Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/x0xx00000x

Biodegradable dextran vesicles for effective hemoglobin encapsulation

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Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Biodegradable dextran vesicles were developed based on dextran and poly(L-lactide) (PLA). Amphiphilic graft copolymer dextran-g-poly (L-lactide) (dextran-g-PLA) was obtained by coupling carboxyl-terminated PLA to dextran. The copolymer could self-assemble to form nanoparticles using a dialysis method. The morphology and size of nanoparticles were examined by Transmission electron microscopy (TEM) and Dynamic light scattering (DLS). Different morphology could be obtained by varying the relative mass ratio of dextran and PLA. The formed vesicles showed high stability. In order to encapsulate hemoglobin (Hb), we need to disperse the lyophilized dextran-g-PLA into Hb phosphate buffer solution (PBS). Free Hb was removed by centrifugation. The oxygen affinity of hemoglobin vesicles (HbVs) is close to pure Hb. Thus, the HbVs have great potential as blood substitute in the future.

Introduction

Due to serious blood shortage all over the world and the risks of modern blood transfusion, blood substitutes were initiated and driven by the medical and life science research communities. Blood substitutes mainly refer to artificial oxygen carriers. Pioneering work to mimic the cellular structure of erythrocyte was performed by Chang in 1957.[1, 2] Then various artificial oxygen carriers, such as conjugated micelles[3, 4], spheres[5, 6] and hydrogel capsules[7, 8], have been synthesized and used as red blood cell (RBC) substitutes. Encapsulation was to protect Hb from the external environment, thereby helping to increase circulation time, reduce nephrotoxicity.[9] Great progress has been made in liposome encapsulated Hb decades ago.[10, 11] Compared with liposome, polymersome shows better mechanical strength and structural stability.[12-14] However, the development of biodegradable polymers offers us new clinical applications. Thus, biodegradable polymersomes indicate great potential.

The biodegradable polymers used in previous work mainly focus on the amphiphilic block copolymers, like poly(ethylene glycol)-co-polylactide (PEG-b-PLA) and poly(ethylene glycol)-co- polycaprolactone (PEG-b-PCL).[15] Chang's group first tried to prepare artificial blood cells with PLA in 1976 and reported some approaches to nanoscale Hb encapsulation with PEG-PLA.[9, 16] Poly (ethylene glycol) (PEG) usually functions as the hydrophilic block, but the low protein adsorption of PEG limits the Hb content in vesicles. Alternatively, dextrans possess a number of appealing characteristics as drug carriers, and have been incorporated into a variety of nanoparticle formulations.[17-19] Compared with PEG, dextran is easier to be modified because there are abundant functional hydroxyl groups.[20-22] Aldehyde dextran nanocarriers were developed by M. Kavallaris to carry doxorubicin, which improved tumor penetration.[23] Dextran was first used as vesicles for dual encapsulation of hydrophilic rhodamine-B and hydrophobic camptothecin by M. Jayakannan.[24] Very recently, dextran is gradually explored in drug delivery system, but there is no report using in encapsulating Hb. It is important to encapsulate biomacromolecules into vesicles, but it is still a challenge to prevent protein from denaturation during the encapsulation process.[28]

In this work, the vesicles were made through self-assembly of the dextran-g-PLA, and used to encapsulate Hb as the blood substitutes. The encapsulated Hb is likely to hold better bioactivity. Our strategy has shown obvious advantages due to its green and facile, which is generally applicable to load protein drugs.

Experimental

Materials

L-lactide (Zhejiang Hisun Biomaterials Co.Ltd) was recrystallized from ethyl acetate. Tin (Ⅱ) 2-ethylhexanoate ($Sn(Oct)_2$) was purchased from Aldich. 4-(Dimethylamino) pyridine (DMAP) and dicyclohexylcarbodiimide (DCC) were from Tci. Bovine hemoglobin was purchased from Shanghai Kayon Biological Technology Co. Ltd. and stabilized under CO atmosphere to afford CO-binding hemoglobin (COHb). Toluene was purified by distillation from sodium with benzophenone. Dimethyl Sulfoxide (DMSO) was stored over CaH_2 and distilled under reduced pressure. Other reagents were analytical grade and used as received.

