Chapter 5
Hemoglobin-Based Molecular Assembly

Li Duan, Yi Jia and Junbai Li

Abstract Development of protein-based molecular devices is an active area of research due to their broad applications in biotechnology, biorelated chemistry, bioelectronics, and biomedical engineering. Hemoglobin (Hb) is a physiologically important oxygen-transport metalloprotein present in the red blood cells. In this chapter, we present the recent development in fabrication and tailoring of a variety of hemoglobin protein shells via covalent layer-by-layer (LbL) assembly combined with template technique. Also, the developed strategy is effective and flexible, advantageous for avoiding denaturation of proteins. The as-fabricated Hb shells have better applications in drug delivery and controlled release, biosensors, biocatalysis, and bioreactors due to the enhancement of biological availability. In view of the carrying-oxygen function of Hb protein in blood, we particularly focus on the potential applications of hemoglobin-based nanoarchitectonic assemblies as artificial blood substitutes. These novel oxygen carriers exhibit advantages over traditional carriers and will greatly promote research on reliable and feasible artificial blood substitutes.

Keywords Hemoglobin protein · Layer-by-layer assembly · Template technique · Covalent cross-linking · Carrying-oxygen function · Artificial blood substitutes

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5.1 Introduction

Biomimetics has proved very useful in the design and fabrication of new functionally structured materials on the micro- and nanoscale [1]. Biomimetics refers to human-made processes, devices, or systems that mimic or imitate certain aspects of biological systems and have proven useful in providing biological inspiration from natural efficient designs [2–4]. Engineering biomimetic materials encompass a wide variety of research, from nanomaterials to the mechanics of how biological molecules such as proteins and enzymes can function as analogous man-made structures. With the development of modern biology, biomimetics is not limited to just copying nature. Scientists can directly utilize biological units themselves to construct hybrid nanostructured materials. In this chapter, we mainly focus on how molecular biomimetic applied to engineering functional materials, particularly fabricating the hemoglobin protein shells via covalently Layer-by-Layer (LbL) assembly, and its application in biomedical fields. This method to fabricate protein capsules is simple and inexpensive, advantageous for avoiding denaturation of proteins and is applicable to nearly any proteins, which shows a wide range of application.

5.2 Hemoglobin—An Oxygen-Carrying Protein

Hemoglobin (Hb) plays an important role in vital activities. It's the main functional constituent of the red blood cell that causes blood as a red color, and transports oxygen to the cells from the lungs and carbon dioxide away from the cells to the lungs. Red blood cells must contain adequate hemoglobin. A deficiency of hemoglobin would lead to various diseases, such as anemia. In mammals, the Hb protein makes up about 96% of the red blood cells' dry content (by weight), and around 35% of the total content (including water). Each erythrocyte contains 200–300 molecules of hemoglobin. Hemoglobin is also found outside red blood cells and their progenitor lines. Other cells that contain hemoglobin include the A9 dopaminergic neurons in the substantia nigra, macrophages, alveolar cells, and mesangial cells in the kidney. In these tissues, hemoglobin has a non-oxygen-carrying function as an antioxidant and a regulator of iron metabolism [5].

Hemoglobin (Hb) is a conjugated iron–protein compound in which each molecule is a tetramer composed of four monomers held together by weak bonds (Fig. 5.1). It is made up of four globular polypeptide subunits ($\alpha 1$, $\beta 1$, $\alpha 2$, $\beta 2$), noncovalently bound to each other by salt bridges, hydrogen bonds, and the hydrophobic effect. Each globular subunit contains an attached heme molecule composed of iron plus a protoporphyrin molecule. This porphyrin ring consists of four pyrrole molecules cyclically linked together (by methine bridges) with the iron ion bound in the center. The iron ion, which is the site of oxygen binding,

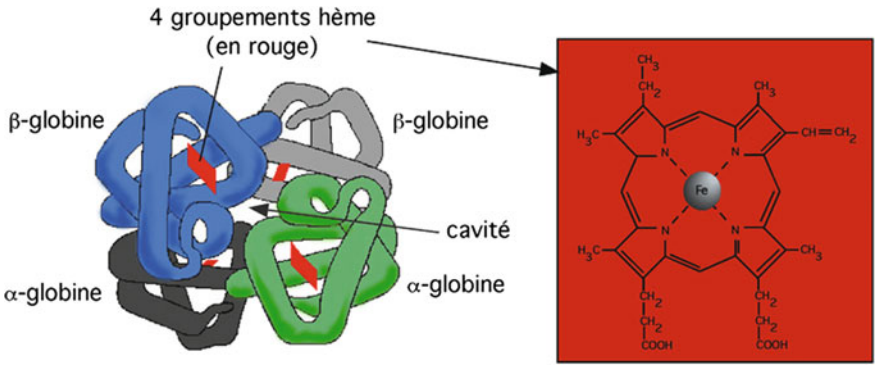

Fig. 5.1 Structure of hemoglobin

coordinates with the four nitrogen atoms in the center of the ring, which all lie in one plane. The iron is bound strongly (covalently) to the globular protein via the N atoms of the imidazole ring of F8 histidine residue (also known as the proximal histidine) below the porphyrin ring. A sixth position can reversibly bind oxygen by a coordinate covalent bond, completing the octahedral group of six ligands. Oxygen binds in an "end-on bent" geometry where one oxygen atom binds to Fe and the other protrudes at an angle. When oxygen is not bound, a very weakly bonded water molecule fills the site, forming a distorted octahedron. The iron ion may be either in the Fe^{2+} or in the Fe^{3+} state, but methemoglobin (Fe^{3+}) cannot bind oxygen [6]. In binding, oxygen temporarily and reversibly oxidizes Fe^{2+} to Fe^{3+} while oxygen temporarily turns into the superoxide ion, thus iron must exist in the +2 oxidation state to bind oxygen. If superoxide ion associated to Fe^{3+} is protonated, the hemoglobin iron will remain oxidized and incapable of binding oxygen. In such cases, the enzyme methemoglobin reductase will be able to eventually reactivate methemoglobin by reducing the iron center.

Several diseases are related to heme and hemoglobin disorders. Gene mutations result in a group of hereditary diseases termed hemoglobinopathies, among which the most common are sickle-cell disease and thalassemia. Decreased levels of hemoglobin and heme synthesis lead to symptoms of anemia, whereas alterations of heme metabolic pathways generate porphyrias syndromes. The decrease of hemoglobin's oxygen-binding capacity affected by molecules such as carbon monoxide (CO) (for example, from tobacco smoking, car exhaust, and incomplete combustion in furnaces) can result in hypoxia and asphyxiation. It is because carbon monoxide competes with oxygen at the same heme binding site on Hb. Hemoglobin binding affinity for CO is 200 times greater than its affinity for oxygen [7], meaning that small amounts of CO dramatically reduce hemoglobin's ability to transport oxygen.

Thus, we have recently devoted to the molecular assembly of hemoglobin shells and its application study to imitate in some sense hemoglobin structural function in the living system and better understand its capacity to select ions.

