Highly Loaded Hemoglobin Spheres as Promising Artificial Oxygen Carriers

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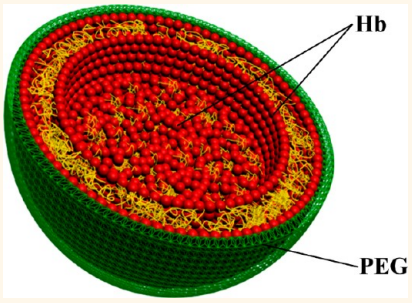
ABSTRACT



Seeking safe and effective artificial blood substitutes based on hemoglobin (Hb) as oxygen carriers is an important topic. A significant challenge is to enhance the loading content of Hb in a well-defined structure. Here we report a facile and controllable avenue to fabricate Hb spheres with a high loading content by templating decomposable porous $CaCO_3$ particles in collaboration with covalent layer-by-layer assembly technique. The surface of the Hb spheres was further chemically modified by biocompatible polyethylene glycol to protect and stabilize the system. Multiple characterization techniques were employed to reveal the loading and density of Hb in an individual $CaCO_3$ particle. The results demonstrate that the strategy developed in this work is effective and flexible for construction of the highly loaded Hb spheres. More importantly, such Hb spheres retain their carrying−releasing oxygen function. It may thus have great potential to develop Hb spheres with highly loaded content as realistic artificial blood substitutes in the future.

KEYWORDS: hemoglobin spheres · porous $CaCO_3$ · LbL assembly · high loading · artificial oxygen carriers

Allogeneic donor blood transfusion has been a routine and safe procedure for patients with conditions of serious blood deficiencies (*e.g.*, acute anemia, severe thrombocytopenia, hemophilia) over the last century. However, donor blood is a limited resource with a worldwide acute shortage.[1] Scientists are attempting to seek artificial blood substitutes for red blood cells without destroying their oxygen-carrying capability. Until now, there have been two types of blood substitutes under development: those derived from hemoglobin, called hemoglobin-based oxygen carriers (HBOCs), and those that use perfluorocarbon emulsions.[2] Hemoglobin (Hb) in red blood cells (RBCs) is responsible for the transport of oxygen. However, cell-free Hb is too toxic to serve as a blood substitute.[3,4] Therefore, it is challenging to modify cell-free hemoglobin purposefully. Several types of Hb modification approaches were developed in the last few decades.[1,5−9] The first generation is the glutaraldehyde (GA) cross-linked polyhemoglobin that has been tested clinically in patients.[10,11] The second generation are the conjugates of Hb with polymers (such as dextran,[12] polyethylene glycol,[13] or polyoxyethylene[14]), intramolecularly cross-linked Hb and recombinant Hb. The PolyHb and conjugated Hb are useful at clinical conditions where only oxygen carriers are requested. To meet more needs or conditions excluding oxygen carriers, a new generation of well-defined Hb containing antioxidant enzymes has to be developed. Such well-defined Hb opens possibilities to construct real artificial red blood cells.[15] Encapsulation of Hb can be achieved by biodegradable polymer capsules[16−20] or lipid vesicles,[21,22] but a significant challenge remains to enhance the loading concentration of Hb in a well-defined structure and to prolong the retention time in intravascular circulation.

Templating by porous calcium carbonate ($CaCO_3$) particles has been a novel strategy to fabricate monodisperse additive-free protein microspheres[23−25] due to its uniformity and monodispersity, easy preparation, controllable size from micrometer to nanometer,[26] mild decomposition conditions, and especially its high loading capacity. The layer-by-layer (LbL) assembly technique can be extensively applied to fabricate protein-dominant biomaterials. We have successfully constructed the hemoglobin-based capsules by covalent LbL assembly using $MnCO_3$ as well as calcium carbonate particles as templates.[27,28]

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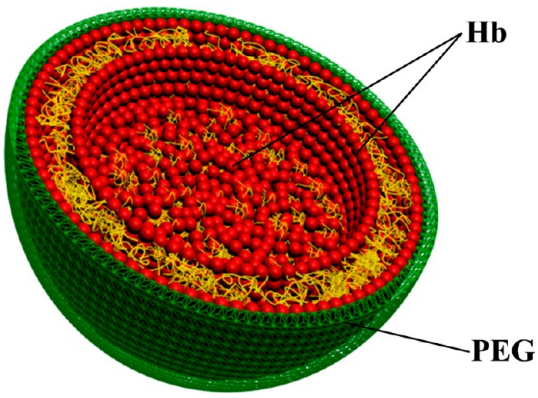
In combination with covalent LbL assembly, the co-precipitation of protein and calcium carbonate with porous structures can greatly increase the content of protein and present adjustable permeability,[27] which is of great importance for maintaining the normal functionalities of natural red blood cells, allowing life-sustaining small molecules such as oxygen, carbon dioxide, glucose, and metabolic products to diffuse into/out of the system.