Characterization

^1H NMR and ^{13}C NMR spectra were recorded with a Bruker AV400 spectrometer at 25 ^oC. Gel permeation chromatography (GPC) measurements were conducted with a Waters 410 GPC instrument equipped with Waters Styragel column. Dimethylfomamide was used as eluent at a flow rate of 1 mL/min at 35 ^oC. The molecular weights were calibrated with polystyrene standards. TEM measurements were performed on a JEOL JEM-1011 electron microscope operating at an acceleration voltage of 100 kV. DLS experiments were carried out with a DAMN EOS instrument equipped with a He-Ne laser at a scattering angle of 90^o. Sodium dodecylsulfate/ polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 5% polyacrylamide in the stacking gel and 12%

in the separation gel. Staining was accomplished using Coomassie blue R-250. UV spectra were recorded on a UV-vis spectrophotometer (Shimadzu UV-2450) at room temperature. Blood cell counting was performed using an ABX Micros 60 counter.

Synthesis of dextran-g-poly(L-lactide)

Carboxyl-terminated poly(L-lactide) (C-PLA) was synthesized according to previous work.[29] In brief, bis（hydroxymethyl propionic acid was used as initiator, $Sn(Oct)_2$ was used as catalyst. The reaction was heated at 130 ^oC under nitrogen atmosphere for 10h. Purification was performed by precipitating the reaction mixture against large volumes of cold ether. Dextran-g-PLA was prepared according to traditional DCC chemistry. Dextran (M_W = 10 000, 0.1 g, 0.01 mmol) and C-PLA (n=25, 0.18 g, 0.1 mmol) were dissolved in anhydrous DMSO. DMAP and DCC were added two equivalent followed by nitrogen-purging. The reaction was stirred for 1h at 25 ^oC and then gradually heated to 90 ^oC for another 48h. Purification was performed by precipitating the reaction mixture against large volumes of cold ethanol and dialysis against distilled water. The product was collected by freeze drying under vacuum to give a yellowish white solid, yield 50%. ^1H NMR (DMSO-d6，400 MHz,)：δ 5.20 (q, methine), 4.68 (s, dextran anomeric proton), 4.89, 4.82, 4.46 (s, hydroxyl of dextran), 4.33 (t, end group of methine), 3.92-3.11 (m, dextran glucosidic protons), 1.29 (s, methyl). ^{13}C NMR (DMSO-d6, 100MHz) δ 173.93， 168.95 (C＝O), δ 98.71 (dextran anomeric carbons), δ 73.82, 72.34, 70.87,70.60, 66.54 (dextran glucosidic carbons), δ 19.96，14.47（aliphatic carbons）.

Preparation of nanoparticles and hemoglobin vesicles

The dextran-based nanoparticles were prepared using dialysis method. Dextran-g-PLA (100 mg) was dissolved in 5 mL DMSO and added into 50 mL deionized water. After being stirred at 30 ^oC for 1 h, the suspension was dialyzed against distilled water using commercial cellulose ester semipermeable for 48 h. It was subjected to lyophilize for 48h before use.

Hemoglobin vesicles (HbVs) were made by dispersing the lyophilized copolymer into Hb PBS solution under CO atmosphere. In brief, 5mL Hb PBS (10mg/mL) was injected into the reaction with 5mg dextran-g-PLA3. The solution was stirred for 6 h at room temperature. Free Hb was removed via centrifugation (10000rpm×30min). Then, moderate ascorbate was added to the retentate, and purged with CO. Finally, the vial was sealed and stored at 4 ^oC.

Loading efficiency and loading content

The Hb was measured at 540 nm after conversion into cyanomethemoglobin as reported elsewhere.[10] The amount of entraped Hb was measured indirectly, which was determined by measuring the concentration of initial Hb (Hb_{total}) and Hb in the supernatant (Hb_{free}). The loading efficiency and loading content of Hb was calculated by the following equations.