5.3 Covalent LbL Assembly of Hemoglobin Protein

Development of protein-based molecular devices has become an active area of research due to their broad applications in biotechnology, biorelated chemistry, bioelectronics, and biomedical engineering [8]. Up to now, a variety of methods has been used to construct protein molecular films, including LB technique, sol-gel method, physical adsorption, and covalent cross-linking strategies, etc. [9–11]. Also, the LbL approach has been applied widely in constructing protein multilayer assemblies [12]. The LbL technique was based on the alternating adsorption of charged species onto an oppositely charged substrate via electrostatic interactions, which was introduced firstly by Decher and his coworkers [13]. The procedure is simple and basically applicable to many different kinds of the substrate (1-D, 2-D, or 3-D). Now, different types of films containing charged and uncharged species have been successfully prepared, including polypeptides, polysaccharides, DNA, proteins, viruses, nanoparticles [14–16]. As a molecular assembly technique, LbL assembly has been extensively used in biology, spanning biomimetics, biomedicine, and tissue engineering [17–19].

Since 1996, multi-protein assemblies has been successfully built up on planar solid surface or on 3D colloidal particles through alternated electrostatic adsorption between charged proteins and oppositely charged polymers [20]. And their applications in catalysis and immune sensing have also been demonstrated. However, in all these reports, the deposition of proteins was based on the electrostatic interactions between them and oppositely charged polyelectrolytes. The existence of the other polymers may result in a lack of desirable properties or functionality of such proteins multilayer [8, 21]. In 2004, a novel type of protein nanostructures was constructed with the LbL assembly technique, which was introduced by Martin and his coworkers [22]. They developed a method with glutaraldehyde (GA) as a covalent cross-linker to fabricate hemoglobin (Hb) nanotubes and glucose oxidase (GOX) nanotubes, respectively. The studies showed that the protein nanotubes prepared in this way could remain the electroactivity and catalytic activity. Later, our group extended this strategy to sacrificial 3-D colloidal particles and hollow protein capsules were obtained without the use of any polymer [23–26], different from the assembly of proteins and oppositely charged polyelectrolytes. Here, we mainly introduce the fabrication of this kind of microcapsules and their applications in biomedical engineering and biologically related chemistry, in which they can be flexibly designed to be stimuli-responsive under physiological conditions and allow for the incorporation of uncharged species, which is important for capsule's multifunctionalization.

5.3.1 *Hemoglobin Protein Hollow Shells Fabricated Through Covalent LbL Assembly*

Our group has recently prepared hemoglobin (Hb) protein microcapsules by using a covalent LbL technique [24]. The use of covalent bonds to assemble LbL microcapsules can provide significant advantages compared to traditional electrostatic assembly [27]. In particular, they have high stability due to the covalent bonds formed, and therefore do not disassemble with changes in pH or ionic strength. In this work, GA is used as a chemical cross-linker because it has less effect on the protein activity. Poly(ethylenimine) (PEI) was firstly adsorbed on template particles to produce an amino-functionalized surface. Then the GA and Hb were alternately adsorbed. Figure 5.2a shows a schematic representation of a covalently cross-linked hemoglobin protein capsules. Direct information on the capsules formation can be obtained from the measurements by TEM and CLSM (Fig. 5.2b). The results show that the wall thickness of capsules can be controlled by the adsorption cycles of alternate GA/Hb. The UV-visible spectra of GA/Hb capsules show the absorption

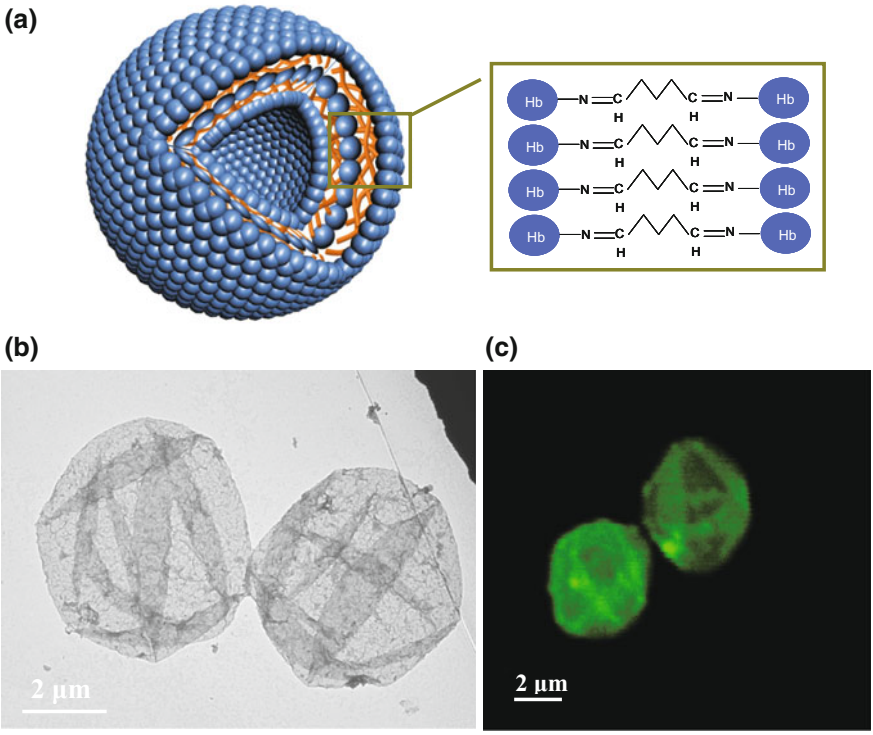

Fig. 5.2 **a** Schematic representation of the assembled hemoglobin protein microcapsules via covalent layer-by-layer assembly. **b** TEM and **c** CLSM images of $(GA/Hb)_5$ capsules at a dried state. Reprinted from Ref. [24], Copyright 2007, with permission from Elsevier

band of heme at 411 nm, indicating that Hb essentially remains in the capsules. Cyclic voltammetry and potential-controlled amperometric measurements confirm that cross-linked Hb capsules keep their heme electroactivity and are not denatured. The typical amperometric response toward the successive additions of H_2O_2 shows the electrocatalytic property of GA/Hb capsules. The permeability of the assembled GA/Hb microcapsules was tested by using fluorescein isothiocyanate (FITC)-dextran with different molecular weight as fluorescent probes. The results show that the $(GA/Hb)_5$ capsules are impermeable to FITC-dextran with molecular weights of 2000 kDa and 500 kDa, while FITC-dextran with molecular weights below 70 kDa can partly, and even completely permeate into the capsules interior. In comparison with traditional $(PAH/PSS)_5$ capsules, the Hb protein shells have a selective permeability. Similarly, the permeability decreases with the increase of wall thickness. Using FITC-dextran with a molecular weight of 20 kDa as a fluorescent probe, the fluorescence recovery in the capsules interior as a function of time is observed at lower excitation intensity. As a consequence, $(GA/Hb)_5$ microcapsules have a permeability of 4×10^{-8} m/s. The improved permeability of Hb capsules is helpful to better tune the storage and release of encapsulated small molecules. The fabrication of Hb capsules could imitate in some sense its structural function in the living system, and may help us to understand its properties and can be expected to have better applications in medicine, catalysis, and cosmetics, as well as biotechnology.

5.3.2 *Glucose-Sensitive Microcapsules from Glutaraldehyde Cross-Linked Hemoglobin and Glucose Oxidase*

Loading a therapeutic agent into LbL films/capsules is fundamental for developing these systems for drug delivery applications. Also, engineering release of the cargo from the multilayer systems is essential for therapeutic delivery applications. An ideal method to load and release drug is by incorporating components within multilayer films that are responsive to specific environmental stimuli [28].