Herein, we explored a facile and controllable avenue to fabricate Hb spheres with a high loading content as promising oxygen carriers. Hb spheres were prepared by templating decomposable porous $CaCO_3$ particles in combination with covalent layer-by-layer assembly. The surface of the Hb spheres was further chemically modified by biocompatible polyethylene glycol (PEG) to protect and stabilize the system. Scheme 1 shows a schematic representation of the assembled Hb microspheres with the surface modified by PEG. On the one hand, we obtained a high loading of Hb in an individual particle by a facile method, which is very favorable for application in an urgent need for large amounts of artificial blood, such as natural disasters and battlefield. On the other hand, the surface of the resulting particles can readily be modified chemically and biologically for prolonging the retention time in the body and introducing new functions such as targeting, antibacterial, antibody function, and decreasing cytotoxicity. In previous reports,[15−22] the common method to encapsulate Hb into controlled field is by an emulsion technique, which entraps Hb only in the emulsion shell and fails in further coating of Hb. In the present work, Hb spheres are fabricated by a porous template strategy in combination with covalent layer-by-layer assembly. Apparently, loading concentration of Hb spheres would be higher with regard to those prepared by an emulsion method because a number of micro- and nanopores of the $CaCO_3$ templates are accessible for Hb molecules. In addition, the LbL technique can further help the loading of Hb by coating the external surface of $CaCO_3$ with encapsulated Hb inside. Both factors contribute to the high loading of Hb in the sphere.

RESULTS AND DISCUSSION

To exploit the special structures of $CaCO_3$ particles, such as porous channel-like structures with high surface area and degradability under mild conditions, we chose $CaCO_3$ as templates to fabricate Hb spheres with a high loading content. Figure 1a,b compares the surface morphology between pure $CaCO_3$ and $CaCO_3$ particles loaded with Hb. Apparently, the pore size of particles is clearly decreased after Hb is encapsulated into $CaCO_3$, indicating the presence of Hb inside. The effective encapsulation of Hb into $CaCO_3$ is attributed to the interaction between Ca^{2+} and Hb during the co-precipitation. The porous structures allow the loading of the hemoglobin molecules as much as possible due