Loading efficiency (%) = (Hb_{total} - Hb_{free}) / Hb_{total} × 100

Loading content (%) = (weight of Hb in vesicles / weight of Hb loaded vesicles) × 100

SDS-PAGE

SDS-PAGE was performed to confirm Hb was encapusalated in the cavity of vesicles rather than adsorbed on the surface of vesicles. Triton X-100 was added to the sample to destroy the vesicles. After adequate mixing, the sample was subjected to centrifugation (10000 rpm × 30 min), and the supernatant was analyzed. The sample without addition of Triton X-100 and pure Hb were also analyzed as the control.

Gas-binding capacity and oxygen affinity

Different gas-binding state of Hb would be transformed reversibly, which could be monitored by UV. The CO-binding hemoglobin (COHb) was used during the encapsulation process. The COHb could convert to N_2-binding state (O_2-binding state) by exposing the solution to visible light under N_2 (O_2) atmosphere with the shift of maximum absorption peak. Pure Hb and HbVs solution (0.1mg/mL, 0.2M phosphate, 0.9% NaCl, pH 7.4) were used in this section.

The HbVs' oxygen affinity was quantitated by half-saturation pressure (P_{50}) and Hill coefficient. These two parameters were calculated according to oxygen dissociation curve, which reflect the relationship with O_2 partial pressure and O_2 saturation. In order to monitor the value of O_2 saturation with different oxygen partial, the HbVs solutions should first convert into complete N_2-binding state (deoxyHb). Then different volumes of air were introduced into the cuvette through a syringe, deoxyHb gradually changed into O_2-binding state (oxyHb). O_2 saturation was calculated according to literature.[30]

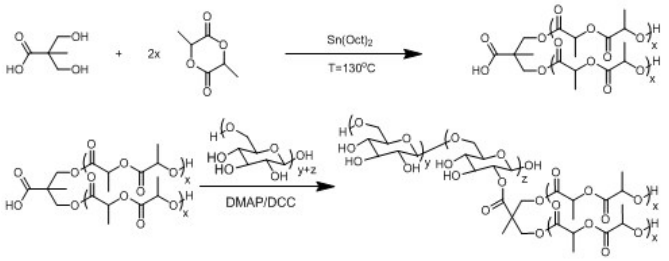
Hemocompatibility of HbVs *in vitro*

Whole blood was withdrawn from wistar rats. All of the study protocol was performed according to the Guidelines of the Committee on Animal Use and Care of Chinese Academy of Sciences. 500 μL, 400 μL, 300 μL, 200 μL of fresh whole blood was stored in heparinized eppendorf tubes and mixed with 0 μL, 100 μL, 200 μL ,300 μL of HbVs (0.1mg/mL, 0.2M PBS, 0.9% NaCl, pH 7.4) respectively. Afterwards, the mixture was incubated immediately at 37 ^oC in a water bath incubator with constant shaking. The blood cell number was counted after a period of time. Whole blood without HbVs was tested as positive control. The morphology changes were recorded by optical microscope. All of the experiments were carried out in triplicate, and the counted cell number was shown as mean ± SD of independent groups.

Results and discussion

Synthesis of Dextran-g-PLA

The grafting onto strategy has been applied for the synthesis of dextran-g-PLA copolymer as described in Scheme 1. The C-PLA was synthesized by ring-opening polymerization of L-lactide in the presence of Sn(Oct)2 using 2,2-bis(hydroxymethyl)propionic acid as initiators. Then the dextran-g-PLA was made through coupling C-PLA to the pendant of dextran in the presence of DCC/DMAP.



Scheme 1 Synthesis of dextran-g-PLA.

The ^{1}H NMR spectrum of dextran, C-PLA, dextran-g-PLA was shown in Fig. 1. The characteristic peaks at 1.3 and 5.2 ppm for C-PLA segments, and signals at 4.5~4.8 ppm for dextran appeared in ^{1}H NMR spectra of dextran-g-PLA, which indicated the successful synthesis of dextran-g-PLA. The number-average molecular weight (Mn) of the C-PLA calculated by comparing the integrals of the methine proton (δ_e) and methyl protons (δ_h) on the initiator is 1800 g/mol. The mass ratio of dextran to PLA in copolymers could also be obtained by comparing the integral area of 5.2 ppm and 4.63 ppm.