Encouraged by the successful assembly of Hb capsules via GA cross-linking, the above approach was extended to fabricate a type of comparative, two-enzyme microcapsules including those composed of hemoglobin and glucose oxidase (Hb/GOD capsules), and those composed of catalase and glucose oxidase (CAT/GOD capsules). In this section, we described the fabrication of glucose-sensitive protein multilayer microcapsules by the alternate assembly of Hb and GOD with glutaraldehyde (GA) as cross-linker (Fig. 5.3a) [29, 30]. GOD catalyzes the oxidation and hydrolysis of β-D-glucose into gluconic acid and H_2O_2. Hb can catalyze the reduction of H_2O_2 due to its certain intrinsic peroxidase activity. This reduction can be monitored by nonfluorescent Amplex red, which is oxidized by H_2O_2 into resorufin (a fluorescence dye). Thus, the system involves two enzymatic catalysis reactions, and it offers two advantages. One is that the

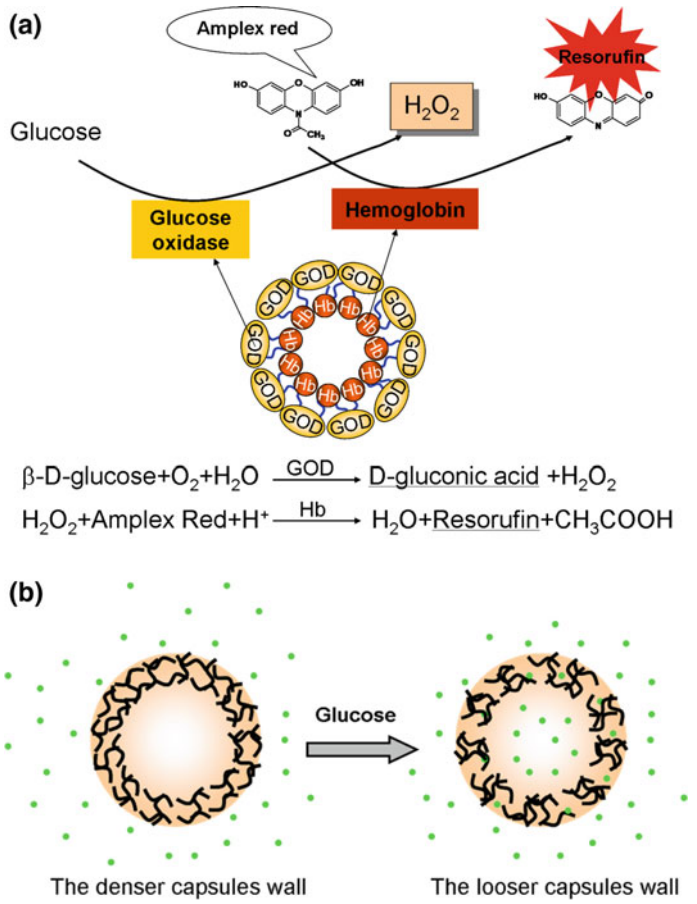

Fig. 5.3 **a** Schematic illustration of coupled enzymatic process based on glucose oxidase (GOx) and hemoglobin (Hb) coimmobilized as LbL microcapsules components; **b** illustration of glucose-stimulated FITC-dextran release (*green circles*). The consumption of glucose enhanced the release of the encapsulated materials. Reprinted with the permission from Ref. [29]. Copyright 2009 American Chemical Society

fluorescence of resorufin makes it convenient to monitor the reactions process, which may facilitate the development of an approach to design new fluorescence sensors for glucose. The other is that the consumption of glucose enhanced the permeability of the capsule wall and increased the release of the encapsulated drugs (Fig. 5.3b), which is highly attractive for the fabrication of glucose-responsive release systems. So, it is anticipated to apply this system in developing a fluorescence sensor to detect glucose qualitatively in vitro. In addition, it is also believed the observed glucose-stimulated enhancement of the wall permeability could play an important role in the development of drug delivery vehicles and may find widespread application in drug loading and release in the coming future.

5.3.3 *Assembly of Lipid Bilayers on Covalently LbL-Assembled Hemoglobin Capsules as a Biomimetic Membrane System*

In our subsequent work, Hb protein multilayer-supported liposomes or lipid bilayer-coated Hb protein microcapsules were fabricated through the conversion of liposomes into lipid bilayers to cover the capsule surface in analogy to the cell membrane [25]. These lipid-coated protein microcapsules can be considered as an ideally supported biomimetic membrane system to mimic the real cell membrane. It can not only conveniently tune their morphological properties, including the exterior shape, interior space, and shell structure, but also realize their multifunctionalization. Protein multilayer-supported liposome systems should be useful for the understanding of the principles of the interaction between membranes and biopolymers enzyme, and enable the design and application of new biomimetic structured materials.

It is well known that the production of adenosine 5′-triphosphate (ATP) is one of the most important chemical reactions in living organisms. The enzyme primarily responsible for the production of ATP is ATP synthase, which is a rotary motor protein. This enzyme drives the generation of ATP from adenosine 5′-diphosphate (ADP) and inorganic phosphate by utilizing proton gradients. ATP synthase contains two domains, the membrane- embedded F_0 part and the soluble F_1 domain, and is hence also called F_0F_1-ATPase. The F_0 part is involved in proton transport across the membrane, which is accompanied by a rotation of the intramembrane subunits (rotor), whereas the F_1 part catalyzes the synthesis and hydrolysis of ATP. The integrated F_0 and F_1 complex drives protons toward the F_1 side of the membrane, resulting in the synthesis of ATP [31]. Over the last decade, understanding of the functioning of ATPase has advanced dramatically to the point where the enzyme is now being studied by scientists interested in developing nanomachines for information storage and energy interconversion. Several groups have been working on the reconstitution of CF_0F_1 in liposomes to drive the motor proteins to carry out ATP biosynthesis [31–35]. Recently, we have created a route to generate a proton gradient by varying the pH values within and outside polymer microcapsules, with the CF_0F_1 species reconstituted within the walls of the capsules [36]. In this section, we performed the reconstitution of chloroplastic F_0F_1-ATPase (CF_0F_1-ATPase) within lipid bilayer-coated hemoglobin (Hb) protein microcapsules (Fig. 5.4a) to imitate the system in living cells governed by molecular motors [25].

Commonly, the first step of this biomimetic membrane fabrication process consists of preparing the hollow Hb microcapsules by covalent LbL assembly technique, as above-mentioned [36]. Then, CF_0F_1-proteoliposomes were prepared by the incorporation of CF_0F_1-ATP synthase into liposomes according to the method described previously in the literature [37]. Briefly, phosphatidylcholine/phosphatidic acid liposomes have been added into a Triton X-100-solubilized CF_0F_1-ATPase buffer solution, followed by the slow removal of Triton X-100 by