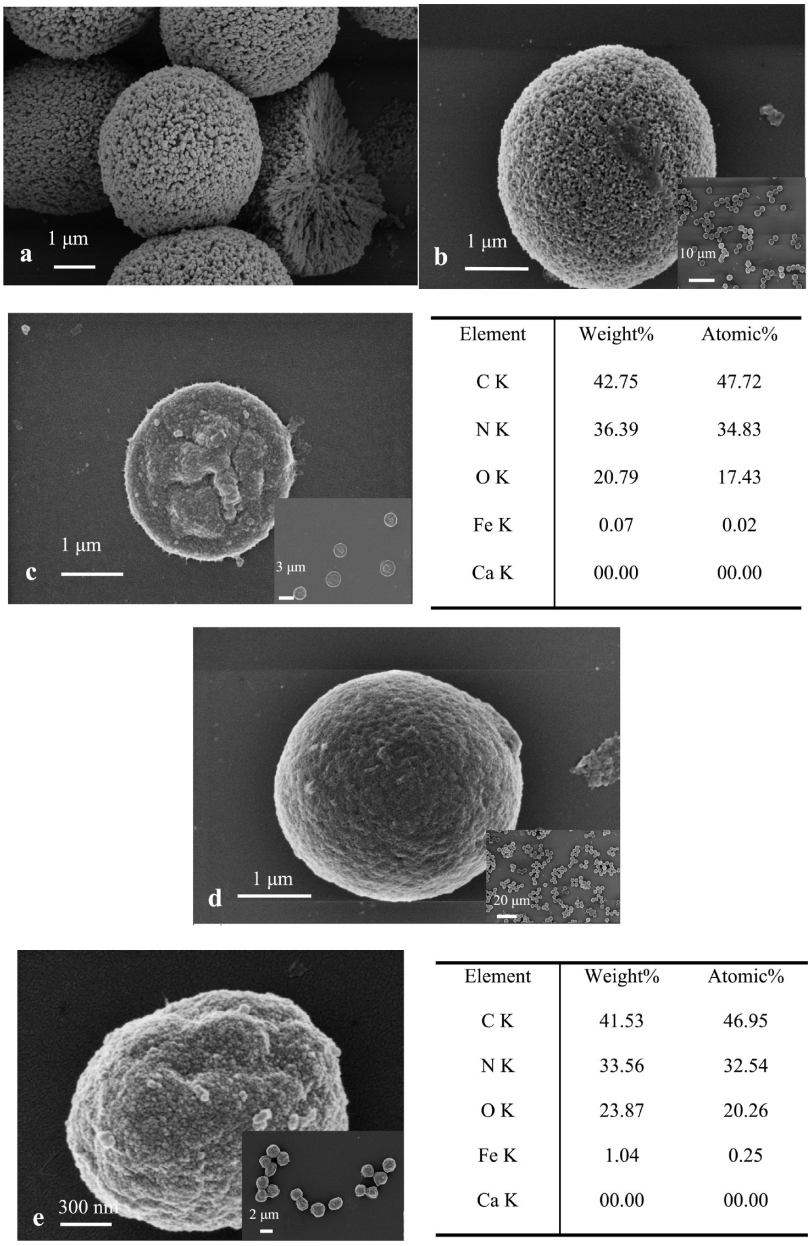


Scheme 1. Schematic representation of the assembled Hb microspheres with the surface modified by PEG.

to the larger surface area compared to that of the nonporous templates. Moreover, due to the porous structures of the templates, we can somehow control the loading content of Hb by merely changing the initial Hb concentration, adsorption time, or cycle, *etc*. Also, the co-precipitation makes the $CaCO_3$ particles more stable with the vaterite phase not with cubic crystallites. The inset image in Figure 1b reveals that the obtained particles are uniform and monodisperse. A cross-linker, glutaraldehyde (GA), was applied to stabilize the Hb spheres by the formation of covalent bonds between the aldehyde groups of GA and free amino groups of Hb. Covalent cross-linking can stop the rapid decomposition of Hb tetramers into dimers, prolong its vascular retention, and eliminate nephrotoxicity.[1,4]

After removal of the $CaCO_3$ templates with 0.1 M Na_2EDTA (pH 7.0), uniform and monodisperse Hb spheres can be obtained, shown in Figure 1c. The Hb spheres have few folds, and the EDX spectrum shows there is no Ca in the Hb spheres. This means that the prepared spheres are completely composed of Hb. Topology analysis of the Hb spheres by atomic force microscopy (AFM) also confirmed the loading. The AFM image together with the height profile in the cross section marked by a line on the Hb sphere image is shown in Figure S1 of the Supporting Information. The double wall thickness of Hb spheres determined from the height profile is about 286 $\pm$ 15 nm. The large thickness of Hb spheres is attributed to the presence of a large number of Hb inside. It should be noted that the effect of EDTA on the Hb can be neglected because the six binding sites of the iron ion in hemoglobin have been occupied (Figure S2), and the concentration of the calcium ion is much higher than that of the iron ion, as EDTA preferentially coordinates with the calcium ion and not with the iron ion. Thus, the EDTA dissolution would not harm Hb functionality.

In order to obtain a higher loading of Hb in an individual particle, additional Hb was assembled on the outer shell of the Hb-loaded $CaCO_3$ particles by covalent layer-by-layer assembly with GA, similar to the previous method.[27] The surface morphology of the



Element	Weight%	Atomic%
C K	42.75	47.72
N K	36.39	34.83
O K	20.79	17.43
Fe K	0.07	0.02
Ca K	00.00	00.00

Element	Weight%	Atomic%
C K	41.53	46.95
N K	33.56	32.54
O K	23.87	20.26
Fe K	1.04	0.25
Ca K	00.00	00.00

Figure 1. SEM images: (a) pure $CaCO_3$ particles; (b) Hb-loaded $CaCO_3$ particles; (c) Hb spheres after removing $CaCO_3$ and its EDX analysis; (d) Hb-loaded $CaCO_3$ particles with additional Hbs as the outer shell; (e) Hb spheres with additional Hbs as the outer shell after removing $CaCO_3$ and its EDX analysis. Inset is the reduced image of uniform and monodisperse particles.

obtained particles (Figure 1d) clearly shows a more compact structure than Hb-loaded $CaCO_3$ particles (Figure 1b). Interestingly, after the $CaCO_3$ templates were removed, the Hb spheres showed no folds and even a high surface roughness, as seen in Figure 1e. EDX data demonstrate that the particles are pure Hb spheres. The inset image also shows that the obtained Hb spheres are uniform and monodisperse. So it can be concluded that we fabricated a higher loading Hb sphere by additional Hb assembly on the outer shell than only by Hb encapsulated inside. This is greatly advantageous for clinical application.