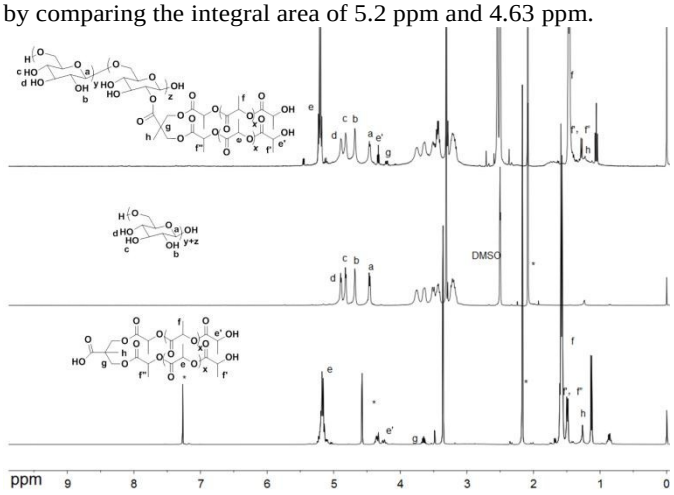


Fig. 1 ^{1}H NMR spectra (400 MHz) dextran and dextran-g-PLA were dissolved in DMSO, and C-PLA was dissolved in $CDCl_3$.

As shown in Table 1, the molecular weight and distribution were determined by GPC using DMF as the eluent. GPC results showed a polydispersity of ~ 1.20 for these copolymers (Table 1 and Fig. S1). We synthesized four copolymers with various molecular weights by changing the ratio of dextran to C-PLA in the feed. The detailed information for copolymers dextran-g-PLA was displayed in Table 1.

Self-assembly of copolymers dextran-g-PLA

The nanoparticles were made using solvent exchange with DMSO/H_2O. Meanwhile, the intact nanoparticles could be obtained by dispersing lyophilized powder into aqueous solution, which was examined by TEM (Fig. 2).

