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Construction and Evaluation of Hemoglobin-Based Capsules as Blood Substitutes

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Hemoglobin-based capsules for use as blood substitutes are successfully fabricated by covalent layer-by-layer assembly. Dialdehyde heparin (DHP) is used both as one of the wall components and a cross-linker without employing other extraneous or toxic crosslinking agents. The biocompatibility of $(Hb/DHP)_6$ microcapsules is evaluated through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay and cell experiments. The hemocompatibility of $(Hb/DHP)_6$ microcapsules is characterized in terms of prothrombin time, thrombin time, activated partial thromboplastin time, and hemolysis rate. The oxygen-carrying capacity of the microcapsules is demonstrated by converting the deoxy-Hb state of the microcapsules into the oxy-Hb state. All these results demonstrate that the hemoglobin-based microcapsules exhibit oxygen-carrying capacity as well as biocompatibility and hemocompatility, indicating that the as-prepared capsules have great potential to function as blood substitutes.

1. Introduction

Blood transfusion is currently an indispensable and common clinical procedure, widely used for saving lives as well as maintaining the normal function of human organs. However, the donated blood used in medical practice often meets with various difficulties, such as blood-type mismatching, short shelf-life, severe shortage of blood inventory, and virus transmission (for example, HIV, malaria, or hepatitis) through the blood product. From this point of view, research on blood substitutes has aroused great concern and rapid progress have been made in this field.[1–6]

Among the blood substitutes developed to date, hemoglobin-based oxygen carriers (HBOCs) have attracted the most attention. Hemoglobin (Hb) possesses both the osmotic activity and the ability to deliver and release oxygen. However, stroma-free Hb was not a good candidate as a viable oxygen carrier since it dissociated into dimers that have a short circulation time, renal toxicity, high oncotic pressure, and high O_2-affinity.[7,8] Since the 1950s, various approaches have been developed to overcome these problems, such as surface modification,[9,10] polymerization,[11,12] polymer conjugation,[13,14] intramolecular crosslinking,[15,16] intermolecular crosslinking,[17] and recombination.[18–21] But side effects also occurred in some cases due to the strikingly different structure in comparison with red blood cells (RBCs).[22] An alternative route is to encapsulate Hb with polymer membrane to solve all the problems of molecular Hb. Chang performed the pioneering work of microencapsulation of Hb and enzymes using nylon and collodion to mimic the structure of RBC.[23] After that, the encapsulation of Hb within a phospholipid vesicle was studied by Djordjevich and Miller.[24,25] Other scientists and groups have also investigated encapsulation of Hbs with gelatine, lipid, polymersome, and so forth.[4,6,26] However, it has remained challenging to make Hb-based blood substitutes with a regulated diameter, nontoxicity, biodegradability, biocompatibility, hemocompatibility, and good O_2-transporting capacity.

In this work, we fabricated capsules that mimic artificial red blood cells using hemoglobin and oxidized heparin (dialdehyde heparin, DHP) by applying a layer-by-layer assembly strategy. DHP was used both as one of the wall components and as a cross-linker because of its nontoxicity, biodegradability, biocompatibility, and hemocompatibility. In addition, the negative charges of DHP on the microcapsules' surfaces would be beneficial to prolong the blood retention time of microcapsules in vivo. Combining with the advantages of the layer-by-layer technique, the size and behavior of as-prepared capsules could be well controlled and adjusted to have similar properties to RBCs by changing the templates and wall components; this endows these capsules with great potential to function as blood substitutes.

2. Results and Discussion

Spherical $MnCO_3$ particles with an average diameter of 3 μm served as templates for hollow capsule assembly. Hb was firstly adsorbed onto the template cores to produce amino group surfaces. Then Hb-coated particles were added into DHP solution. Hb was cross-linked with DHP through imine linkage

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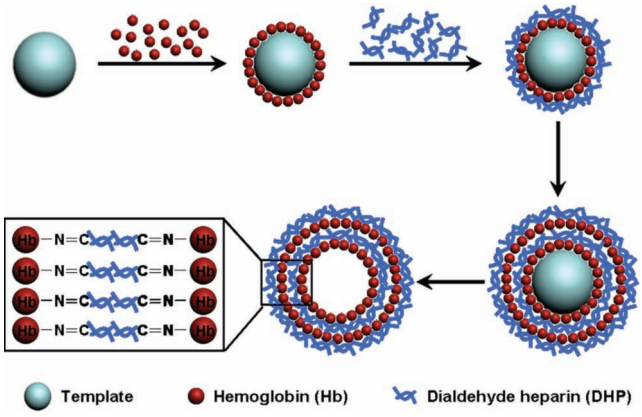

Scheme 1. Schematic illustration to show the fabrication process of Hb/DHP microcapsules through Schiff's base bond.

between amino groups of Hb and aldehyde groups of DHP; thus, the DHP was immobilized. The Hb and DHP were alternately adsorbed until the assembly of a desired number of Hb/DHP layers was complete. Hb/DHP multilayer were stabilized by employing DHP both as one of the wall components and a cross-linker, without employing any extraneous crosslinking agents. Then, $MnCO_3$ templates were dissolved in a Na_2EDTA solution (pH 7.0) and hollow microcapsules were obtained as shown in **Scheme 1**. It should be noted that selecting 3 μm spherical $MnCO_3$ particles as templates was only to facilitate characterization and observation. In consideration of the advantages of layer-by-layer assembly, many kinds of particles ranging in size from nanometers to micrometers could be used as templates to adjust the sizes of the Hb-based capsules. In this paper, Hb-based nanocapsules with an average diameter of 500 nm were also fabricated on $CaCO_3$ nanoparticles (Figure S1 and S2 in the Supporting Information).

