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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

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Abstract

Poly(ethylene glycol)-Poly(lactic acid) block-copolymer (PEG-PLA) was prepared and characterized using Fourier transform infrared spectrophotometer (FTIR). Glutaraldehyde was used to crosslink stroma-free hemoglobin (SFHb), superoxide dismutase (SOD), catalase (CAT), and carbonic anhydrase (CA) into a soluble complex of PolySFHb-SOD-CAT-CA. PEG-PLA was then used to nanoencapsulated PolySFHb-SOD-CAT-CA by oil in water emulsification. This resulted in the formation of PLA-PEG-PolySFHb-SOD-CAT-CA nanocapsules that have enhanced antioxidant activity and that can transport both O_2 and CO_2. These are homogeneous particles with an average diameter of 100 nm with good dispersion and core shell structure, high entrapment efficiency (EE%), and nanocapsule percent recovery. A lethal hemorrhagic shock model in rats was used to evaluate the therapeutic effect of the PLA-PEG-PolySFHb-SOD-CAT-CA nanocapsules. Infusion of this preparation resulted in the lowering of the elevated tissue PCO_2 and also recovery of the mean arterial pressure (MAP).

Keywords: artificial cells, carbon dioxide, catalase, carbonic anhydrase, oxygen, PLA-PEG nanocapsules, PolySFHb-SOD-CAT-CA, polyhemoglobin, transport, transfusion, superoxide dismutase

Introduction

Blood transfusion is a life-saving procedure, but in some situations there may be concerns related to the lack of sufficient donors, delays due to the need for typing and crossmatching, and the risk of infection. To overcome these challenges, the first artificial red blood cell was reported (Chang 1964). This was followed by glutaraldehyde crosslinking of hemoglobin and enzymes (Chang 1971) and other approaches. However, international research and development only started to become more serious during the crisis of tainted blood in the 1980s and '90s, when over 60,000 people in the Western world infected with the AIDS virus (Winslow 2006). Thus, a number of groups (Moore et al. 2009, Jahr et al. 2008) have independently developed the glutaraldehyde-crosslinked hemoglobin method (Chang 1971) into products that have been tested extensively in clinical trials. One of these has been approved for routine use in South Africa and Russia. Polyhemoglobin has the advantages that it can be treated to remove infective agents; it has no blood group antigens, and thus can be administered without typing and crossing and is immediately available. Furthermore, it can be stored at room temperature for more than a year (Moore et al. 2009, Jahr et al. 2008) as compared to about 40 days for donor blood which also requires refrigeration.

Risk/benefits analysis is essential in any medical therapy especially in different clinical conditions (Chang 2005, 2007). Thus, in clinical situations with oxidative stress (D'Agnillo and Chang 1998, Alayash 2004, Biro et al. 1995) or endothelial dysfunction (Yu et al. 2008) the use of polyhemoglobin may result in some adverse events. To resolve these adverse events, glutaraldehyde has been used to crosslinked Hb, SOD and CAT into soluble nanodimension PolySFHb-SOD-CAT (D'Agnillo and Chang 1998). It prevents ischemia-reperfusion injuries in a combined hemorrhagic shock-cerebral ischemia rat model (Powanda and Chang 2002) and in liver and kidney transplantation in rats (Chang et al. 2004). This did not induce immunological reaction (Zhu et al. 2010). Hsia's group has extended this to result in a product that is an oxygen carrier with synthetic antioxidant properties (Buehler et al. 2004).

Carbonic anhydrase (CA) is the enzyme in RBC responsible for the transport of CO_2. CA inhibitor can block RBC–CA activity, resulting in marked decrease in CO_2 transport (Geers and Cros 2000). The result is an increase in the tissue CO_2 partial pressure (PCO_2) rising to 80 mmHg from the normal of 45 mmHg. The importance of CA is such that recently Chang's

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group prepared a glutaraldehyde-crosslinked PolySFHb-SOD-CAT-CA (Bian et al. 2011). The incorporation of CA resulted in a soluble nanodimension complex of PolySFHb-SOD-CAT-CA that has enhanced antioxidant properties in addition to acting as carrier for both O_2 and CO_2 (Bian et al. 2011). In vivo study shows that this can lower the elevated tissue CO_2 level in a hemorrhagic shock rat model more effectively than RBC (Bian et al. 2013).

Another approach is to develop the micro-dimension artificial RBC (Chang 1964) into nanodimension RBC with biodegradable PLA polymeric membrane (Yu and Chang 1994) or PEG-PLA membrane (Chang et al. 2003). The enclosing membrane prevents vasopressor effects, avoids the Hb colloidal osmotic effect, prolongs circulation half-life, and makes the direct modification of Hb unnecessary (Chang et al. 2003, Piras et al. 2008). Other reports on the nanodimension membrane enclosed RBC have concentrated on the use of different membranes, such as lipid membrane liposomes (Djordjevich and Miller 1980, Tsuchida et al. 2009), synthetic polymer (Piras et al. 2008), or natural polymer (Gao et al. 2011).

This paper combines our nanobiotechnological approaches of PolySFHb-SOD-CAT-CA (Bian et al. 2011) with PEG-PLA nanocapsules (Chang et al. 2003, Chang 2007, Fustier and Chang 2012). The details have been described in these references. We nanoencapsulated PolySFHb-SOD-CAT-CA into PEG-PLA nanocapsules to form PEG-PLA-PolySFHb-SOD-CAT-CA nanocapsules (Figure 1).