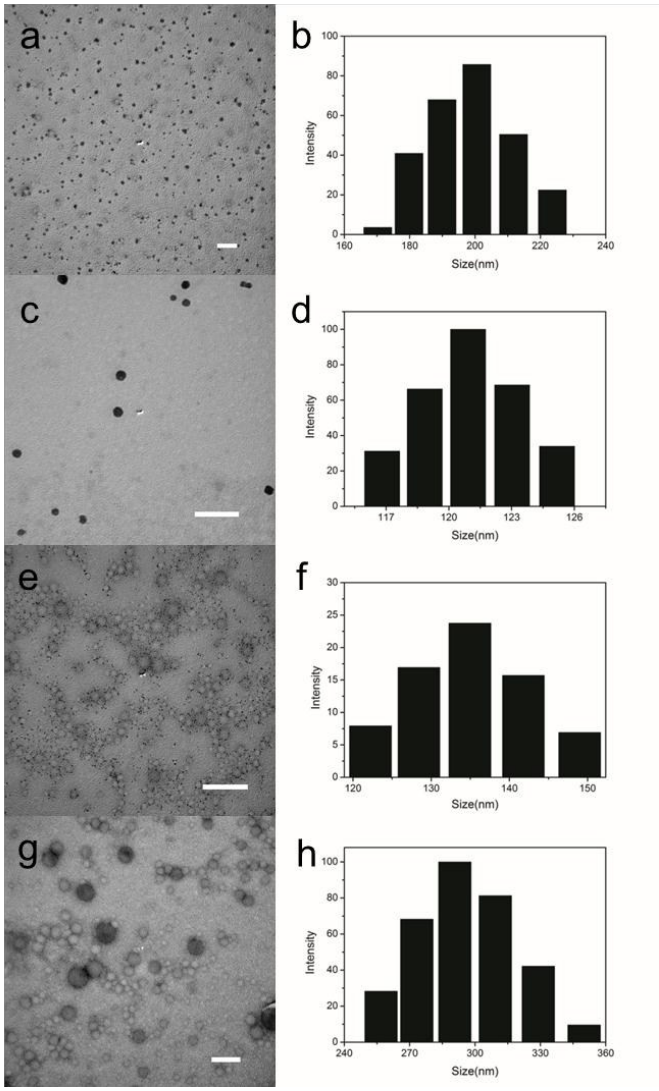


Fig. 2 The morphology of dextran-g-PLA in TEM, Dextran-g-PLA1 (a), Dextran-g-PLA2 (c), Dextran-g-PLA3 (e), Dextran-g-PLA4 (g) and the size distribution of dextran-g-PLA monitored by DLS, Dextran-g-PLA1 (b), Dextran-g-PLA2 (d), Dextran-g-PLA3 (f), Dextran-g-PLA4 (h). The scale bar stands for 500 nm with each TEM image.

TEM images showed copolymer dextran-g-PLA1 and dextran-g-PLA2 formed spherical nanoparticles with diameter of 156 and 101 nm respectively. Dextran-g-PLA3 and dextran-g-PLA4 gave vesicular structure with diameter of 125 and 252 nm. The size and size distribution of nanoparticles were determined by DLS as shown in Fig. 2 and table 2. The size obtained by DLS was hydrodynamic diameter, so it was larger than that determined by TEM.

Table 1. GPC and ^{1}H NMR characterizations of dextran-g-PLA

Copolymer	Mass ratio in feed	Mass ratio in copolymer[a]	Mn[b]	PDI[b]
Dextran-g-PLA1	1:05	1:0.3	7800	1.09
Dextran-g-PLA2	1:1	1:0.6	8200	1.21
Dextran-g-PLA3	1:1.5	1:0.75	10400	1.17
Dextran-g-PLA4	1:2	1:1	11200	1.17

[a] Mass ratio of dextran to PLA in the copolymers dextran-g-PLA calculated by ^{1}H NMR;
[b] Determined by GPC in DMF using polystyrene standards.

Table 2. Characterizations of nanoparticles formed by dextran-g-PLA.

Copolymer	f^a(%)	Morphology	Size(nm)		PDI^c
			TEM^b	DLS^c	
Dextran-g-PLA1	76.9	sphere	156±30	195±25	0.179
Dextran-g-PLA2	62.5	sphere	102±10	121±4	0.247
Dextran-g-PLA3	57.1	vesicle	125±20	135±13	0.190
Dextran-g-PLA4	50	vesicle	252±50	295±50	0.217

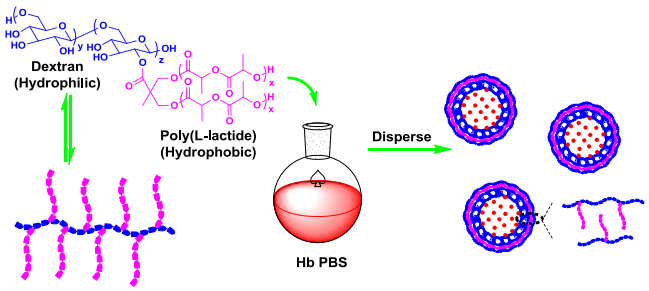
f^a Mass fractions of dextran segment in the copolymers dextran-g-PLA calculated by ^{1}H NMR.^{b} determined accoding to TEM. ^{c} determined by DLS.

Stability of polymeric vesicles is important for their application in biomedical field, especially for drug encapsulation and delivery. Herein, the vesicles formed by dextran-g-PLA3 were used to study the stability. Fig. S2 gave the size stability with time monitored by DLS. The formed vesicles possess high stability, which is vital for their following application in encapsulating Hb.