2.1. Optical Images of Microcapsules

A transmission electron microscopy (TEM) image of $(Hb/DHP)_6$ microcapsule is shown in **Figure 1**a. It can be seen that the hollow microcapsule preserved its structural integrity and showed typical folds and creases in a dried state. Figure 1b–d are CLSM images of $(Hb/DHP)_6$ microcapsules excited at 488 nm. It is clear that the $(Hb/DHP)_6$ microcapsules displayed an intriguing autofluorescent property without conjugating to any external fluorochromes. This phenomenon can be attributed to the n–π^* transition of C–N bonds in the Schiff's bases formed during the crosslinking reaction between amino groups of Hb and aldehyde groups of DHP.[27–30] The autofluorescence of these capsules would be beneficial in predicting and monitoring the safety and efficacy of Hb-based capsules in humans, while avoiding the use of external fluorochromes for biological tracing that may influence the functionality of the capsules. In addition, the crosslinking reaction between amino groups of Hb and aldehyde groups of DHP prevented the dissociation of the Hb tetramer into dimers (Figure S3 and S4 in the Supporting Information),[31] which would improve the half life of Hb and avoid renal toxicity.

2.2. Biocompatibility and Biodegradability of Microcapsules

In order to assess the biocompatibility of microcapsules, the in vitro cytotoxicity of the microcapsules on human umbilical vein endothelial cells (HUVEC) was examined using an MTT assay. The results demonstrated that no significant cytotoxic activity for the microcapsules on their own was observed (**Figure 2**). Additionally, with CLSM, we can directly observe the status of the cells incubated with microcapsules. **Figure 3** shows CLSM images and the corresponding bright-field image of $(Hb/DHP)_6$ microcapsules. The cell membrane stained with Alexafluro 488 appears green in color, whereas microcapsules with Rhodamine B isothiocyanate labeled Hb are red. It can be seen clearly that the cell coexisted with microcapsules without any abnormalities, which confirmed the biocompatibility of the microcapsules. It is also noteworthy that microcapsules with red fluorescence are distributed around cells without being uptaken by the cells. This can be attributed to the electrostatic repulsion between negative charges on the cell membrane surface and the microcapsule surface (Table S1 in the Supporting Information), which will help to prolong the blood retention time of microcapsules in vivo.[32]

To access the degradation behavior of $(Hb/DHP)_6$ microcapsules, CLSM images were taken after incubated with trypsin solution for 24 h. No microcapsule was observed after degradation (Figure S5 in the Supporting Information), confirming that $(Hb/DHP)_6$ microcapsules were biodegradable.

2.3. Opsonization and Phagocytosis of Microcapsules

When particles are injected intravenously, they are rapidly opsonized by plasma proteins. Serum albumin and immunoglobulin G (IgG) represent, respectively, the most abundant protein (35–52 g L^{-1}) and immunoglobulin (7–16 g L^{-1}) in the blood.[33] It has been suggested that albumin may exert a dysopsonic effect, whereas IgG is thought to act as a potent opsonin that promotes recognition and uptake by cells of the mononuclear phagocyte system (MPS).[34] Therefore, reduction or inhibition of opsonization is a necessary prerequisite to developing blood-contacting materials, especially for blood substitutes with long plasma retention time. In this work, we have investigated whether the use of covalently bound periodate-oxidized heparin (dialdehyde heparin, DHP) to prepare Hb-based microcapsules could reduce opsonization and thus reduce uptake by macrophages. The $(Hb/DHP)_5Hb/PEG$ microcapsules were used as a comparison. **Figure 4**a presents the percentages of bovine serum albumin (BSA) and Immunoglobulin G (IgG) adsorbed onto the surfaces of $(Hb/DHP)_6$ microcapsules and $(Hb/DHP)_5Hb/PEG$ microcapsules following an incubation period of 24 h. It can be seen that DHP-coated microcapsules adsorbed more BSA and less IgG compared to PEG-coated microcapsules in terms of percentage of adsorbed protein. It is well documented that PEG has protein-rejecting properties and could enhance the plasma residence time.[33] Our data suggest that DHP at the surface of the microcapsules promoted dysopsonization and reduced opsonization with respect to PEG, which would be more favorable to reduce uptake by macrophages and prolong the blood retention time of microcapsules in vivo.