To protect the immobilized Hb spheres from direct contact with body tissues and also to prevent Hb leakage from the interior substrates, biocompatible polyethylene glycol (PEG) was bound to the surface of the Hb spheres as the previous method.[29] PEG is a good membrane material for encapsulating the blood Hb, as the polymer is stronger and more permeable compared to lipid and less membrane material is required. The surface modification with PEG yields a protective barrier for Hb spheres and substantially increases the circulation time as opposed to plasma-free Hb. In addition, as reported in our previous work, the Hb shell membranes are semipermeable[27] and the PEG membranes are also permeable, which can allow small molecules such as O_2 and CO_2, the life-sustaining glucose in plasma, reducing agents, and metabolic

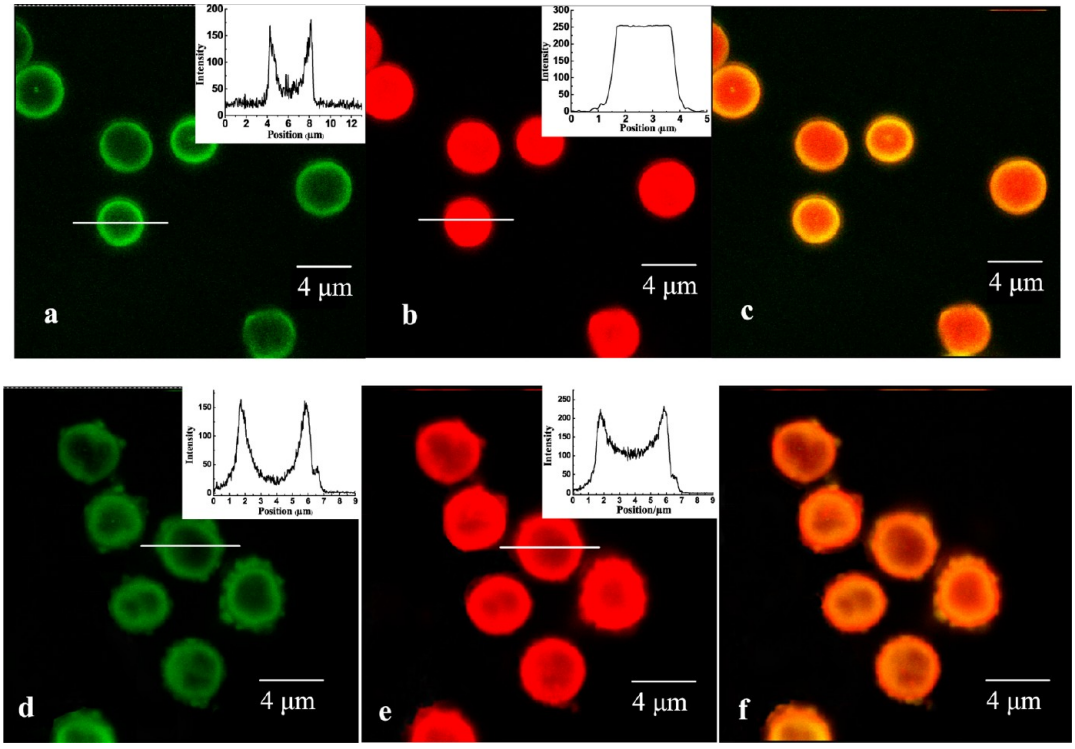


Figure 2. CLSM images of (a–c) Hb/$CaCO_3$ particles with the surface modified by PEG: (a) green DTAF-PEG modified well around the particles; (b) red RBITC-Hb protein uniformly distributed into the whole particles; (c) overlapping image of (a) and (b). CLSM images of (d–f) Hb spheres modified by PEG with additional Hbs assembled on the outer shell of Hb/$CaCO_3$ particles: (d) green DTAF-PEG; (e) red RBITC-Hb protein; (f) overlapping image of (d) and (e). Inset is the fluorescence intensity profile image around the sphere.

products to diffuse into/out of the system, just more like the active behavior of red blood cells. Direct observation of the successful coupling of PEG on the surface of Hb spheres can be achieved by confocal laser scanning microscopy (CLSM). Hb was labeled with red fluorescent RBITC and PEG labeled with green fluorescent DTAF. As shown in Figure 2a–c, the outer shell is green (Figure 2a) and the interior of the spheres is red (Figure 2b). Figure 2c shows the overlapping of (a) and (b). This result indicates the presence of Hb inside and of PEG on the surface. From the fluorescence intensity profile in the inset, it can be concluded that Hb is distributed uniformly over the whole $CaCO_3$ particles, and PEG also coats well the Hb spheres.