Characterization of hemoglobin vesicles

As shown above, dextan-g-PLA3 and dextan-g-PLA4 can form vesicles with hollow structure in their core. In order to avoid the serious side effects caused by vasoconstriction, the size of HbVs should be preferably less than 1 μm and more than 100 nm.[6] In detail, oxygen carriers that are 20-50 nm in diameter can readily clear by renal, and particles with size of larger than 1 μm can be rapidly removed by the reticuloendothelial system.[1] In addition, there is evidence that particles greater than 200 nm in the circulation can induce pulmonary hypertension.[31] Herein, copolymer dextan-g-PLA3 was used to study their ability for encapsulating Hb as a potential blood substitutes.

Both emulsion process[32, 33] and lyophilisation-rehydration method[34] have its own disadvantages. In this work, HbVs were obtained by the simple mixing Hb solution with lyophilized powder of dextan-g-PLA3 at room temperature as shown in Scheme 2. Free Hb was removed via centrifugation. The morphology of HbVs was characterized by TEM and DLS (Fig. S3).



Scheme 2 Schematic representation of the Hb encapsulation in the vesicles.

The loading efficiency and the Hb content in the vesicles were shown in Fig. 3. The loading efficiency decreased with increasing initial Hb, but rose slightly with increasing temperature. Meanwhile, loading content increased with increasing initial Hb amount and temperature. It is not necessary to load Hb at 60°C as the efficiency increase slightly.

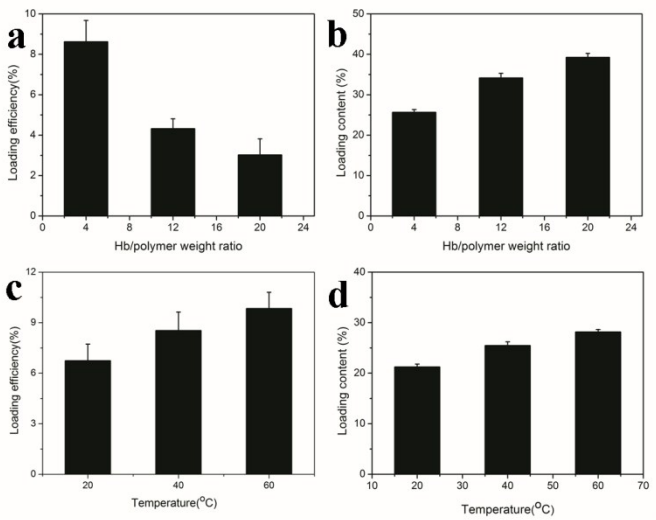


Fig. 3 Loading efficiency (a) and loading content (b) with different Hb/polymer weight ratio at room temperature, loading efficiency (c) and loading content (d) at different temperature with fixed Hb/polymer weight ratio. (dextan-g-PLA3).

To demonstrate that Hb was encapsulated in the cavity of vesicle, SDS-PAGE was performed afterwards. Samples with and without Triton X-100 treatment was shown in Fig. S4. The sample without Triton X-100 treatment showed no signal (Fig. S4a). Due to the release of encapsulated Hb, the sample treated with Triton X-100 appeared major band migrating at ~15 kDa. These results clearly confirmed that Hb was successfully encapsulated into the vesicles.

The ability of HbVs to bind oxygen was investigated by UV. As shown in Fig. 4a, the maximum absorption peak of COHb at 419 nm disappears, and the absorption peak of deoxyHb appears at 430 nm after degassing with nitrogen. When the deoxyHb was exposed to air, the characteristic peak of oxyHb appears at 415 nm. This result also indicates that the Hb encapsulated in the dextran-g-PLA3 vesicles can bind and release oxygen reversibly, and retains its own bioactivity after encapsulation like free Hb (Fig. 4b).

The degree of Hb interact with the polymer matrix influences the oxygen affinity of the oxygen carrier, which was influenced by changing conformations of Hb between the R and T states.[31] P_{50} is an indicator of oxygen affinity and refers to the partial pressure of oxygen at which half of the hemoglobin based oxygen carrier is half saturated with oxygen.[35] The P_{50} and Hill coefficient were both calculated by fitting equation as described elsewhere.[10, 30] In brief, P_{50} was the oxygen partial pressure (P) when O_2 saturation (Y) reached 0.5 fitted as sigmoidal curve. Hill coefficient was the slope of the Hill plot (Log(Y/(1-Y)) versus LogP) fitted linearly. Fig. 6a shows the oxygen dissociation curves of encapsulated Hb. P_{50} and Hill coefficient

values (Fig. 5) of HbVs were about 20 mmHg and 2.59, respectively, which was close to that of free Hb (26.6 mmHg and 2.8 in theory). These results indicated that the cooperative binding of O_2 to Hb was not yield after being encapsulated by the vesicles.