This paper describes the preparation and characterization of PEG-PLA-PolySFHb-SOD-CAT-CA nanocapsules. To evaluate its special function of CO_2 carrier, we also monitor the changes of tissue PCO_2 in a lethal hemorrhagic shock model. This is the first step to be followed by much more needed research.

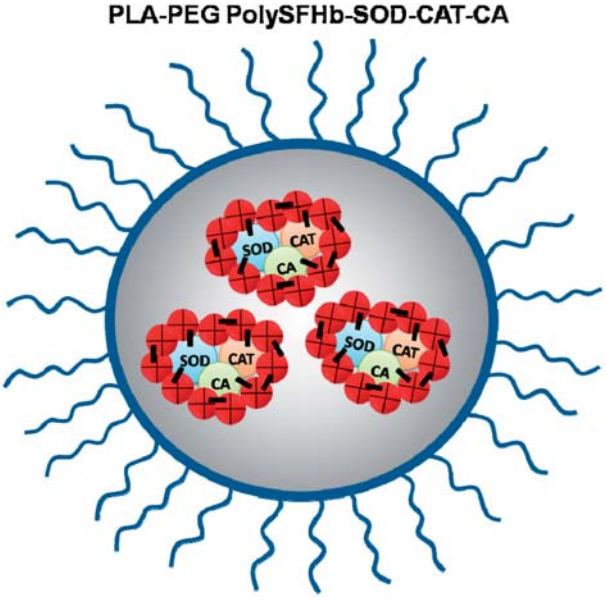

Figure 1. Schematic representation of Poly(ethylene glycol)-poly(lactic acid) block-copolymer encapsulated Polystroma-free hemoglobin-superoxide dismutase-catalase-carbonic anhydrase (PEG-PLA-Poly SFHb-SOD-CAT-CA) nanocapsules.

Materials and methods

Materials

Poly(dl-lactic acid) (PLA) (M.W. 15,000 g/mol) was purchased from Polysciences Inc. Tween 20 was obtained from Fisher. Tin 2-ethylhexanoate, methoxypolyethylene glycol (PEG) (M.W. = 5000 g/mol), Hydrogenated L-α-Phosphatidylcholine, and other analytical reagents were purchased from Sigma. Carbon film-coated TEM Grids were purchased from Science Services. The stroma-free hemoglobin (SFHb) was Hb with the original RBC enzymes extracted from bovine blood was purchased from the McGill University McDonald Campus Cattle Complex (Sainte-Anne-de-Bellevue, Canada). The ultrapure Hb with all enzymes removed was bought from Biopure to prepare PolyHb. Polyethersulfone ultrafiltration membranes with a cut-off molecular weight of 500 kDa were purchased from Millipore.

Preparation of PEG-PLA copolymer

The procedures used were referred to the methods by TMS (Chang 2007). Briefly, 1.5 g of DL-PLA (M.W. 15,000 g/mol) and 0.75 g of methoxypolyethylene glycol (M.W. 5,000 g/mol) were placed in a 125 mL Erlenmeyer flask and dried under vacuum overnight in a vacuum oven (Fisher isotemp) at room temperature in the presence of phosphorus pentoxide. They were dissolved in 7.5 mL of acetone. The mixture was heated in an oil bath under nitrogen. The acetone evaporated completely in 2–4 min and the flask was left in the oil bath at 180°C for two more hours. 10 μL of the catalyst stannous-2-ethylhexanoate were then added and the flask was heated for three more hours at 180°C under nitrogen. The copolymer solidified slowly at room temperature. It was collected in a vial and stored in desiccators.

Preparation of PolySFHb-SOD-CAT-CA, PolySFHb and PolyHb

The procedures used were referred to the methods by TMS (Chang 2007).

(1) Polyhemoglogin (PolyHb) was prepared using ultrapure Hb with all enzymes removed.
(2) PolySFHb, was prepared using 7 g/dl of stroma-free hemoglobin SFHb (extracts from red blood cells containing enzymes normally present in the red blood cell).
(3) PolySFHb-SOD-CAT-CA, was prepared using 20 mL SFHb (7 g/dL) with the addition of 5.6 mg of SOD, 110.3 mg of CAT, and 5.3 mg of CA in 50 mM potassium phosphate buffer (pH7.4). The analysis of enzyme activities have been described (Bian et al. 2013).

Before the initiation of the crosslinking reaction, 187 μl of 1.3 M lysine was added at a molar ration of 11.1:1 lysine/Hb, and shaken in the speed of 140 r/min for 1 h. Nitrogen gas was used to flush the reaction vessel to prevent formation of methemoglobin. Crosslinking was started with the addition of 744 μl of 0.5 M glutaraldehyde at a molar ration of 17:1 glutaraldehyde/Hb. Glutaraldehyde was added in four equal aliquots over a period of 15 minutes, and the reaction was

allowed to crosslink for 24 hours at 4°C with constant stirring in the speed of 140 r/min under anaerobic conditions. Crosslinking was stopped by adding 4.47 g of lysine at a molar ratio of 1118:1 lysine/Hb and shaken in the speed of 150 r/min for 1 h. To remove free Hb and enzymes, A a Sephacryl-300 HR column was used to purify PolySFHb-SOD-CAT-CA, PolySFHb and PolyHb. The column was equilibrated with the elution buffer (0.1 M Tris-HCl and 0.15 M NaCl pH 7.4). Samples were passed through the column at a flow rate of 36 ml/hour. The molecular weights of PolySFHb-SOD-CAT-CA, PolySFHb, and PolyHb were higher than 250 kDa, so they were collected by peak identification using UV detector at 280 nm in order to exclude unlinked enzymes, such as catalase (250 kDa), superoxide dismutase (32.5 kDa), Carbonic anhydrase (30 kDa), and free tetrameric Hb (64 kDa). Then the samples were concentrated to 5 g Hb/dL using the spectral absorbent gel and stored at − 80°C.