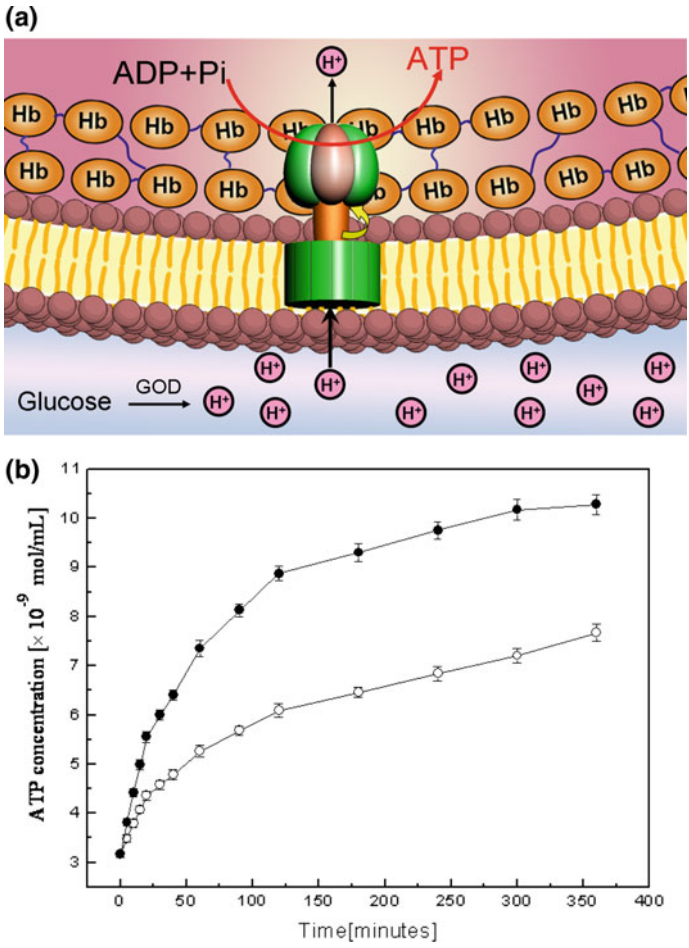

Fig. 5.4 **a** Schematic representation of ATP synthesis catalyzed by CF_0F_1-ATPase reconstituted in lipid-coated Hb microcapsules. **b** ATP synthesis catalyzed by reconstituted CF_0F_1 in proteolipid-coated $(GA/Hb)_5$ microcapsules as a function of the reaction time. Reproduced from Ref. [25] by permission of John Wiley & Sons Ltd

Biobeads SM-2. This leads to the formation of CF_0F_1-proteoliposomes. It is noted that, as phosphatidylcholine lipid is a major component of biological membranes, egg phosphatidylcholine was specially chosen as a lipid model to cover the capsules, but a small fraction of negatively charged lipid, phosphatidic acid, was added in order to promote the adsorption and fusion of vesicles. In other words, the lipid composition on the surface could easily be varied and fine-tuned to specific conditions. The existence and stability of the lipid bilayer were proved by means of CLSM. The fluorescent orb arises from the presence and continuous distribution of lipids on the capsule shells. Following that, a suspension of Hb capsules was first

mixed with pyranine by shaking for several minutes, subsequently followed by washing with buffer solution. Next, CF_0F_1-proteoliposomes were added to the mixture and incubated for 30 min, followed by centrifugation and three washing cycles with buffer solution. As a result, the pyranines were encapsulated within the Hb protein capsules, and ATPase–proteoliposomes were assembled onto the outer shell of Hb capsules. It should be noted that only in the case of F_1 subunits of the CF_0F_1 complex extending toward the interior of the assembled capsules, ATP synthesis inside the capsules could become possible. A standard luciferase assay was used to quantitatively determine the ATP amount. Figure 5.4b show that the ATP production continuously increases as a function of the reaction time under driven by a proton gradient between the interior and exterior of the microcapsules. The results indicated that ATP catalyzed by reconstituted CF_0F_1 in proteolipid-coated $(GA/Hb)_5$ microcapsules have been synthesized inside the microcapsules. Such assembled microcapsules are very stable under physiological conditions. Their longer lifetime will enhance the ATP production efficiency. The well-defined microcapsules may serve as containers for the storage of the synthesized ATP as an energy currency. By using this system, it becomes possible to study the function of ATPase in a biomimetic unit in detail. Furthermore, as vital activities need energy, ATP could be released from the assembled capsules to provide energy on demand. Hence, we have built a micrometer-sized energy-storage device suitable to power biological activity.

5.3.4 Assembled Hemoglobin and Catalase Nanotubes for the Treatment of Oxidative Stress

Oxidative stress can induce the occurrence of diseases like nonalcoholic steatohepatitis (NASH). The increased oxidative stress in patients will cause the pro-oxidation environment against the antioxidant enzyme activities. In that case, the local H_2O_2 concentration will rise up. The excessive H_2O_2 can be converted to reactive oxygen species (ROS) or released into the extracellular environment. Therefore, how to eliminate the oxidative stress is a big challenge. Hemoglobin (Hb), the main oxygen transporter in erythrocytes, can be expressed in non-hematopoietic organs (in hepatocyte or mesangial cells) to balance the oxidative stress [38]. Oxidative stress upregulates hemoglobin expression, and hemoglobin overexpression suppresses oxidative stress in pathological cells [39, 40]. Thus, the drug constituted mostly by hemoglobin can provide an efficient treatment for oxidative stress induced disease like NASH.

Comparing with other nanostructures, the tubular structure presents several advantages to nanospheres. The multifunctionalities can be introduced on the inner and outer surfaces independently [41]. Second, open-end terminals enable quick loading of target molecules without the structural change. Third, nanotubes can have long circulation persistence in the bloodstream [42, 43]. One of the most

efficient methods to prepare the structure-defined nanotubes is using the LbL technique. The flexibility of the LBL assembly allows us to create nanoreactors to scavenge H_2O_2 by an enzymatic reaction.

Hence, our group fabricated biomimetic nanotubes with H_2O_2-scavenging capabilities through LbL assembly of hemoglobin, catalase, and dialdehyde heparin (DHP) (Fig. 5.5a) [44]. Both hemoglobin and catalase are potential candidates to scavenge H_2O_2, and DHP is used as one of the wall components, an auxiliary drug in liver diseases, and also as a cross-linker. With the LbL assembly technique, the