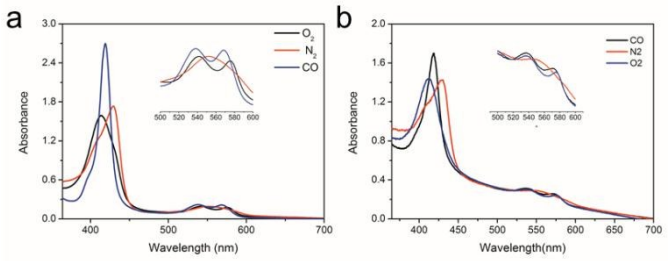


Fig. 4 UV-vis spectra of encapsulated Hb (a) and free Hb (b) in different gas-binding states (CO, O_2, N_2).

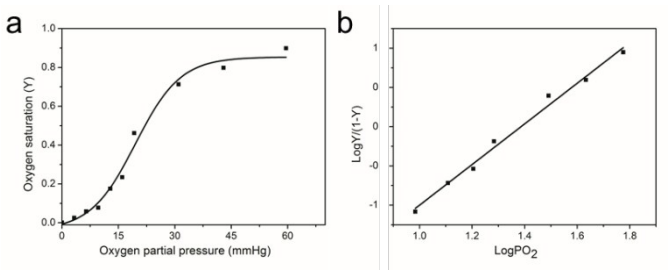


Fig. 5 Oxygen dissociation curve (a) and Hill plots (b) of HbVs from dextran-g-PLA3.

As oxygen carriers, the stability and compatibility with blood components has always been very important. The Hb leakage studies demonstrated that 20% of the Hb was leaked out after one week (Fig S5). There was no obvious burst happened during *in vitro* release. PBS (0.2M, pH 7.4) was used to simulate the physiological environment. All groups showed similar profile with whole blood observed by microscopy, which indicated no obvious hemolysis and further cell damage (Fig. S6). To further confirm the hemocompatibility of our HbVs, blood cell counting was performed. Fig. 6 showed the cell count results of four group (whole blood (1), 20% HbVs (2), 40 % HbVs (3) and 60% HbVs (4)) before and after incubation. As the volume of whole blood decreased each group, the number of RBC indicated a little decrease. The number of PLT in 60% HbVs is much higher than other group. This may cause by aggregates of HbVs comparable to PLT in volume, as no obvious hemolysis was observed. There is no significance difference over time. The result indicated that the HbVs formed from dextran-PLA3 had no detrimental effects on blood components when infused into blood.

