology, nanomedicine, regenerative medicine, blood substitutes, bioencapsulation, cell/stem cell therapy. Singapore/London: World Scientific/Imperial College.
Chen L, Liu T. 2008. Interaction behaviors between chitosan and hemoglobin. Int J Biol Macromol. 42:441–446.
D'Agnillo F, Chang TM. 1997. Reduction of hydroxyl radical generation in a rat hindlimb model of ischemia-reperfusion injury using crosslinked hemoglobin-superoxide dismutase-catalase. Artif Cells Blood Substit Immobil Biotechnol. 25:163–180.
Degim IT, Celebi N. 2007. Controlled delivery of peptides and proteins. Curr Pharm Des. 13:99–117.
Gao W, Bian Y, Chang TM. 2013. Novel Nanodimension artificial red blood cells that act as O_2 and CO_2 carrier with enhanced antioxidant activity: PLA-PEG nanoencapsulated PolySFHb-superoxide dismutase-catalase-carbonic anhydrase. Artif Cells Nanomed Biotechnol. 41:232–239.
Gao W, Sha B, Zou W, Liang X, Meng X, Xu H, et al. 2011. Cationic amylose-encapsulated bovine hemoglobin as a nanosized oxygen carrier. Biomaterials. 32:9425–9433.
LoPresti C, Lomas H, Massignani M, Smart T, Battaglia G. 2009. Polymersomes: nature inspired nanometer sized compartments. J Mater Chem. 19:3576–3590.
Nan A. 2007. Multifunctional nanocarrier for image-guided delivery of bioactive agents. Nanomedicine (Lond). 2:739–743.
Sakai H, Horinouchi H, Masada Y, Takeoka S, Ikeda E, Takaori M, et al. 2004. Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. Biomaterials. 25:4317–4325.
Squires JE. 2002. Artificial blood. Science. 295:1002–1005.