Preparation of PLA-PEG-PolySFHb-SOD-CAT-CA nanocapsules, PLA-PEG-PolySFHb nanocapsules and PLA-PEG-PolyHb nanocapsules

The preparation was performed using the improved methods reported by TMS (Chang 2007). Briefly, the nanocapsules were prepared by injecting an organic phase into an aqueous phase containing PolySFHb-SOD-CAT-CA, PolySFHb, or PolyHb. The aqueous phase consisted of 10 ml of 5 g/dL PolySFHb-SOD-CAT-CA, PolySFHb, or PolyHb with 100 µl of tween 20. While the organic phase consisted of 40 mg of PEG-PLA, 20 mg of phosphatidylcholine, 3.2 ml of acetone, and 1.6 ml of ethanol. Slowly dropped the organic phase through a 26 G needle into the aqueous phase under magnetic stirring with Therm-O-Swirl Stirrer (Precision Scientific Co., Chicago) setting to 6 grade at 4°C. The nanocapsules were formed immediately, but the suspension was kept stirred for 15 min. The organic solvent was partly removed from the suspension prepared by rotary evaporator under vacuum at 0°C for about 3 h. The nanocapsules were purified by a 50 mL Millipore-stirred cells device operating at 4°C with a polyethersulfone ultrafiltration membrane under a nitrogen pressure of 69 kPa.

The FTIR of PLA-PEG and the TEM of nanocapsules

The chemical structure of PLA-PEG was determined using FTIR (Shimadzu Corporation, Japan). Solutions of PLA-PEG-PolySFHb-SOD-CAT-CA nanocapsules, PLA-PEG-PolySFHb nanocapsules, and PLA-PEG-PolyHb nanocapsules were relatively dropped on a carbon film-coated TEM grids and air dried, and then were relatively measured using transmission electron microscopy (TEM) (Philips, Holand).

Measurement of Hb concentration and enzyme activity

Based on the methods of TMS (Chang 2007), the Hb concentration and enzyme activity could be measured using UV-visible spectrophotometer (GE, America) as follows. The Hb concentration was determined by reaction the samples with Drabkin's reagent (Sigma-Aldrich), then measuring the concentration of the resulting cyan-methemoglobin solution by spectrophotometry at 540 nm. The hydration activity of CO_2 by CA was determined by the pH of a 0.02 M Tris buffer decreasing from pH 8.3 to 6.3 per minute. The measurements in seconds were converted into W-A units according to the following formula: 1 W-A unit = [2*(T0 − T)]/T. The activity of CAT was measured by the rate of disappearance of H_2O_2 in UV 240 nm according to the following formula: $U = 1106.301 \times \text{Log}\ (A_0/A_{15})$. The measurement for SOD activity was based on the reduction of cytochrome C.

Determination of entrapment efficiency and nanocapsule recovery

After rotary evaporation, samples (PLA-PEG-PolySFHb-SOD-CAT-CA) were centrifuged at 15000 r/min × 15 min 4°C (Beckman J2-21, USA). The volume of supernatant and its Hb concentration and enzyme activity were measured using UV-visible spectrophotometer. The sediment was lyophilized for 48 h in a Lab Con Co freeze dryer (Freeze Dry 5). The total amount of enzymes in the final PolySFHb-SOD-CAT-CA preparation consists of those normally present in the stroma-free hemoglobin (SFHb) plus the amount added. The entrapment efficiency (EE%) and nanocapsule recovery (%) of nanocapsules were calculated as follows. All procedures and measurements were repeated thrice.

	Entrapment efficiency (%)	Nanocapsules recovery (%)
Hb	(mass of Hb in nanocapsules/ mass of Hb used in formulation) × 100	(mass of nanocapsules recovered/mass of polymer, Hb, SOD, CAT, and CA in formulation) × 100
SOD	(mass of SOD in nanocapsules/ mass of SOD used in formulation) × 100	
CAT	(mass of CAT in nanocapsules/ mass of CAT used in formulation) × 100	
CA	(mass of CA in nanocapsules/ mass of CA used in formulation) × 100	

The effects on a hemorrhagic shock model

McGill University ethics committee on animal research has approved our animal research. A total of 24 male Sprague–Dawley rats with a mean body weight of 250 ± 20 g were used. All rats were obtained from the comparative medicine Animal Research Center of the McGill University. The rats were maintained in the McIntyre animal center with 12 h light/dark cycles. They were provided with food pellets and water ad libitum. The rats were randomly divided into three groups (n = 6). All rats were anesthetized with intraperitoneal injection of 54.7 mg/ml × 5.5 ml/g pentobarbital solution. Then a right inguinal incision was made. Heparinized catheters were inserted into the right femoral vein and right femoral artery. A CO_2 meter probe (lazer research lab, USA) was placed on the surface of the muscle at the incision site to measure tissue PCO_2. Mean arterial pressure (MAP) was observed from the right femoral artery using a Biopac system (BIOPAC Systems Inc., Goleta, CA), and the result of the observation was used as a basis for the modified model of hemorrhagic shock (Chang 2007). When the rats recovered conjunctival reflex, blood was withdrawn from the