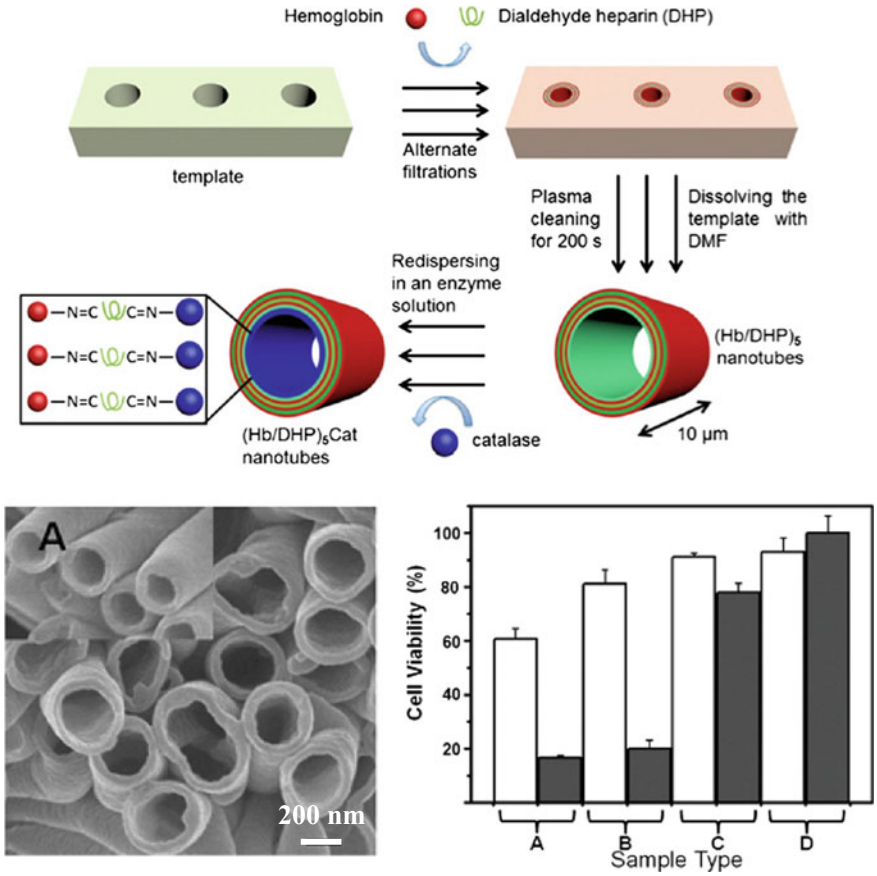

Fig. 5.5 **a** Schematic illustration for the fabrication of $(Hb/DHP)_5$ and $(Hb/DHP)_5Cat$ nanotubes. **b** Scanning electron microscopy (SEM) images of the $(Hb/DHP)_5$ nanotubes. **c** $(Hb/DHP)_5Cat$ nanotubes improved the HepG-2 cells viability against oxidative stress. HepG-2 viability when exposed to H_2O_2 of (*A*) 1000, (*B*) 500, (*C*) 250, and (D) 0 μM for 24 h as extracellular oxidative stress was evaluated by the CCK-8 assays. The white column represents the experiment group, and the gray column represents the control group. Each error bar represents the mean of at least six measurements (±SD). Reprinted with the permission from Ref. [44]. Copyright 2013 American Chemical Society

size, and behavior of as-prepared nanotubes could be well controlled and adjusted by changing the template inner pore size and the wall components (Fig. 5.5b). All these merits endow the nanotubes with great potential in the treatment of oxidative stress. In this work, in order to assess the H_2O_2-scavenging capacities of the as-assembled nanotubes in vitro, we evaluated cell viability under oxidative stress by the CCK-8 assays. As shown in Fig. 5.5c, when they were exposed to the 1000 and 500 μM H_2O_2, respectively, the improvement of the HepG-2 cells viability by the $(Hb/DHP)_5$Cat nanotubes is obvious. With no additional H_2O_2, the viability of the experiment group is about 92% of that of the control group, which further proves the biocompatibility of the nanotubes. These results suggested that the assembled $(Hb/DHP)_5$Cat nanotubes have a positive effect on the HepG-2 cells under oxidative stress. The as-prepared nanotubes were modified with a catalase interior layer after the PC dissolution process, which protects the enzyme from inaction within the loading process and also enhances the capabilities of the nanotubes in the treatment of oxidative stress. With the modification process, the behavior of hemoglobin-based nanotubes is open to a wide range of variations. These variations could provide a basis for maximizing the advantages of hemoglobin-based nanotubes and minimizing its disadvantages by means of systematic and reasonable design, which make the obtained nanotubes promising candidates for applications in the treatment of oxidative stress and other possible biomedical fields.

5.4 Hemoglobin-Based Nanoarchitectonic Assemblies as Artificial Blood Substitutes

Medical interest toward Hb is related to the possibility of its administration as blood substitutes to re-establish oxygen homeostasis in tissues. At present, blood transfusions are mostly applied for this purpose, but the need of the right type of blood and its short shelf life are still serious problems to be overcome. In this case, the use of autologous transfusions prevents the need of cross-matching and virus transmission, although autologous transfusions are considered the safest, they are not always feasible because they may cause perioperative anemia and are more expensive than allogenic transufions [45].

The development of oxygen carriers is particularly indicated in the case of urgent need of oxygen delivery to tissues and to solve the above-mentioned problems related to blood transfusions. The ideal oxygen carrier would deliver oxygen, not transmit disease, not have immunosuppresive effects, would have less strict storage requirements than that for human blood, would not need cross-matching, would be available at reasonable costs, be easy to administer, and able to reach all areas of human body, including ischemic tissues [46].

In the past decades, artificial oxygen carriers have been extensively studied and have gone through a rapid development. Up to now, there are mainly two types of

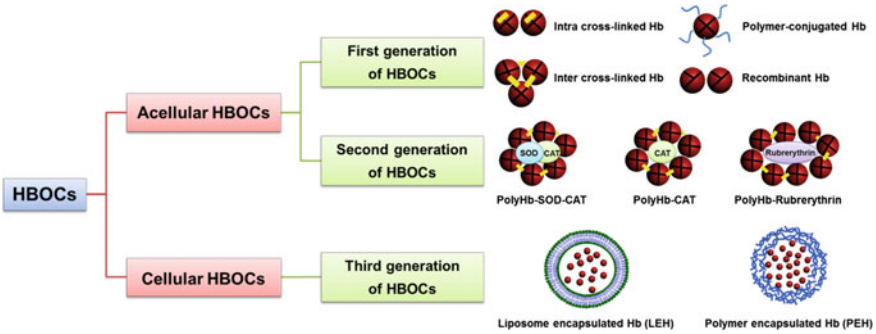

Fig. 5.6 A list of hemoglobin-based oxygen carriers (HBOCs). Reproduced from Ref. [50] by permission of John Wiley & Sons Ltd

oxygen carriers: totally synthetic oxygen carriers (including perfluorochemicals and heme hybrids) [47] and hemoglobin-based oxygen carriers (HBOCs) [48]. Among these oxygen carriers, HBOCs attracted the most attention owing to unique oxygen-transport ability and normal metabolic pathways of Hb [49]. However, stroma free Hb could not directly be used as an oxygen carrier since it dissociated into dimers that have a short circulation time, renal toxicity, high oncotic pressure and high O_2-affinity. To overcome these problems, hemoglobin must be chemically modified or microencapsulated to prevent its dissociation and thus evolved two types of HBOCs: acellular hemoglobin-based oxygen carriers (HBOCs) and cellular hemoglobin-based oxygen carriers, as shown in Fig. 5.6 [50].

Acellular HBOCs refer to chemically modified Hb and can be separated into two generations. The first generation of HBOCs aims to prevent the dissociation of Hb tetramer into a dimer, and mainly includes intra-molecular cross-linked Hb, inter-molecular cross-linked Hb, polymer-conjugated Hb and recombinant Hb [48]. The second generation of HBOCs is on the basis of the first generation that co-assembly of Hb with antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) [51, 52] to further stabilize the assemblies and meanwhile avoid the formation of inactive methemoglobin (metHb). Hb is also intramolecularly cross-linked with ATP, and intermolecularly cross-linked with adenosine, and conjugated with reduced GSH [53] to prevent the dissociation of Hb and the formation of homogeneous polymers, regulate the blood vessel tone, and protect heme from reactive oxygen species. Up to date, some of the above-mentioned Hb-based assemblies have gone through different stages of clinical trials and a few have been approved for routine clinical uses in South Africa or anemia treatment in canines.

Despite acellular HBOCs have made great progress, side effects still occurred in some cases due to the striking different structure in comparison with red blood cells (RBCs). Therefore, the importance of cellular structures like RBCs, that is, the third generation of HBOCs, became apparent. For cellular HBOCs, Hb molecules are encapsulated inside oxygen carriers of various structures. Cellular HBOCs

could well protect Hb molecules from direct contact with surrounding tissues and blood components, and avoid the leakage of Hb molecules from the vessel walls. In addition, an appropriate size of cellular HBOCs could enable Hb-based assemblies with long-term circulation in the body and ensure oxygen availability to all body compartments. Generally, there are mainly two types of cellular HBOCs: liposome encapsulated hemoglobin (LEH) and polymer encapsulated hemoglobin (PEH) [47].