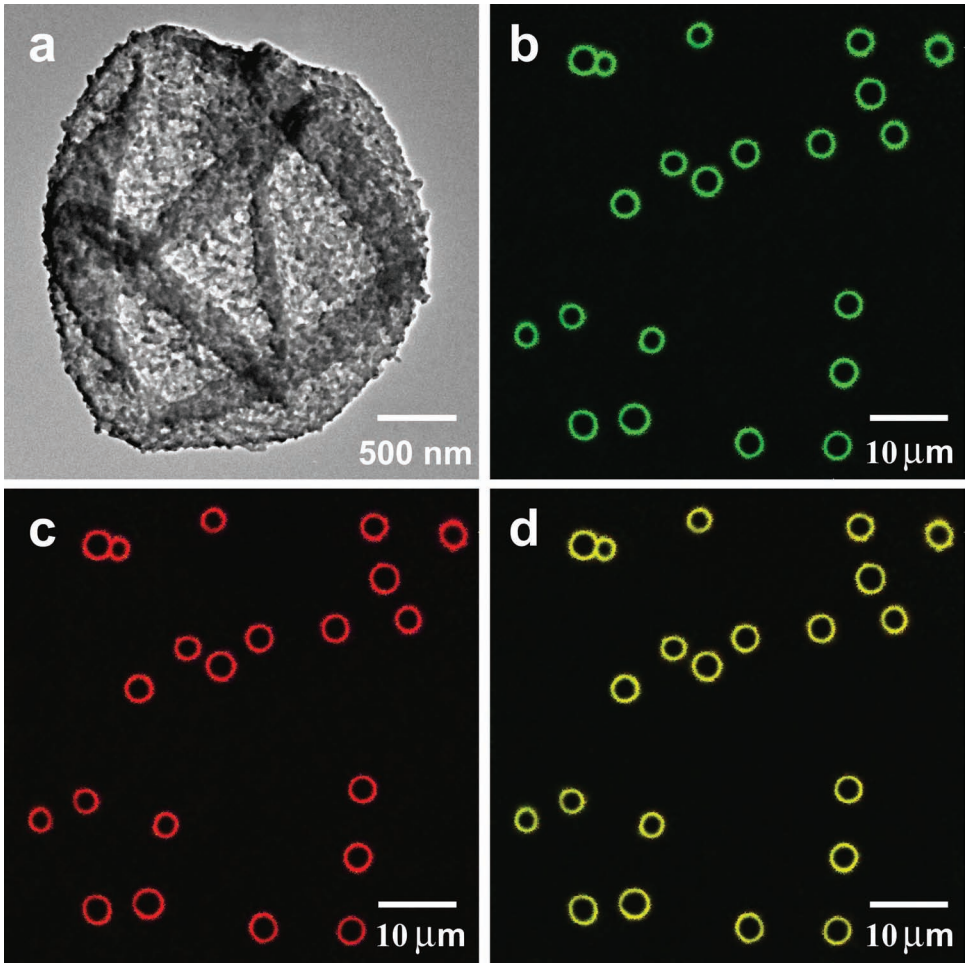

Figure 1. a) TEM image of $(Hb/DHP)_6$ microcapsule, the scale bar represents 500 nm. b–d) CLSM images of $(Hb/DHP)_6$ microcapsules. The samples were excited at 488 nm and three fluorescent images obtained: b) 510–540 nm; c) 570–600 nm; and d) overlay. The scale bars represent 10 μm.

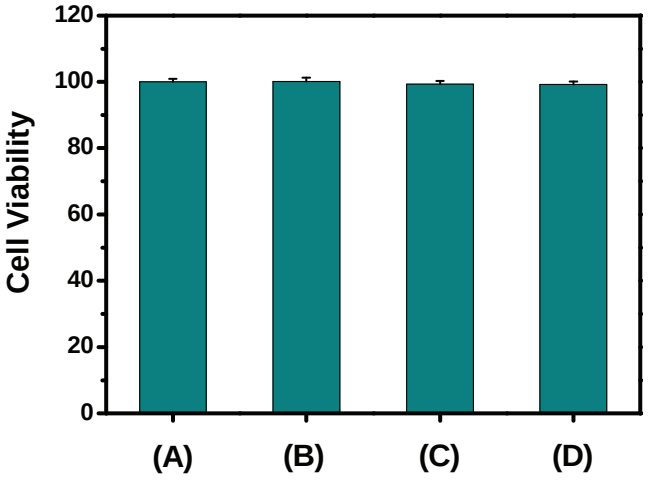

Figure 2. In vitro cytotoxicity of microcapsules on HUVEC cells: A) a negative control, the cells were incubated under normal conditions without microcapsules; B, C, and D) cells were incubated with 20, 40, 60 μL 0.11 mg mL^{-1} $(Hb/DHP)_6$ microcapsules, respectively. Each error bar represents the mean of at least six measurements (±S.D.).