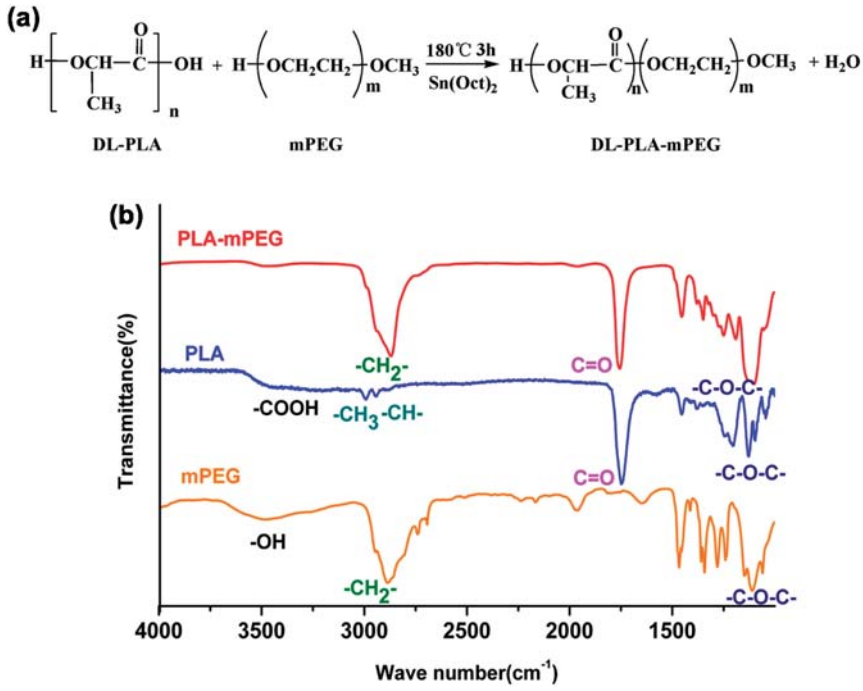

Figure 2. Synthesis and characterizations of PLA-PEG (a) Chemical reaction equation to synthesize PLA-PEG: the –COOH in DL-PLA reacted with –OH in mPEG, dehydrating into PLA-PEG. (b) FTIR spectra of mPEG, PLA and PLA-PEG. 0.25 mg of the dry sample was mixed with IR-grade KBr (0.1 g) and pressed (10 ton) into tablet form. The spectrum of the tablet was then recorded by an FTIR spectrometer.

right femoral artery and MAP was reduced to 30 mmHg and maintained for 60 min. The rats were infused from the femoral vein with the same volume of liquid that was shed from the femoral artery. The rats were divided into four groups. The first group of rats were treated with PLA-PEG encapsulated 5 g/dL of PolyHb nanocapsules (n = 6). The second group of rats were treated with PLA-PEG encapsulated 5 g/dL of PolySFHb nanocapsules (n = 6). The third group of rats was treated with 5 g/dL of PolySFHb-SOD-CAT-CA (n = 6). The fourth group of rats were treated with PLA-PEG encapsulated 5 g/dL of PolySFHb-SOD-CAT-CA nanocapsules (n = 6). After an hour of resuscitation and follow up, the rats were sacrificed using intraperitoneal nembutal.

Statistical analysis

Test data were 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 FTIR of PLA-PEG and the TEM of nanocapsules

As shown in Figure 2a, PLA-PEG was prepared by PLA and PEG at 180°C in the presence of the catalyst stannous-2-ethylhexanoate. FTIR spectra were shown in Figure 2b, PEG had the peaks at 3492 cm^{-1}, 2889 cm^{-1}, and 1110 cm^{-1}, which were respectively assigned to at –OH, $–CH_2–$, and –C–O–C– groups. While the peaks at 3485 cm^{-1}, 2995 cm^{-1}, 2947 cm^{-1}, and 1751 cm^{-1} in the spectrum of PLA were due to –COOH, $–CH_3$, –CH-, and C = O groups, respectively. Moreover, both peaks at 1125 cm^{-1} and 1096 cm^{-1} corresponded to –C–O–C–. PLA-PEG had all the characteristic peaks of PLA and PEG, thus proving the presence of the two component blocks in the copolymer. Meanwhile, the peaks of –OH and –COOH end groups were much smaller than those of PLA and PEG for the dehydration reaction. These results demonstrated that PLA-PEG agreed with previous literature in terms of its chemical structure (Chang 2007).

As observed through TEM in Figure 3, all nanocapsules of PLA-PEG-PolyHb, PLA-PEG-PolySFHb, and PLA-PEG-PolySFHb-SOD-CAT-CA had a homogeneous particle diameter, good dispersion, and core shell structure. The mean diameters were 100 nm, and there was no difference in sizes for the different preparations.

EE and nanocapsule recovery

There was good EE% of Hb, SOD, CAT, and CA in PLA-PEG-PolySFHb-SOD-CAT-CA at 68.34 ± 0.85%, 68.52 ± 1.44%, 68.27 ± 1.07%, and 71.85 ± 2.51%, respectively. Nanocapsule recovery was 71.30 ± 2.97%.