After LbL assembly of additional Hbs on the $CaCO_3$ particles loaded with Hb, PEG modification was also observed by CLSM. The CLSM images and the corresponding fluorescence intensity profiles are shown in Figure 2d–f. Apparently, red microspheres were encapsulated by green fluorescent PEG, and moreover, the shell of Hb spheres shows stronger red fluorescence than their interior. That is, Hb protein existed not only in the interior but also on the shell membrane. Also, PEG successfully covered the surface of the Hb spheres.

In the present work, experiments with different initial concentrations of Hb from 1 to 40 mg mL^{-1} were performed. The percentage of Hb loaded in $CaCO_3$ particles can be obtained by thermogravimetric analysis (TGA) (Figure 3). It can be seen that the thermal decomposition of Hb is completed at around 325 °C, and the weight loss for Hb particles with different initial concentration of 1, 4, 10, and 40 mg mL^{-1} was about 6.6, 13.2, 17, and 28%, respectively. That is, with the increase of initial Hb concentration, the content of Hb loaded in $CaCO_3$ particles is greatly increased, and the content of Hb loaded in spheres can be easily controlled by adjusting the initial concentration of Hb. Considering the fact that the high viscosity at high protein concentration can affect the loading efficiency of protein in spheres, higher initial Hb concentration beyond 40 mg mL^{-1} was not used in the present work.

To provide further insight into the loading content of Hb in the template particle, the density of Hb in an individual $CaCO_3$ particle, ρ (g cm^{-3}), was determined and calculated according to eq 1

$$\rho = \frac{\dfrac{m \times \mathrm{TGA}\%}{N}}{\dfrac{4\pi}{3}\left(\dfrac{d}{2}\right)^3 10^{-12}} \qquad (1)$$

where m is 1 g, N is the number of $CaCO_3$ spheres per gram, TGA% is the percentage of Hb in $CaCO_3$ spheres, d (μm) is the mean diameter of $CaCO_3$ particles, and π is Ludolph's number. The mean diameter of particles was determined by both dynamic light scattering (Figure S3)

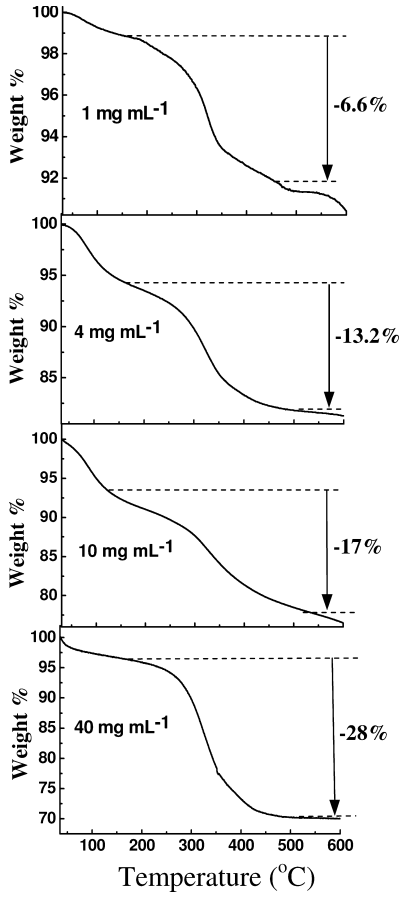


Figure 3. TGA curves between 35 and 600 °C of hemoglobin spheres with different initial concentration (1, 4, 10, and 40 mg mL^{-1}).

and SEM. For the initial Hb concentration of 1, 4, 10, and 40 mg mL^{-1}, the corresponding density is around 0.29, 0.5, 0.76, and 1.36 g cm^{-3}, respectively. The results indicate that the relatively high loading of Hb (1.36 g cm^{-3}) is readily achieved by the present approach. Hb spheres with high density are favorable for artificial blood substitutes, especially for an urgent need of large amounts.