We then investigated the phagocytic capacity of murine macrophage cells (J774A.1) for the $(Hb/DHP)_6$ microcapsules. As shown in Figure 4b,c, when J774A.1 cells were incubated with $(Hb/DHP)_6$ microcapsules, only a few microcapsules were found in the macrophages. Such a minimal cellular uptake by macrophages exhibits a similar stealth effect compared to PEGylation, which might also be beneficial to prolong the blood circulation time of these microcapsules.[35,36]

2.4. Hemocompatibility of Microcapsules

Hemocompatibility is a key property of biomaterials that come in contact with blood. When a foreign material is exposed to blood, the intrinsic coagulation process would be initiated either on the surface of aggregating platelets or by activation of clotting factors on the foreign material which would eventually lead to the formation of an insoluble fibrin network or thrombus.[37] The most popular technique used to improve hemocompatibility is to modify the materials with antithrombogenic materials.[38,39] Heparin is widely used as an antithrombotic agent, but its dose has been limited due to its strong intrinsic

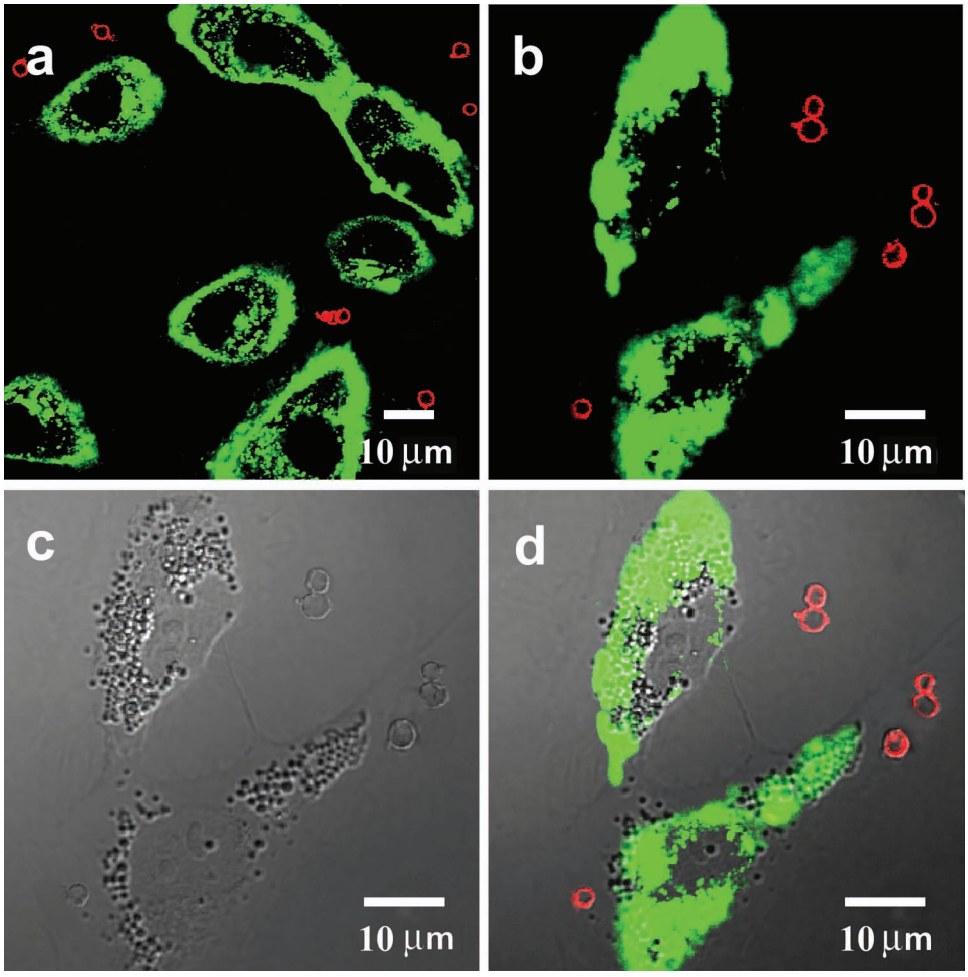

Figure 3. a,b) CLSM images of HUVEC cells stained with Alexafluor 488 incubated with $(Hb/DHP)_6$ microcapsules by excitation at 488 and 559 nm, respectively. c) Corresponding bright-field image of (b). d) The overlapped image of (b) and (c).

anti-coagulant property, which may cause severe bleeding complications. It is reported that periodate-oxidized heparin (dialdehyde heparin, DHP) do not have a specific pentasaccharide structure to interact with antithrombin III, therefore its anti-coagulant activity is much lower than heparin.[40] In addition, DHP contains abundant aldehyde groups which could easily react with other groups. Therefore, in this paper, we employed DHP both as one of the wall components and a cross-linker to fabricate $(Hb/DHP)_6$ microcapsules using the layer-by-layer assembly technique. The hemocompatibility of $(Hb/DHP)_6$ microcapsules was characterized in terms of prothrombin time (PT), thrombin time (TT), and activated partial thromboplastin time (APTT). The platelet poor plasma (PPP) from a healthy volunteer blood donor was used as a control. As shown in **Figure 5**, the PT, TT, and APTT values of $(Hb/DHP)_6$ microcapsules were (12.3 ± 0.3), (20.5 ± 0.2), and (34.3 ± 0.2) s, respectively, which were higher than those of the control ((11.8 ± 0.2), (15.1 ± 0.1), and (25.7 ± 0.3) s) but still wthin acceptable normal values of blood ((9.8–13.1), (14.0–21.2), and (25.4–38.4) s). The results indicated that $(Hb/DHP)_6$ microcapsules had an