Tissue PCO_2 in muscle on a lethal hemorrhagic shock model (Figures 7 and 8)

The concentrations of Hb and enzymes activities in PLA-PEG-PolyHb, PLA-PEG-PolySFHb, PolySFHb-SOD-CAT-CA, and PLA-PEG-PolySFHb-SOD-CAT-CA are shown in Figure 4. There were no significant differences in MAP and PCO_2 among any of the groups during the control period. The rats in all groups responded to bleeding of 67% of the total blood volume (56 mL/kg) with a decrease in MAP to 30 mmHg, which was maintained for 1 hour. After resuscitation, the MAP in all groups recovered initially. The recovery of MAP was well maintained in the PLA-PEG-PolySFHb-SOD-CAT-CA

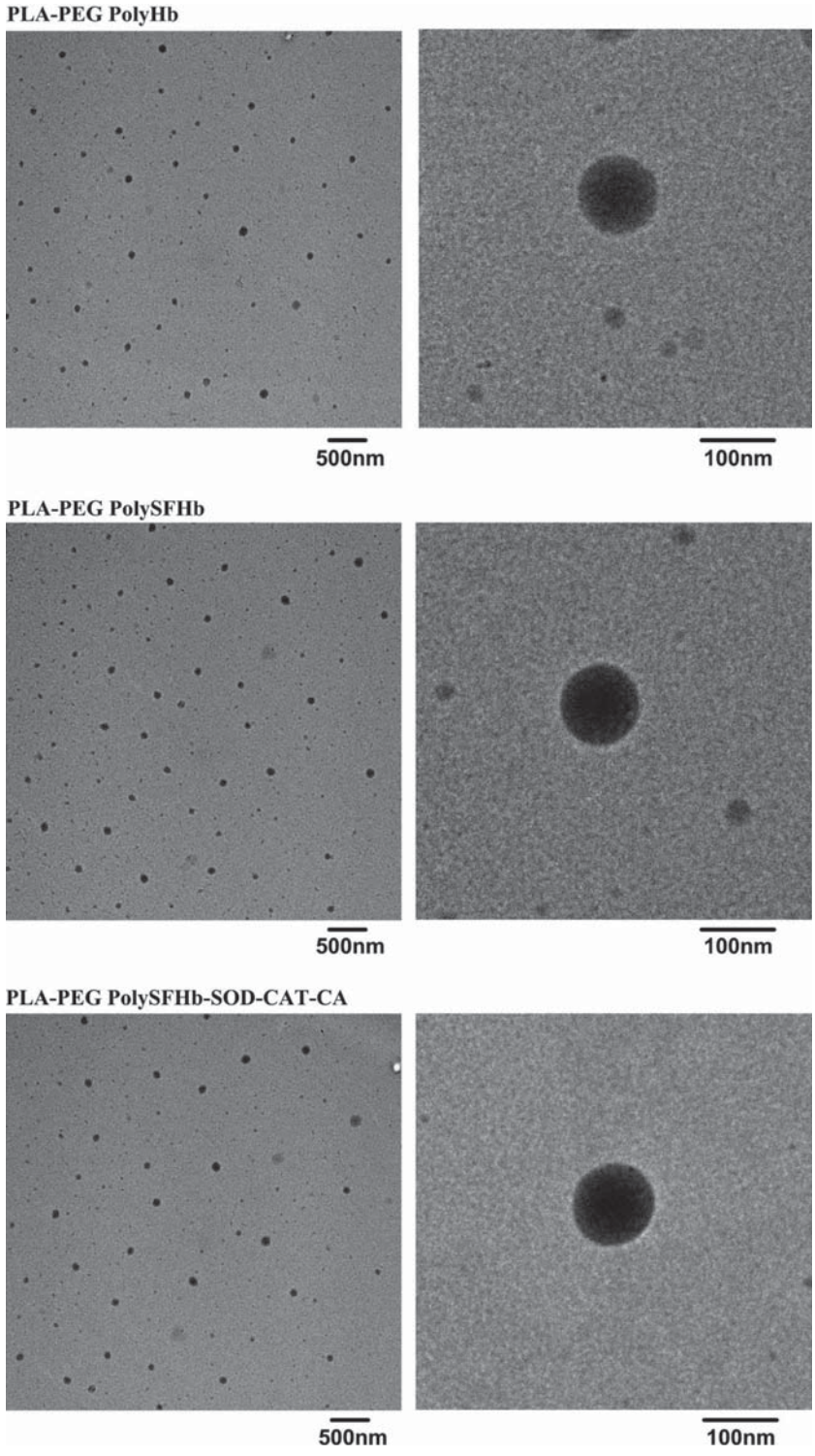

Figure 3. TEM pictures without metal spraying of PLA-PEG-PolyHb nanoparticles, PLA-PEG-PolySFHb nanoparticles, and PLA-PEG-PolySFHb-SOD-CAT-CA nanoparticles.

group, whereas this was not as well maintained in the PolySFHb-SOD-CAT-CA, PLA-PEG-PolySFHb, and PLA-PEG-PolyHb groups (Figure 5). Meanwhile, the tissue PCO_2 in all groups increased rapidly during shock. With resuscitation, the tissue PCO_2 in all the groups decreased, but the PCO_2 in all groups were not lowered to the same levels. Thus, PLA-PEG-PolyHb was least efficient in lowering the tissue PCO_2 level. PLA-PEG-PolySFHb was lower than PLA-PEG-PolyHb. However, PolySFHb-SOD-CAT-CA and PLA-PEG-PolySFHb-SOD-CAT-CA were the most effective of the four tested. ($n = 6$, $P < 0.05$) (Figure 6).