LEH has been proposed and investigated as early as the 1970s. The size, surface properties, in vivo efficacy and safety evaluation of LEH have been thoroughly studied in the past decades. Despite the properties of LEH have been well improved through adjusting the components of lipid or surface modification with PEG, some defects still exist. For example, phospholipids are expensive and may induce peroxidation in ischemia reperfusion. In addition, lipid membrane is impermeable to some reducing agents present in the circulating blood that is important for depressing the formation of metHb [54].

Compared to LEH, the researches on PEH have aroused increasingly concerns due to the easy availability, low price, broad variety, and biocompatibility of polymers. Theoretically, PEH is more like a red blood cell than LEH. It has a better physical strength, a better permeability, and ease of adjustment than LEH. Up to date, various strategies were developed to fabricate PEHs and great progress have been made. For instance, a double emulsion-solvent diffusion/evaporation technique was utilized for the encapsulation of Hb in polymers [54]. Chang et al. employed polylactic acid (PLA), poly(lactic acid-co-glycolide) (PLGA) and poly-isobutycyanoacrylate as the matrix polymer and obtained Hb-loaded particles or capsules with a diameter of 70–200 nm. As a natural process that spontaneously organizes molecular units into well-ordered structures, molecular self-assembly technique is emerging as a powerful method for functional materials fabrication. It is well documented that amphiphilic block copolymers or grafted copolymers could self-assembly into nano/micro-vesicles or capsules in aqueous solutions. Palmer and coworkers [55] successfully employed amphiphilic diblock copolymers poly (butadiene-*b*-ethylene oxide) (PBD-PEO), poly(ethylene oxide-*b*-lactide) (PEO-PLA) and poly(ethylene oxide-*b*-caprolactone) (PEO-PCL) as building blocks to prepare polymersomes for Hb encapsulation through rehydrate and extrusion method.

LbL assembly technique is a versatile method for fabricating assemblies with a specific shape, size, material composition, and functionality [56]. As mentioned above, our group has successfully fabricated Hb-based hollow microcapsules through covalent LbL assembly technique combined with templating porous particles. It is worth to note that LbL assembly technique enables many conceivable ways to adjust and improve the behaviors of these Hb-based assemblies. First, the size and properties of Hb-based assemblies could be easily controlled by adjusting the size of the template and the assembled composition. Second., some functional particles or reagents like Fe_3O_4 nanoparticles, allosteric effector or methemoglobin reductase could be readily loaded into Hb-based assemblies for further functionalization and optimization of Hb-based oxygen carriers. In our subsequent works

[57–60], the assembly and application of hemoglobin-based microspheres/capsules as artificial blood substitutes were deeply researched. The integration of modern nanobiotechnology and self-assembly technique has greatly facilitated the development of artificial oxygen carriers. The as-prepared Hb spheres may have potential to be developed as useful artificial blood substitutes in future.

5.4.1 *Highly Loaded Hemoglobin Spheres as Promising Artificial Oxygen Carriers*

In order to enhance the loading content of Hb in a well-defined structure, we explored a facile and controllable avenue to fabricate Hb spheres with a high loading content as promising oxygen carriers [57]. In this work, Hb spheres were prepared by templating decomposable porous $CaCO_3$ particles in combination with covalent LbL assembly. Hb proteins were firstly encapsulated in the porous template particles by the co-precipitation of protein and $CaCO_3$. A biomedical cross-linking agent, glutaraldehyde (GA), was then 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 [61]. In the following, additional Hb was furtherly assembled on the outer shell of the Hb-loaded $CaCO_3$ particles by covalent LbL assembly with GA to obtain a higher loading of Hb in an individual particle. Finally, the surface of the Hb spheres was further chemically modified by biocompatible polyethylene glycol (PEG) to protect and stabilize the system, prolong the retention time in intravascular circulation and also provide a more stable and semipermeable system, which can allow small molecules such as O_2 and CO_2, the life-sustaining glucose in plasma, reducing agents and metabolic products to diffuse into/out of the system, just more like the active behavior of RBCs.

Figure 5.7a 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 way, 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. Compared to the previous reports [62–65], the loading concentration of Hb spheres assembled in the present work would be apparently higher, because a number of micro- and nano-pores of the $CaCO_3$ templates are accessible for Hb molecules (Fig. 5.7b). In addition, the LbL technique can further help the loading of Hb by coating the external surface of $CaCO_3$ with encapsulated Hb inside (Fig. 5.7c). Both factors contribute to the high loading of Hb in the spheres, and also present adjustable permeability [24], which is of great importance

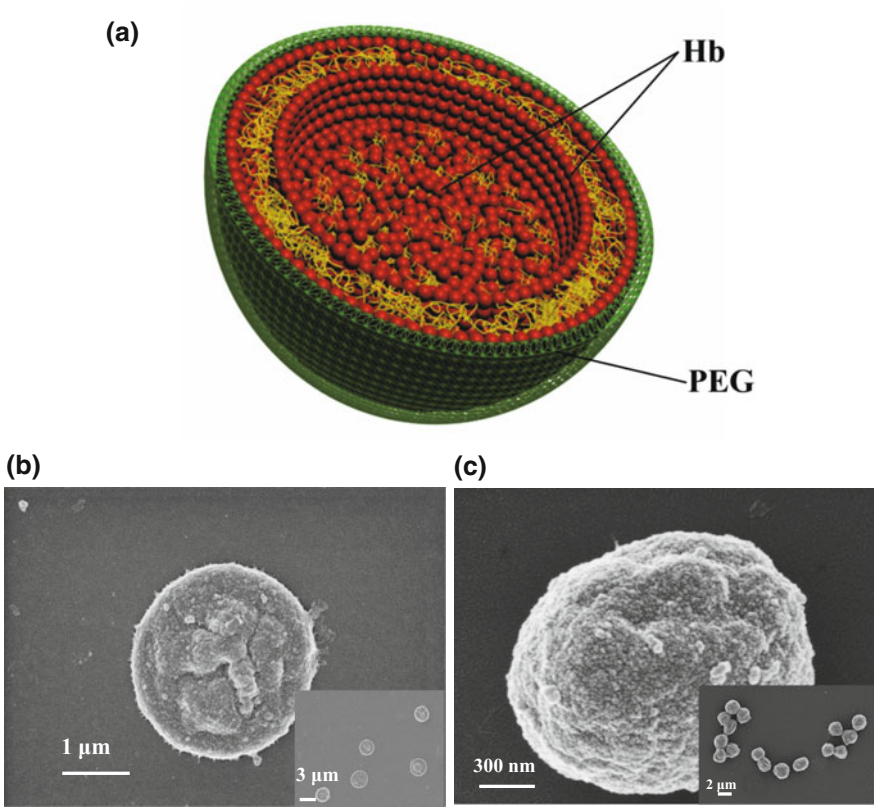

Fig. 5.7 **a** Schematic representation of the assembled Hb microspheres with the surface modified by PEG. **b** SEM images of Hb spheres after removing $CaCO_3$. **c** SEM images of Hb spheres with additional Hbs as the outer shell after removing $CaCO_3$. Reprinted with the permission from Ref. [57]. Copyright 2012 American Chemical Society

for maintaining the normal functionalities of natural RBCs, allowing life-sustaining small molecules such as oxygen, carbon dioxide, glucose, reducing agents and metabolic products to diffuse into/out of the system. More importantly, such Hb protein after covalently cross-linked and modified by PEG retains well its whole structure and especially its oxygen-carrying function. Figure 5.8 shows the results from UV-vis absorption measurement on the carrying and release of oxygen by Hb. It could be obviously seen that 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. All these results demonstrated that the LbL-assembled Hb-based microspheres are much more suitable to function as artificial oxygen carriers, particularly for an urgent need of large amounts.