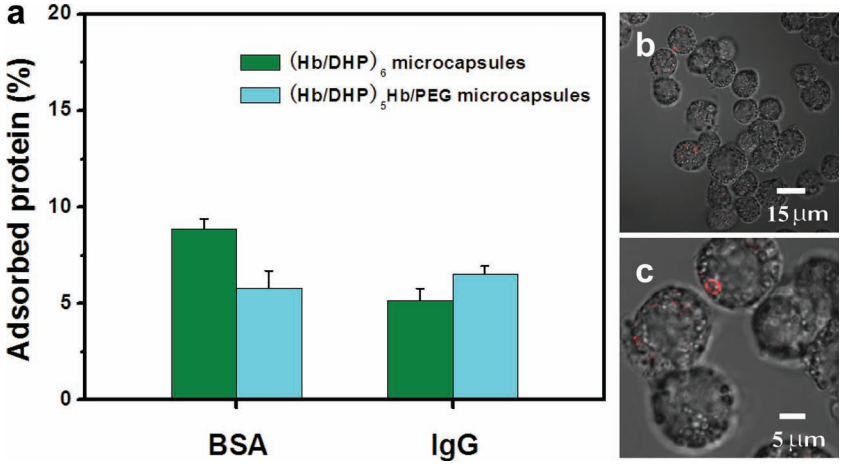

Figure 4. a) The percentages of BSA and IgG adsorbed on the microcapsules with respect to the total amount of protein. b,c) CLSM images of murine macrophage cells (J774A.1) co-incubated with 20 μL 0.11 mg mL^{-1} $(Hb/DHP)_6$ microcapsules.

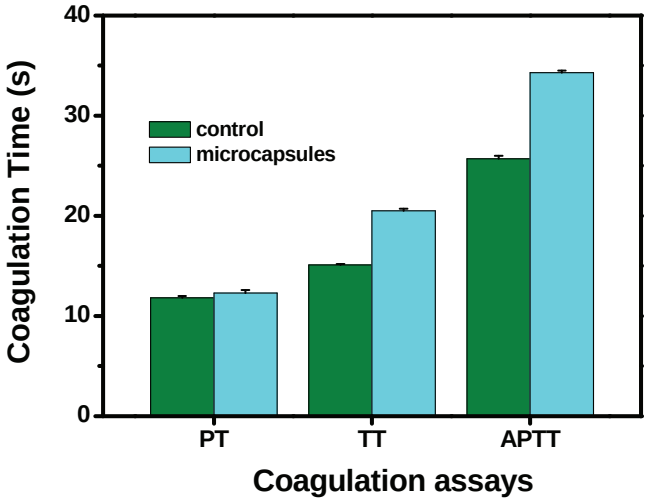

Figure 5. The effects of $(Hb/DHP)_6$ microcapsules on plasma coagulation times, including PT, TT, and APTT. Data are presented as the mean of three measurements.

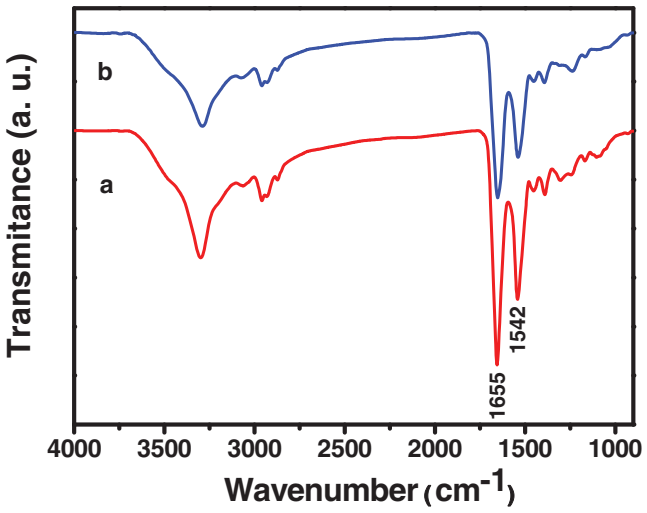

Figure 6. FTIR spectra of: a) native Hb and b) $(Hb/DHP)_6$ microcapsules.

anticoagulant property at a certain extent and exhibited excellent blood compatibility.[41]

Besides no coagulation, a low hemolysis rate is also an important factor for the hemocompatibility of a material. **Table 1** shows the hemolysis rates of the microcapsules and the controls. It can be seen that the hemolysis rate of the $(Hb/DHP)_6$ microcapsules was well within 1%, which is far below the accepted threshold value of 5%,[42–44] implying a good hemocompatibility.

Taken together, the cell experiments, coagulation assays and hemolysis data suggested that $(Hb/DHP)_6$ microcapsule was a biocompatible and blood-compatible material warranting further application for use as a blood substitute.