Discussion

Most of the current RBCs substitutes just work as O_2 carriers. For example, PLA-PEG-encapsulated Hb has shown

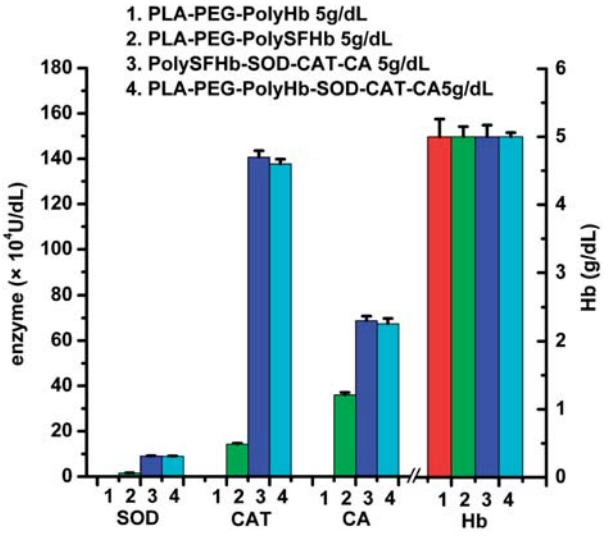

Figure 4. The concentrations of Hb and enzymes activities in PLA-PEG-PolyHb nanoparticles, PLA-PEG-PolySFHb nanoparticles, PolySFHb-SOD-CAT-CA, and PLA-PEG-PolySFHb-SOD-CAT-CA nanoparticles.

good oxygen dissociation curve (Zhang et al. 2009) and long circulation time (Chang et al. 2003). Poly SFHb-SOD-CAT has shown the dual function of an oxygen carrier and the ability to remove oxygen radicals, so it can carry oxygen to ischemic brain tissue without causing significant injury in an ischemia-reperfusion rat brain model (Powanda and Chang 2003). This basic research has been extended to a product which is an oxygen carrier with synthetic antioxidant properties (Buehler et al. 2004). However, real RBCs are not only O_2 carriers, but also are CO_2 carriers. As a result, a three functional O_2 and CO_2 carriers with enhanced antioxidant activity in the form of PolySFHb-SOD-CAT-CA were prepared (Bian et al. 2011) and shown to be effective in lowering PCO_2 in a hemorrhagic shock rat model (Bian et al. 2013). We now report an extension of this to prepare nanodimension artificial RBC in the form of PLA-PEG nanocapsules containing PolySFHb-SOD-CAT-CA.

The FTIR spectrum of the PLA-PEG contained the characteristic peaks of PLA and PEG, while lowered peaks of –OH and –COOH for the dehydration reaction. These results show that PLA-PEG agrees with previous literature in terms of its chemical structure (Luo et al. 2002), indicating the PLA-PEG had been successfully prepared. As shown in the TEM micrographs in Figure 3, all nanocapsules of PLA-PEG-PolyHb, PLA-PEG-PolySFHb, and PLA-PEG-PolySFHb-SOD-CAT-CA had a nucleus shell structure with good dispersion without aggregation. The mean diameter of the nanocapsules was 100 nm. PLA-PEG-PolySFHb-SOD-CAT-CA showed good EE% of Hb, SOD, CAT, and CA and