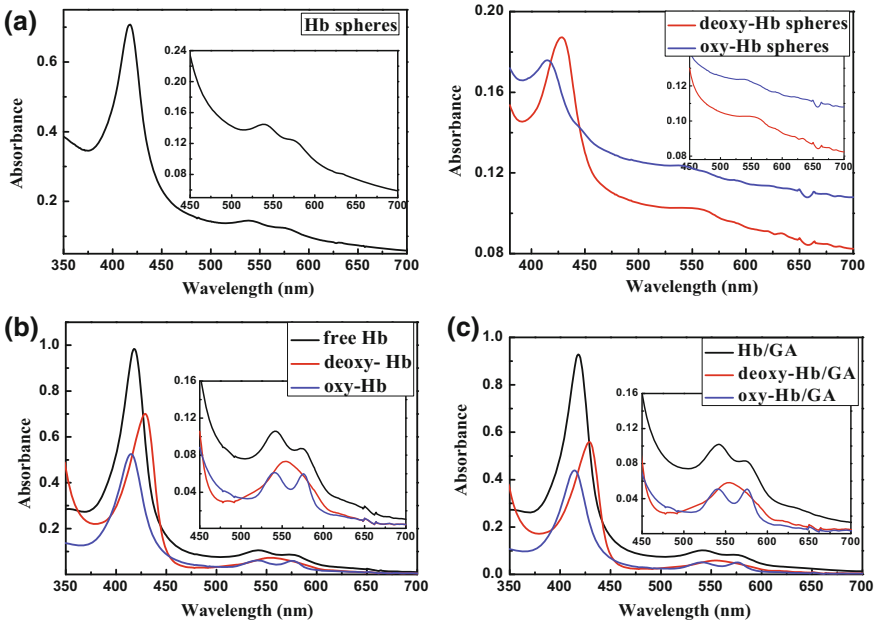

Fig. 5.8 UV-vis absorption spectra of deoxy-Hb and oxy-Hb. **a** Hb spheres; **b** free Hb; **c** cross-linked Hb. Inset image in detail shows the absorption between 450 and 700 nm. Reprinted with the permission from Ref. [57]. Copyright 2012 American Chemical Society

5.4.2 *Construction and Evaluation of Hemoglobin-Based Capsules as Blood Substitutes*

In the preceding section, we prepared the $(Hb/DHP)_6$ microcapsules to mimic artificial RBCs via covalent LbL assembly technique and template method (Fig. 5.9) [58]. The oxidized heparin (dialdehyde heparin, DHP) was specially used both as one of the wall components and a cross-linker because of its nontoxicity, biodegradability, biocompatibility and hemocompatibility. In addition, the negative charges of DHP on microcapsules' surface would be beneficial to prolong the blood retention time of microcapsules in vivo. The amino groups of Hb react easily with aldehyde groups of DHP through Schiff's base reaction. The formation of Schiff's base bonds among the Hb/DHP multilayers enabled the $(Hb/DHP)_6$ capsules with autofluorescence, which attributed to the n–π* transition of C=N bonds. This property would beneficial in predicting and monitoring the safety and efficacy of $(Hb/DHP)_6$ capsules in humans, while avoiding the use of external fluorochromes for biological tracing. In addition, the cross-linking reaction between amino groups of Hb and aldehyde groups of DHP effectively prevented the dissociation of the Hb tetramer into dimers.

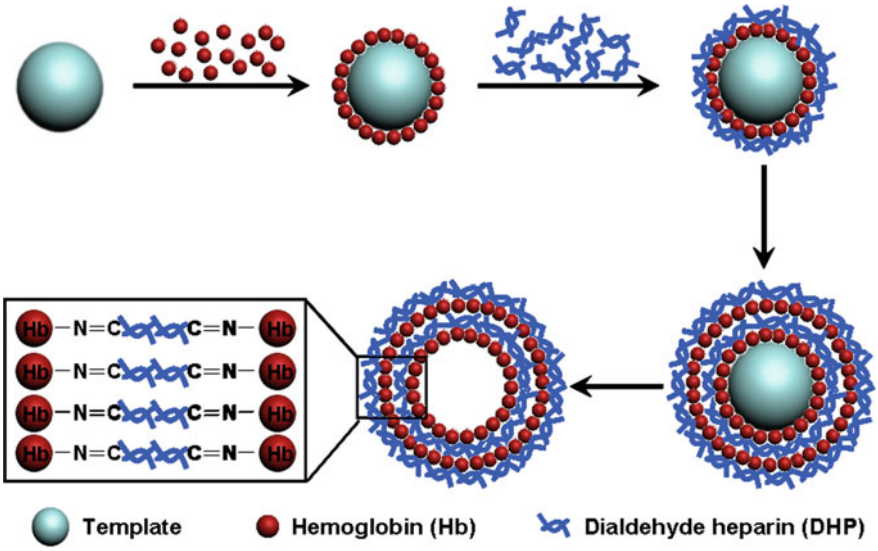

Fig. 5.9 Schematic illustration to show the fabrication process of Hb/DHP microcapsules through Schiff's base bond. Reproduced from Ref. [58] by permission of John Wiley & Sons Ltd

In this work, the biocompatibility of $(Hb/DHP)_6$ microcapsules is evaluated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay and cell experiment. Figure 5.10 clearly shows that the cell coexisted with microcapsules without any abnormalities, which confirmed the biocompatibility of the microcapsules. It is also worth to note that microcapsules with red fluorescence are distributed around cells without being uptaken by the cells. It can be attributed to the electrostatic repulsion between negative charges on cell membrane surface and microcapsules surface, which will help to prolong the blood retention time of microcapsules in vivo [66]. To access the degradation behavior, the $(Hb/DHP)_6$ microcapsules were incubated with trypsin solution for 24 h. The results show that no microcapsule was observed after degradation, confirming that $(Hb/DHP)_6$ microcapsules were biodegradable. Hemocompatibility of $(Hb/DHP)_6$ microcapsules is also characterized in terms of prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (APTT) and hemolysis rate. Oxygen-carrying capacity of the microcapsules is demonstrated by converting the deoxy-Hb state of the microcapsules into oxy-Hb state. All these results demonstrated that the Hb assembled in the $(Hb/DHP)_6$ microcapsules still maintained its own bioactivity and possessed the essential oxygen-transporting function of an oxygen carrier, as well as nontoxicity, biodegradability, biocompatibility, and hemocompatibility. It is indicated that the as-prepared hemoglobin-based capsules have great potentials to function as blood substitutes.