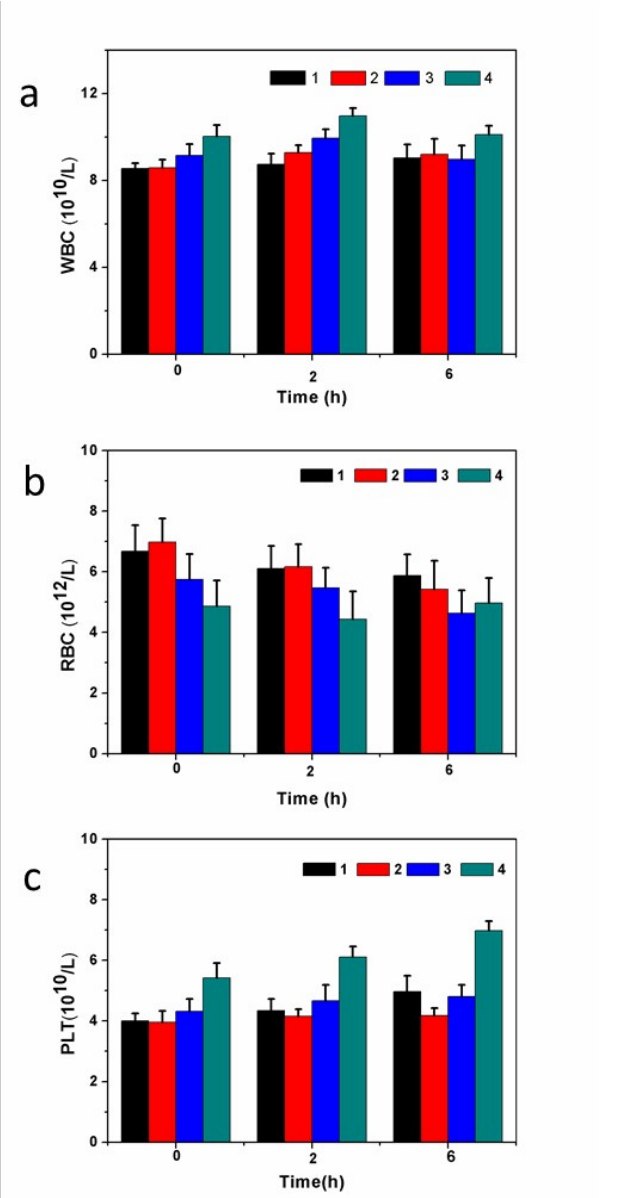


Fig. 6 The blood cell counting of the blood mixed with the HbVs dispersion *in vitro* at different incubation time, WBC (a), RBC (b), PLT (c). whole blood (1), 20% HbVs (2), 40 % HbVs (3) and 60% HbVs (4)

Conclusions

In conclusion, a series of graft copolymer based on dextran and PLA were successfully synthesized. They could self-assemble into nanoscale particles in aqueous solution. Different morphologies were obtained by adjusting the ratio of hydrophilic/hydrophobic segments. Due to the ultra-stable feature of the formed vesicles, it could serve as a delivery vehicle for encapsulating Hb. The encapsulated Hb retained its biological activity and showed excellent stability and compatibility with blood components. Importantly, the process is facile, green and amenable to scale-up.

Acknowledgements

Financial support was provided by the National Science Foundation of China (Project no. 91227118 and 51373167).

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†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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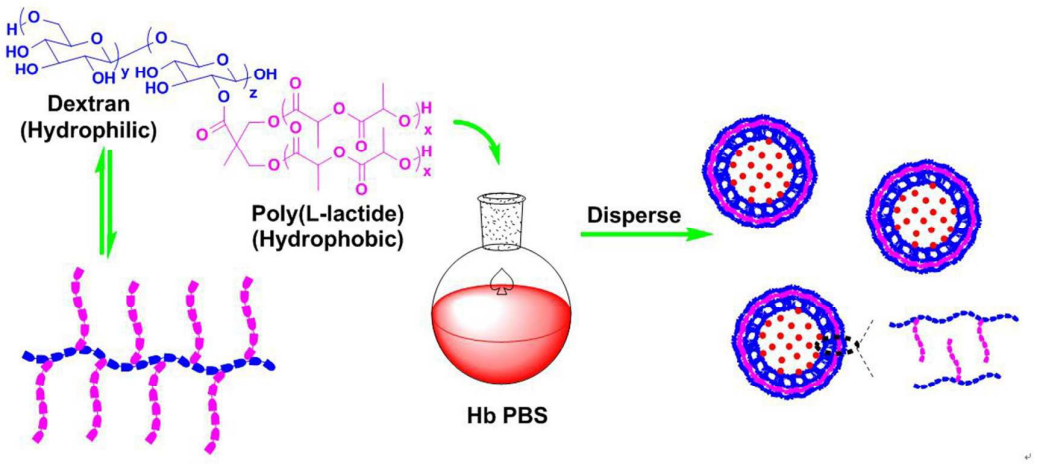

Biocompatible and biodegradable dextran-PLA copolymer self-assembled into polymeric vesicles, which could encapsulate the hemoglobin. The encapsulated hemoglobin retained biological activity and could be potentially used as blood substitute.