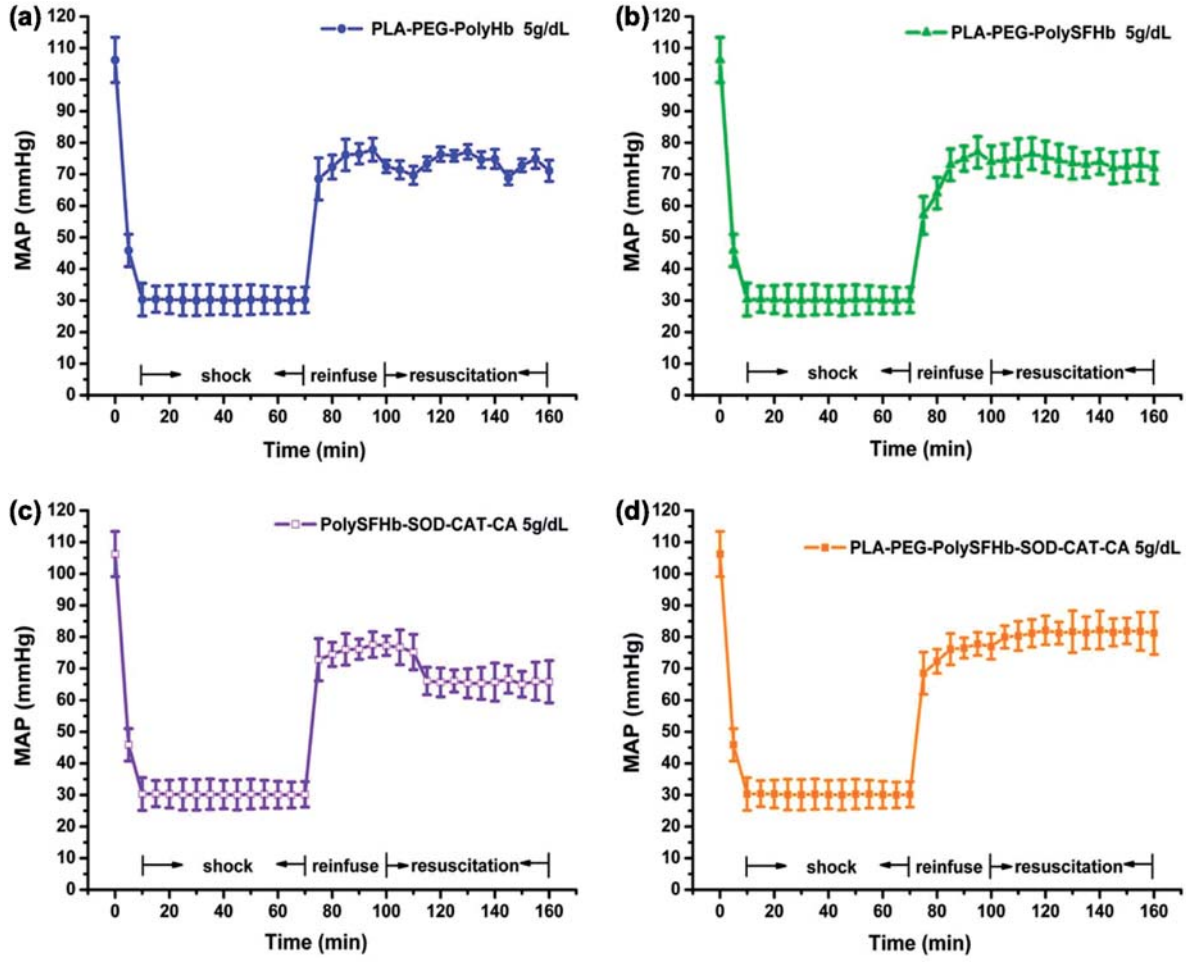

Figure 5. MAP of lethal hemorrhagic shock and resuscitation for each of the four groups.

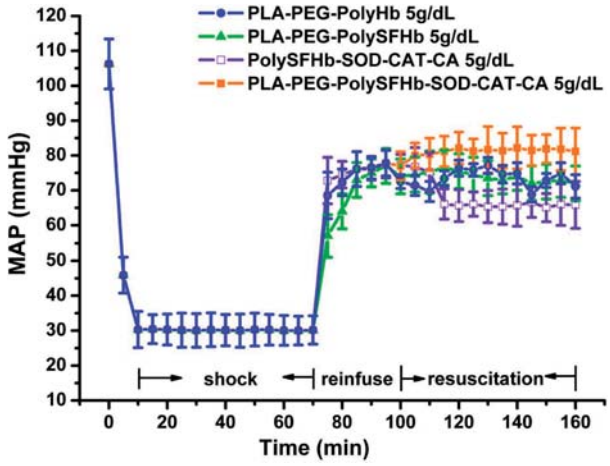

Figure 6. Combined figure of MAP: There were no significant differences between the MAP in PLA-PEG-PolySFHb group (—▲—) and PLA-PEG-PolyHb group (—●—). PLA-PEG-PolySFHb-SOD-CAT-CA group (—■—) had significantly highest MAP in all groups at any time of resuscitation, PolySFHb-SOD-CAT-CA group (—□—) had significantly lowest MAP in all groups after 15 min of resuscitation (n = 6, $P < 0.05$).

recovery of nanocapsules. This is likely because it is easier to nanoencapsulate the larger size PolyHb-SOD-CAT-CA and the proportion of hemoglobin and enzymes remained unchanged in the crosslinked form.

Compared with arterial pressure, end-tidal CO_2, cardiac index, arterial blood lactate, and base deficit, tissue PCO_2 was shown to be more predictive of outcome after rapid bleeding, so it likely to serve as a useful guide for the immediate management of hemorrhagic shock (Cammarata et al. 2009). For rats, skeletal muscles showed better compliance than others. To optimize the compliance, PCO_2 probe was placed on the surface of skeletal muscles at inguinal incision. As shown in Figure 6, both the PolySFHb-SOD-CAT-CA group and the PLA-PEG-PolySFHb-SOD-CAT-CA group were as effective in lowering the elevated PCO_2 at any time after resuscitation. Thus, the encapsulated procedure had not decreased CA activity. These two groups were able to lower the elevated PCO_2 level much better than the PLA-PEG-PolySFHb group that had no added CA or the PLA-PEG-PolyHb group.