2.5. Oxygen-Carrying Capacity of Microcapsules

The oxygen-carrying capacity is the most important issue for Hb-based blood substitutes. The maintenance of the protein structure is a prerequisite for the fulfillment of this physiological function. Therefore, a preliminary study of the effect of the encapsulating process on Hb chemical structure was conducted. FTIR spectra of native Hb and $(Hb/DHP)_6$ microcapsules are shown in **Figure 6**. The N–H bending vibrations (amide II) were located at 1542 and 1540 cm^{-1} for Hb and $(Hb/DHP)_6$ microcapsules, respectively, while those for the C–O stretching vibration (amide I) were located at 1655 and 1653 cm^{-1}, respectively.[45,46] The comparison of FTIR spectra between native Hb and $(Hb/DHP)_6$ microcapsules indicated that the chemical structure of the Hb assembled in $(Hb/DHP)_6$ microcapsules was maintained, since the characteristic absorption bands of the amide I and amide II were almost identical. Thus, it was demonstrated that the encapsulation process had no influence on the chemical structure of Hb in $(Hb/DHP)_6$ microcapsules.

To verify whether the Hb assembled in the $(Hb/DHP)_6$ microcapsules maintains its own bioactivity and is capable of reversibly binding and releasing oxygen, the oxygen-carrying capacity of $(Hb/DHP)_6$ microcapsules was studied in detail. In general, ferrous iron in Hb molecules could be easily oxidized by oxygen in the water or air and form methemoglobin in which the central ferric ion cannot coordinate with molecular oxygen. For this reason, the $(Hb/DHP)_6$ microcapsules solution was first reduced with L-ascorbic acid for 20 min to obtain ferrous Hb. Then N_2 and O_2 gas were successively flowed over the $(Hb/DHP)_6$ microcapsules solution to obtain deoxy-Hb and oxy-Hb based microcapsules, respectively. The reactions were monitored through scanning from 350 to 600 nm by using a UV-vis spectrometer. **Figure 7**a shows the characteristic peak of deoxy-Hb at 415 nm after feeding N_2 for 1 h. When O_2 gas was flowed over the deoxy-Hb based microcapsules, the state of the Hb assembled in the $(Hb/DHP)_6$ microcapsules was successfully transformed to an O_2-binding state with the characteristic peak of oxy-Hb at 406 nm in Figure 7b.[47,48] After that, N_2 gas was again flowed over the oxy-Hb based microcapsules to demonstrate that whether the Hb assembled in the $(Hb/DHP)_6$ microcapsules have the ability to release oxygen. It can be seen from Figure 7c that after N_2 gas was fed for 1 h, the characteristic absorption peak shifted from 406 nm back to 415 nm, indicating the Hb assembled in the $(Hb/DHP)_6$ microcapsules was transformed from the oxy-Hb to the deoxy-Hb state. It was demonstrated that the $(Hb/DHP)_6$ microcapsules could reversibly bind and release oxygen, and so possess the essential function of an oxygen carrier.

Table 1. Hemolysis rate of the $(Hb/DHP)_6$ microcapsules.

Group	Absorbance	Hemolysis rate [%]
Microcapsules	0.010 ± 0.001	0.68
Negative control	0.004 ± 0.001	0.00
Positive control	0.885 ± 0.003	100.00

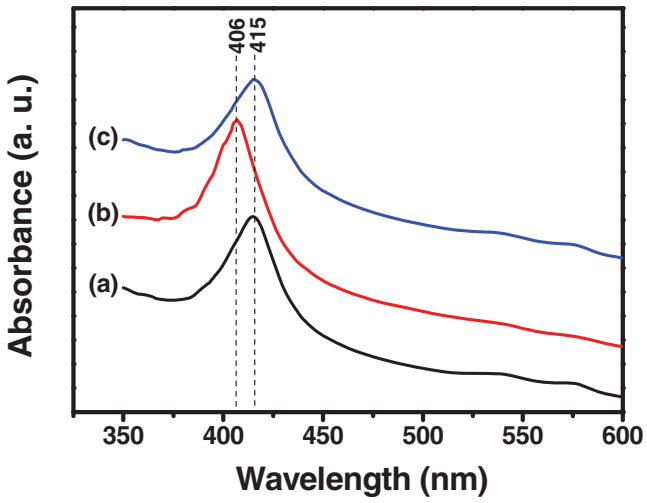

Figure 7. UV-vis absorption spectra of $(Hb/DHP)_6$ microcapsules after successively feeding: a) N_2, b) O_2, and c) N_2 for 1 h each.

3. Conclusions

$(Hb/DHP)_6$ microcapsules were successfully fabricated by covalent layer-by-layer assembly. The as-prepared microcapsules were biocompatible, biodegradable, and blood compatible. DHP was used both as one of the wall components and a crosslinker without employing other extraneous or toxic crosslinking agents. The crosslinking reaction between DHP and Hb prevented the dissociation of Hb tetramer into dimers, which should improve the half life of Hb and avoid renal toxicity. The electrostatic repulsion between negative charges on the microcapsule surfaces and cell membrane surfaces would help to prolong the blood retention time of microcapsules in vivo. More importantly, the Hb assembled in the $(Hb/DHP)_6$ microcapsules maintained its own bioactivity and possessed the essential function of an oxygen carrier. It is noteworthy that, in view of the advantages of the layer-by-layer assembly technique, there are many conceivable ways to adjust and improve the behavior of the Hb-based capsules; functional groups or reagents (such as allosteric effector, superoxide dismutase, or methemoglobin reductase) may be chemically modified or covalently coupled to hemoglobin, in addition to the possibility of replacing DHP itself by other polysaccharides or their derivatives, such as dialdehyde starch, dialdehyde alginate, etc.[27] Consequently, the construction and behavior of hemoglobin-based capsules are open to a wide range of variations. These variations could provide a basis for maximizing the advantages of hemoglobin-based capsules and minimizing its disadvantages by means of systematic and reasonable design, which make the obtained microcapsules promising candidates for applications in blood substitutes, oxygen carriers, and other biomedical fields.