The oxygen-carrying capacity of the encapsulated and cross-linked Hb spheres with an initial concentration of 10 mg mL^{-1} was studied in detail at room temperature in pH 7.4 PBS. Hb possesses oxygen-carrying ability only in the ferrous state ($HbFe^{2+}$). So in our system, met-hemoglobin (metHb) reducing agents, ascorbic acid (H_2A), were added to obtain ferrohemoglobin delivering oxygen to tissues. Ferrous Hb can exist in oxy and deoxy forms. The oxyHb has four oxygen molecules bound to the four oxygen binding sites. The binding sites of deoxyHb are empty. UV–vis absorption measurement on the carry and release of oxygen by Hb was performed according to the following procedure. First, for complete deoxygenation of Hb spheres, CO_2 gas was inflown into oxy-Hb spheres to remove most of the oxygen. Then the use of sodium dithionite ($Na_2S_2O_4$) (concentration less than 0.1% w/v)[30] modified the bimodal spectrum (maximum absorption peaks at 540 and 575 nm) to a single peak with a maximum absorption at 565 nm, representing 100% deoxygenation. After that, O_2 gas was drawn into the cell to obtain the oxyHb spheres with bimodal spectrum again. The optical absorption of Hb spheres is shown in Figure 4a. The left image is for Hb spheres with a bimodal spectrum in air (black curve), and the right image is for the deoxyHb spheres with a single peak (red curve) and oxyHb spheres with a bimodal spectrum (blue curve). For comparison, the deoxygenation and oxygenation processes of free Hb and cross-linked Hb were also studied. The corresponding absorption spectra are shown in Figure 4b,c, respectively. The absorption spectrum of the as-prepared Hb spheres shows changes similar to those of deoxygenation and oxygenation of free Hb and cross-linked Hb. The results demonstrate that, after encapsulation, covalent layer-by-layer assembly, and modification by PEG, Hb retains well its whole structure and especially its oxygen-carrying function. It has been reported that PEG-conjugated Hb exhibits high oxygen affinity, reduced cooperativity, and desensitization to the influence of allosteric effectors, which are either a direct consequence of the reductive alkylation of the four α-amino groups of Hb or the influence of the long PEG chains linked to the amino termini, or a combination of the two.[29] In the present work, however, PEG was merely grown onto the surface of Hb spheres as a protective layer that does not directly interact with a majority of Hb molecules, implying a negligible influence on the oxygen-carrying capacity.

To further evaluate the thermostability and pH stability of the assembled Hb spheres, temperature-dependent experiments ranging from 25 to 60 °C and pH-sensitive experiments from 5.8 to 9.0 were performed. The results for temperature and pH are shown in Figure S4a and Figure S5a, respectively. For comparison, the same experiments for free Hb and cross-linked Hb were also performed, shown in Figure S4, S5b and Figure S4,S5c, respectively. It can be seen that the absorption peaks at 540 and 575 nm both decrease with the increase of temperature (Figure S4a–c). Especially at 60 °C or above, the bimodal spectrum almost disappears for free Hb (Figure S4b), while for cross-linked Hb (Figure S4c) and Hb spheres (Figure S4a), the bimodal spectrum still exists even at high temperature. This demonstrates that the cross-linked Hb with the stable Hb tetramer structure can resist a higher temperature than free Hb. This means, unlike donated red cells which can be stored refrigerated only for a short time, the cross-linked Hb spheres need no special storage requirements and can be kept much longer at room temperature. This is most suitable for field application. Under different pH conditions, the absorption of Hb spheres (Figure S5a) also shows a similar change of absorption intensity with free Hb (Figure S5b) and