As stated in the introduction, the aim of the present study is to analyze the lowering of the elevated PCO_2. The PLA-PEG nanocapsules groups were able to maintain the MAP better than the PolySFHb-SOD-CAT-CA group. This is likely because the circulation half time of PLA-PEG PolyHb nanocapsules is double that of the soluble PolyHb (Chang et al. 2003). PLA-PEG-PolySFHb-SOD-CAT-CA could maintain MAP better than the PLA-PEG-PolySFHb and PLA-PEG-PolyHb

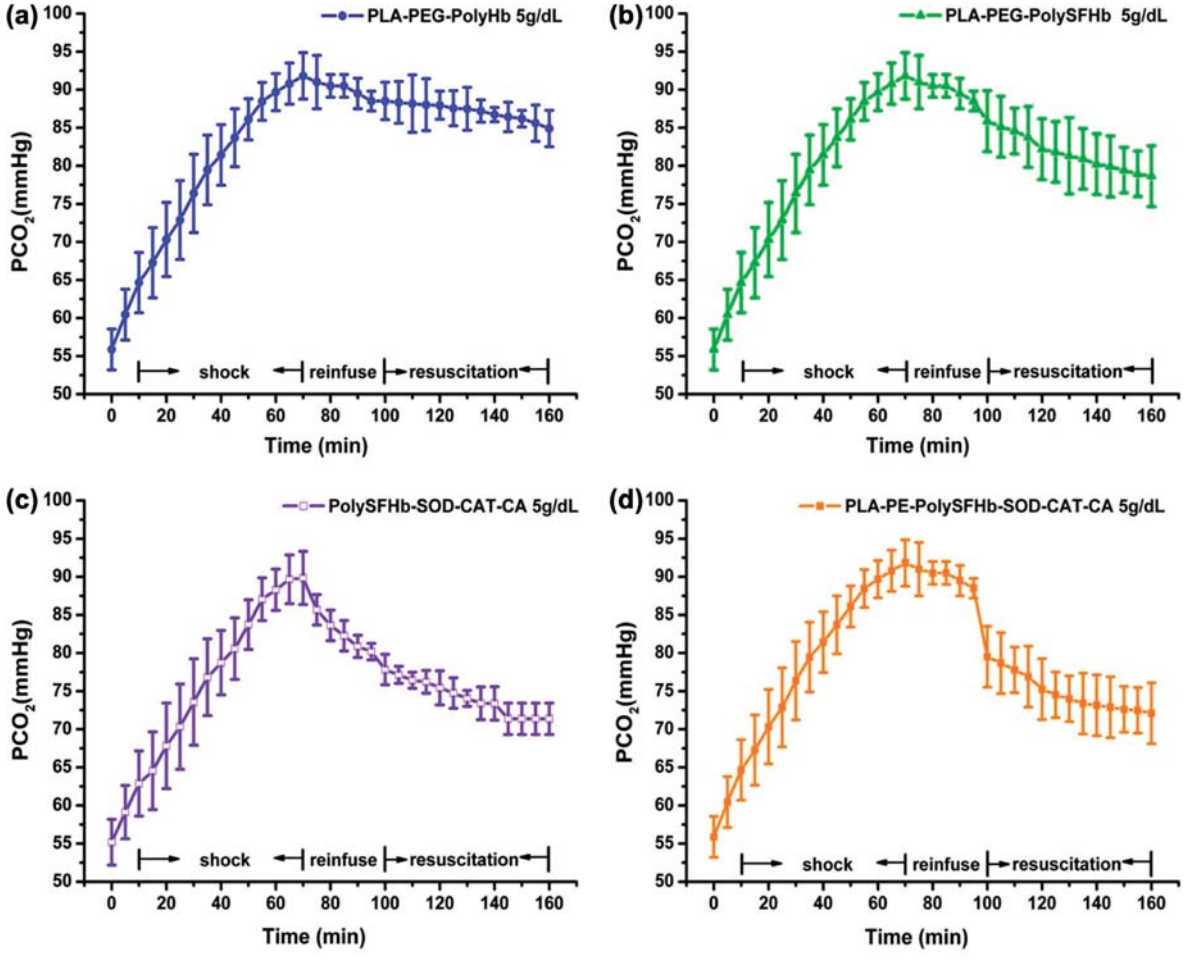

Figure 7. Muscle PCO_2 of a lethal hemorrhagic shock and resuscitation for each of the four groups.

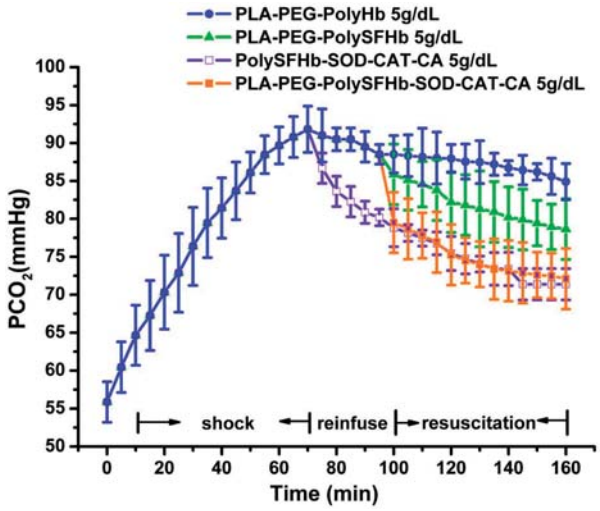

Figure 8. Combined figure of PCO_2: There were no significant differences between the PolySFHb-SOD-CAT-CA group (—□—) and the PLA-PEG-PolySFHb-SOD-CAT-CA group (—■—) in terms of PCO_2 at any time of resuscitation, and they had the lower PCO_2 than the other two groups. PLA-PEG-PolyHb group (—●—) had higher PCO_2 after 20 mins of resuscitation than PLA-PEG-PolySFHb group (—▲—). (n = 6, $P < 0.05$).

group. Further study is needed to see if this is because of the presence of CA resulting in better removal of tissue CO_2 and the lowering of tissue PCO_2.

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Declaration of interest

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

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