4. Experimental Section

Materials: Bovine hemoglobin (Hb, MW 64,500), fluorescein isothiocyanate-labelled bovine serum albumin (FITC-BSA), immunoglobulin G (FITC-IgG), sodium dodecyl sulfate (SDS), and Alexafluor 488 were obtained from Sigma-Aldrich. Sodium metaperiodate ($NaIO_4$), and heparin sodium were obtained from Sinopharm Chemical Reagent Co., Ltd. Trypsin was purchased from Amresce. Sodium chloride (NaCl), ethylene glycol, ethanol, and Na_2EDTA were obtained from Beijing Chemical Corporation. All chemicals were used without further purification. The water used in all experiments was prepared in a three-stage Millipore Milli-Q plus 185-purification system and had a resistivity higher than 18.2 MΩ.

Periodate Oxidation of Heparin Sodium: 0.6 g heparin sodium was dissolved in 50 mL distilled water, then 0.65 g sodium periodate was added and magnetically stirred in the dark at room temperature for 24 h to obtain the product. Then, 5 mL ethylene glycol was added to the above solution in the dark with continuous magnetic stirring and kept for 15 min to neutralize the solution. After reaction, the mixture was dialysed against distilled water (600 mL) for 48 h with several changes of water. The obtained dialyzate was further purified by centriguation and washing with ethanol (repeated three times), before drying at room temperature under vacuum.

Preparation of Capsules: Spherical $MnCO_3$ particles with an average diameter of 3 μm served as templates were synthesized by mixing $MnSO_4$ and NH_4HCO_3 solutions according to a previously reported method.[49,50] Periodate oxidized heparins were prepared according to a previously reported method.[27,51] $MnCO_3$ particles were first dispersed into 4 mg mL^{-1} Hb solution in acetate buffer (pH = 5) for 30 min to produce amino group surfaces, followed by three times centrifugation and washing with water. Then the Hb-coated particles were added into 4 mg mL^{-1} DHP solution in acetate buffer (pH = 5) for 12 h. Hb was cross-linked with DHP through imine linkage between amino groups of Hb and aldehyde groups of DHP and thus the DHP was immobilized. The Hb and DHP were alternately adsorbed until the assembly of a desired number of Hb/DHP layers. Then, $MnCO_3$ templates were dissolved in a Na_2EDTA solution (pH 7.0) and hollow microcapsules were obtained. In the following experiment, we take hollow $(Hb/DHP)_6$ capsules as an example, except where mentioned explicitly. The concentration of $(Hb/DHP)_6$ capsules was expressed by the quantity of Hb loaded on the capsules.

Characterization of Capsules: The samples were characterized by transmission electron microscopy (TEM, JEOL JEM-2011) and scanning electron microscopy (SEM, Hitachi S-4800). Fourier transform infrared (FTIR) spectra and UV-vis spectra were recorded by using a Tensor 27 instrument (Bruker, Germany) and U-3010 UV-vis spectrometer (HITACHI, Japan), respectively. The size distribution and surface charge (Zeta potential) of samples were acquired by using a ZEN3600 Zetasizer nano instrument (Malvern, England). Confocal laser scanning microscopy (CLSM) micrographs were taken with an Olympus FV500 confocal system (Carl Zeiss) equipped with 100× oil-immersion objective and a numerical aperture of 1.4. Individual aliquots of 20 μL were placed on a glass slide and a cover glass was coated before observation. The microcapsules were excited at 488 nm and two fluorescent images were obtained at 510–540 nm (green) and 570–600 nm (red).

SDS-PAGE Gel Electrophoresis: To determin whether the crosslinking reaction between Hb and DHP could prevent the dissociation of Hb tetramer into dimer, SDS-PAGE electrophoresis was conducted.

Cell Culture: Human umbilical vein endothelial cells (HUVEC) and murine macrophage cells (J774A.1) were supplied by the ATCC (American Type Culture Collection). Cells were cultured at 37 °C in a DMEM medium (Gibco BRL, USA) supplied with fetal bovine serum (10%), L-glutamine (2 mM), penicillin (100 U mL^{-1}) and streptomycin (25 mg mL^{-1}) in a humidified atmosphere with 5% CO_2. For the following experiments, cells were detached from culture flasks using phosphate buffered saline (PBS) containing EDTA (0.02%) and trypsin (0.05%) and seeded to 24-well plates or a 35 mm glass-bottom Petri dish.

Biocompatibility Test: Cells were incubated with 20 μL 0.11 mg mL^{-1} $(Hb/DHP)_6$ microcapsules for 12 h and washed three times with PBS. The cell membrane was labeled with Alexafluor 488 for 10 min, followed by washing. Then the cells were examined by CLSM.