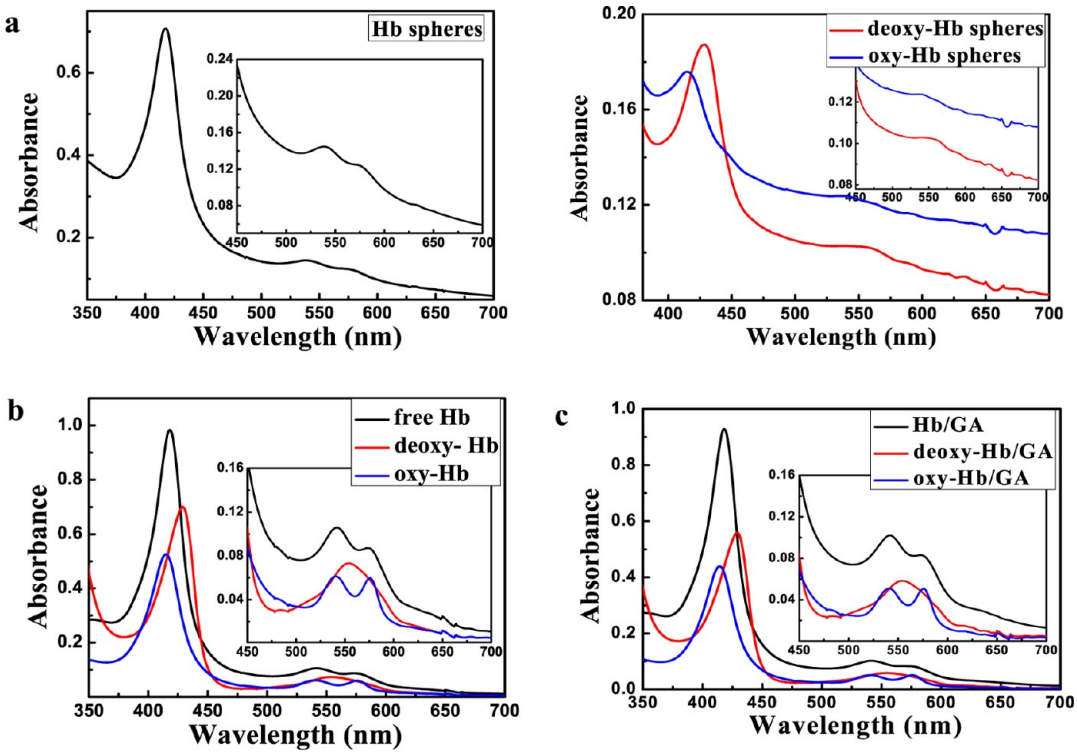


Figure 4. UV−vis absorption spectra of deoxyHb and oxyHb. (a) Hb spheres; (b) free Hb; (c) cross-linked Hb. Inset image in detail shows the absorption between 450 and 700 nm.

cross-linked Hb (Figure S5c). The absorption peak at pH 7.4 is the strongest compared to that in other acidic and alkaline conditions. It demonstrates that the immobilized Hb molecules still keep their bioactivity and structure integrity at relatively high temperature (60 °C) and at neutral pH. However, the protein spheres are unstable under more acidic or basic conditions. It has been proven that the coupling of PEG to Hb does not alter the overall secondary structure of Hb.[29] As PEG is a neutral polymer that is not susceptible to pH variation, its encapsulation onto Hb spheres cannot lead to any change of pH in solution. Therefore, Hb spheres with a coated PEG layer have a similar stability with pure Hb spheres *versus* temperature and pH. Thermostability and pH stability of the Hb spheres with different sizes are studied. The size of particles will not alter the overall secondary structure of Hb; correspondingly, the stability of Hb spheres with different sizes *versus* temperature and pH has a similar change (Figure S6). Namely, the immobilized Hb molecules still retain their bioactivity and structural integrity at relatively high temperature and at neutral pH. For future application in the living system, the stability and functions of the assembled hemoglobin microspheres are under study, and we anticipate we will have an *in vivo* test.

CONCLUSION

In summary, we have presented a high loading capacity of Hb spheres as oxygen carriers based on the decomposable porous $CaCO_3$-templated assembly technique. The Hb spheres have many advantages. The cross-linking action prevents the rapid breakdown of Hb tetramers into dimers, which increases the half-life and also eliminates nephrotoxicity. Additional Hbs were attached on the outer shell of Hb spheres by covalent layer-by-layer assembly, resulting in higher loaded Hb spheres. Biocompatible PEG was exploited to modify the surface of Hb spheres, which can greatly protect the Hb spheres against immune response, prolong its vascular retention time, and also provide a more stable and semipermeable system. Thus, the as-prepared Hb spheres may have potential to be developed as useful artificial oxygen carriers in the future.

EXPERIMENTS

Hemoglobin from bovine blood (Hb, lyophilized powder protein, $M_w \sim 64\,500$), L-ascorbic acid, sodium dithionite ($Na_2S_2O_4$), Rhodamine B isothiocyanate (RBITC), and 5-([4,6-dichlorotriazin-2-yl]amino) fluorescein hydrochloride (DTAF) were purchased from Sigma−Aldrich. Poly(ethylene glycol) (PEG, M_w = 5000) was oxidized to PEG-propionaldehyde (Jiankai Technology Company, Beijing).

Fabrication of Hb Spheres. Preparation of Hb spheres was based on the porous $CaCO_3$ template assembly technique. A mixture of $CaCl_2$ and Hb solution was first vigorously stirred in a beaker. Then the same volume of Na_2CO_3 solution was added quickly to

the above mixture. After 20 s, this system was allowed to stand for 2 min. The products were washed three times by centrifugation. To prevent the dissociation of Hb and prolong the intravascular half-life, the Hb spheres were dispersed in 0.025% glutaraldehyde (GA) in PBS for 2 h, followed by centrifugation and washing. The Hb-loaded $CaCO_3$ particles were collected. Following that, much more Hb was deposited by covalent layer-by-layer assembly on Hb-loaded $CaCO_3$ particles as by the previous method.[27] The 0.025% glutaraldehyde (GA) in pH 7.2 phosphate buffer solution (PBS) was adsorbed by the reaction of the amine groups of Hb molecules with aldehyde groups of GA for 12 h. After three times washing with pH 7.2 phosphate buffer solutions, the particles were dispersed into hemoglobin (Hb) solutions in PBS for 12 h, followed by washing with PBS. The GA and Hb were alternately adsorbed with five bilayers of GA/Hb. DTAF-PEG was incubated with cross-linked Hb spheres for 30 min, followed by three times washing in PBS solution. Finally, 0.1 M Na_2EDTA solution (pH 7.0) was used to remove the $CaCO_3$ templates. Pure Hb spheres were obtained and stored at 4 °C.

Calculation of Hb Density in an Individual $CaCO_3$ Particle. The process to determine the density of Hb in an individual $CaCO_3$ particle is as follows: the mean diameter of particles was measured by both a dynamic light scattering (DLS) and scanning electron microscopy (SEM), where around 200 particles were used to determine the mean diameter. The number of Hb spheres per unit mass was calculated according to the following method. Since the number of $CaCO_3$ particles before and after loading Hb is not changed for a given amount of particles, it is reasonable to calculate the number of Hb spheres by directly calculating the $CaCO_3$ particle number. It is known that the as-prepared $CaCO_3$ particles have vaterite structure[31] with a density of 2.65 g cm^{-3}. In combination with the mean diameter of the $CaCO_3$ particles, the number of $CaCO_3$ particles per unit mass is accessible. Together with the results from TGA, the mean diameter, and the particle number, the density of Hb in an individual $CaCO_3$ particle, ρ (g cm^{-3}), can be calculated according to eq 1 (see the main text).

Blood Hemoglobin Carry and Release of Oxygen. The prepared Hb spheres were injected into the special cell for flow-through measurements (Hellma GmbH & Co. KG, Germany). First, CO_2 gas ran from the inlet tube to the outlet tube under a low flow rate. After about 1 h, sodium dithionite ($Na_2S_2O_4$) was added, and light absorption of deoxyHb was scanned between 350 and 700 nm on a U-3010 UV−vis absorption spectrometer (HITACHI) with PBS as a reference solution. For carrying oxygen by Hb, O_2 gas was then inflown for 1 h, followed by recording the light absorption of oxyHb.

Characterization. Scanning electron microscopy (SEM) was applied to observe the morphology of Hb spheres and measure the mean diameter of spheres. Samples were prepared by dripping a droplet of the sample suspension on a glass slide and then drying overnight. Samples were sputtered with gold and measured using a Gemini Leo 1550 instrument (Carl Zeiss AG, Germany) at an operating voltage of 3.0 keV. Atomic force microscopy (AFM) was carried out to achieve topographic measurements and characterize the roughness and height profile of Hb spheres. Measurements were performed in air at room temperature using a D3100 Nanoscope IIIa MultiMode microscope (Digital instruments/Veeco, Inc., Santa Barbara, CA) in tapping mode with silicon cantilevers (Nanoworld, Neuchâtel, Switzerland). Cantilevers have a typical resonance frequency of 285 kHz and a spring constant of 42 N m^{-1}. Confocal laser scanning microscopy (CLSM) images of Hb spheres with the surface modified by PEG were obtained using a Leica TCS SP5 confocal scanning system (Leica, Germany), equipped with a 63× oil immersion objective. A droplet of sample suspension was dripped on a glass slide and then was covered with a coverslip. Thermogravimetric analysis (TGA) of the Hb spheres was carried out by using a Pyris Diamond TG-DTA (Perkin−Elmer instrument). Samples were heated from room temperature to 600 °C at a constant rate of 10 K min^{-1} in a N_2 atmosphere. Before the thermal analysis, the as-prepared Hb spheres were dried under vacuum until their weights were constant. Dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments) was applied to determine the mean diameter and size distribution of Hb spheres. The absorption of as-prepared Hb spheres dispersed in different pH buffer solutions from 5.8 to 9.0 and under different temperature from 25 to 60 °C was scanned on a U-3010 UV−vis absorption spectrometer (HITACHI) connected with controllable temperature equipment.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Figures S1−S6. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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