In Vitro Cytotoxicty Assay: Cells were allowed to adhere to 48-well plates for 48 h, and then were incubated with 20, 40, or 60 μL 0.11 mg mL^{-1}

$(Hb/DHP)_6$ microcapsules for another 24 h. After incubation, 40 µL sterile filtered MTT in PBS was added to each well and incubated with the cells for 4 h at 37 °C. Then 400 µL dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystals, followed by measuring the absorbance at 600 nm with a PerkinElmer 1420 Multilable Counter.

Biodegradability Test: To prove the biodegradability of the microcapsules, 100 µL 0.11 mg mL^{-1} $(Hb/DHP)_6$ microcapsules were incubated in 10 mg mL^{-1} trypsin solution at room temperature overnight and then investigated by CLSM.

In Vitro Protein Adsorption on Microcapsules from Single Protein Solutions: 100 µL 0.11 mg mL^{-1} $(Hb/DHP)_6$ microcapsules and $(Hb/DHP)_5Hb/PEG$ microcapsules were incubated with FITC-BSA or FITC-IgG (0.5 mg mL^{-1}) in PBS pH 7.4 at 37 °C under constant agitation. After 24 h, samples were centrifuged (2000*g* for 5 min). The supernatants were analyzed by spectrophotometry and using a PerkinElmer 1420 Multilable Counter (λexc./em. 485/525 nm) for BSA and IgG, respectively. Protein adsorption was quantified relative to a control protein solution (0.5 mg mL^{-1}). Results are expressed as the percentage (%) of protein adsorbed on the microcapsules with respect to the total amount of protein.

The Phagocytosis of Microcapsules by Murine Macrophage Cells: Murine macrophage cells (J774A.1) were incubated with 20 µL 0.11 mg mL^{-1} $(Hb/DHP)_6$ microcapsules for 8 h, washed three times with PBS, followed by washing and examination through CLSM.

Coagulation Assays: Coagulation assays of prothrombin time (PT), thrombin time (TT), and activated partial thromboplastin time (APTT) were performed to evaluate material-induced abnormalities in the extrinsic and intrinsic coagulation pathways. PT is a sensitive screening test used to assess material-induced deferment or interdiction through the extrinsic coagulation pathway. TT is used to screening for disorders of fibrin formation or in suspected cases of severe fibrinogen deficiency states. APTT is a global screening procedure used to evaluate coagulation abnormalities in the intrinsic pathway, will also detect severe functional deficiencies in factors II, V, X, or fibrinogen.

For PT, TT, and APTT assays, human whole blood was drawn from a healthy volunteer by venipuncture into vacutainer tubes containing EDTA anticoagulant. Then the blood was centrifuged at 1097*g* at 4 °C for 15 min to separate the blood corpuscles, and the resulting platelet-poor plasma (PPP) was used for the coagulation test. 100 µL PPP was incubated with 20 µL 0.11 mg mL^{-1} $(Hb/DHP)_6$ microcapsules' solution at 37 °C. The time of fibrin (clot) formation was detected automatically by a change in the turbidity of the solution using the Sysmex CA-1500 in Peking University People's Hospital.

Hemolysis Rate Test: Hemolysis rate is an important factor for characterization of the blood compatibility. 4 mL EDTA anticoagulated whole blood of a volunteer was incubated with 5 mL physiologic saline (0.9% w/v) to obtain diluted blood solution. 100 µL 0.11 mg mL^{-1} $(Hb/DHP)_6$ microcapsules solution was dispersed in 10 mL physiologic saline (0.9% w/v) and incubated at 37 °C for 30 min. Then, 0.2 mL diluted blood solution was added into microcapsule physiologic saline solution and incubated at 37 °C for another 1 h. After centrifugation at 122*g* for 10 min, the absorbance at 545 nm of the solution was recorded as D_t. Under the same conditions, the solution containing 0.2 mL diluted blood solution and 10 mL physiologic saline (0.9% w/v) was used as a negative reference, the solution containing 0.2 mL diluted blood solution and 10 mL dH_2O was used as a positive reference. Their absorbencies at 545 nm were recorded as D_{nc} and D_{pc}, respectively. The hemolysis rate α of the samples was calculated via the following formula: $\alpha = (D_t - D_{nc})/(D_{pc} - D_{nc})$

Oxygen-Carrying Capacity: The $(Hb/DHP)_6$ microcapsules were dispersed in PBS (pH 7.4), followed by adding L-ascorbic acid and sodium dithionite solution in PBS. After reaction for 20 min, nitrogen (N_2) gas was allowed to flow over the microcapsules' solution for 1 h to obtain deoxy-Hb based microcapsules and the absorbance was monitored by UV-vis spectroscopy. To verify whether the microcapsules could reversibly bind and release oxygen, the binding and releasing of oxygen was conducted by successively feeding oxygen (O_2) and nitrogen (N_2) for 1 h to obtain oxy-Hb and deoxy-Hb based microcapsules, respectively, followed by recording the UV-vis absorbance.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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