It is worth to note that in view of the advantages of LbL assembly technique, there are many conceivable ways to adjust and improve the behaviors of the

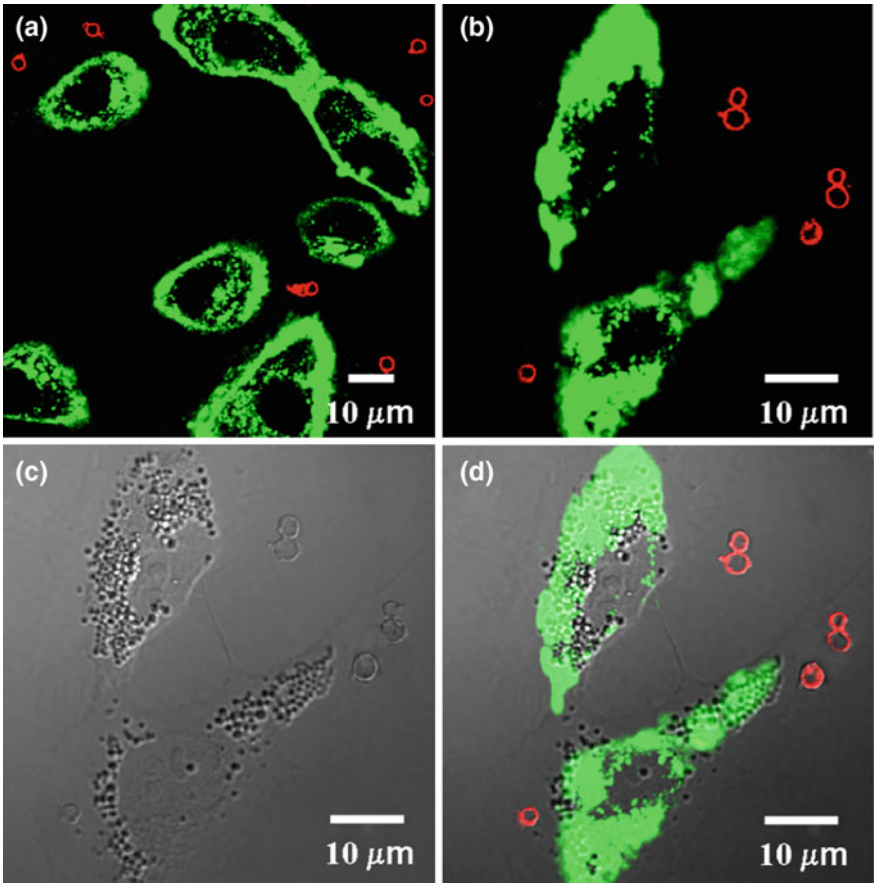

Fig. 5.10 **a**, **b** CLSM images of HUVEC cells stained with Alexafluor 488 incubated with $(Hb/DHP)_6$ microcapsules by excitation at 488 and 559 nm, **c** corresponding bright field image of **b**, **d** the overlapped image of **b** and **c**. Reproduced from Ref. [58] by permission of John Wiley & Sons Ltd

Hb-based capsules that functional groups or reagents (such as allosteric effector, superoxide dismutase, and 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. [67]. 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 biomedicine fields.

5.4.3 High Impact of Uranyl Ions (UO_2^{2+}) on Carrying-Releasing Oxygen Capability of Hemoglobin-Based Blood Substitutes

Recently, our group investigated the effect of radioactive UO_2^{2+} on the structure and carrying-releasing oxygen capability of hemoglobin-based oxygen carriers in vitro [59]. It is well-known, radioactive metal toxins seriously threaten the human health and environments because of their radiological and chemical toxicity. Radioactive uranyl ion (UO_2^{2+}) is an important contamination discharged into environment mainly from nuclear power reactor effluents. However, it is not so clear how the accumulation of radioactive uranyl ions (UO_2^{2+}) in blood cells can cause immune system damage. It is would be helpful to explore the damage of UO_2^{2+} on the biofunction and physical chemistry mechanism of the blood cells. In this work, the magnetic Hb microspheres fabricated through covalent LbL assembly on iron oxide (Fe_3O_4) loaded porous $CaCO_3$ particles were utilized as blood substitutes for the damage study of UO_2^{2+} on the biofunction of blood cells. The carriers with magnetic properties can help to solve some technical operations [68], which facilitate their trapping from the medium with the help of an external magnet. For the separating process of UO_2^{2+} adsorbed on the Hb microspheres, the magnetically assisted chemical separation (MACS) method is introduced as a rapid and easy avenue based on biocompatible magnetic nanoparticles by applying an external magnetic field. Figure 5.11a, b shows a schematic representation of the fabrication process of the magnetic Hb microspheres and their specific adsorption for UO_2^{2+}. Magnetic nanoparticles of iron oxide (Fe_3O_4) are first encapsulated in porous $CaCO_3$ particles after ultrasonic dispersion. Then, hemoglobin proteins are chemically modified on the surface of the Fe_3O_4-loaded $CaCO_3$ microspheres to form the Hb shells based on covalent LbL assembly. To study its chemical toxicity, the UO_2^{2+} is mixed with the as-assembled magnetic Hb microspheres. The results from UV-vis absorption measurement on UO_2^{2+} before and after being adsorbed on magnetic Hb microspheres (Fig. 5.11c) shows that UO_2^{2+} are highly adsorbed on the as-assembled magnetic Hb microspheres. The damage experiments of radioactive UO_2^{2+} on magnetic Hb microspheres (Fig. 5.11d) obviously proved that the presence of UO_2^{2+} in vivo seriously caused Hb microspheres to be inactivated and denaturalized, and resulted in the loss of the oxygen-transporting capacity. It may be attributed to the highly binding coordination of UO_2^{2+} with an iron atom of Hb, and the tight electrostatic interaction between positive UO_2^{2+} and negative Hb. Our works well confirmed that the accumulation of radioactive uranyl ions (UO_2^{2+}) in blood cells can seriously damage the human health, especially the transporting-oxygen capability of blood cells. In addition, in view of the high adsorption capacity of the as-assembled magnetic Hb microspheres, the assembled magnetic Hb-based microspheres may open a new highly effective way for removal of UO_2^{2+} from the radioactive-contaminated body or from the nuclear power reactor effluents before discharge into the environment. It can also be applied in the removing of other metal toxins from blood due to its biocompatibility.

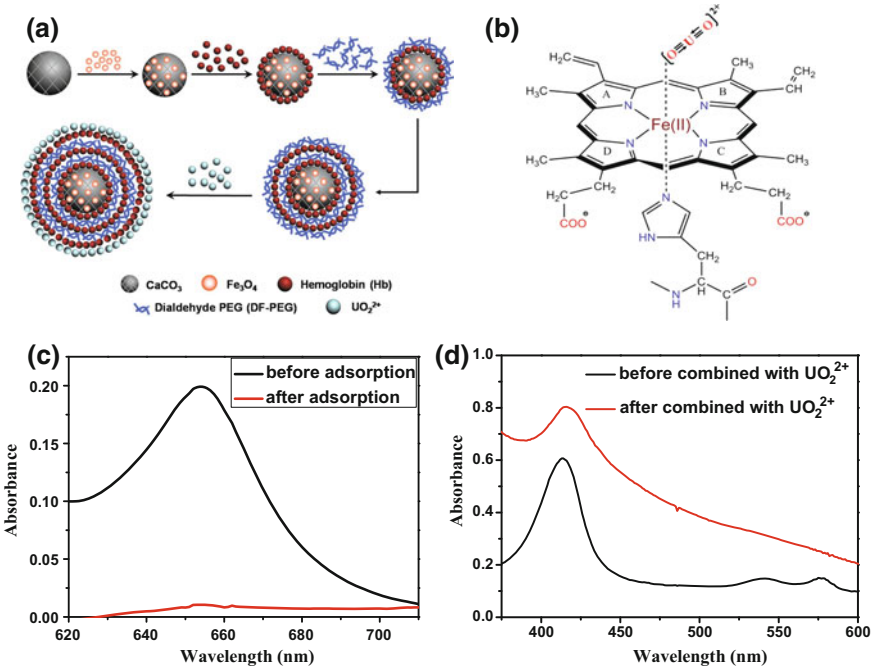

Fig. 5.11 **a** Schematic illustration of the fabrication of magnetic hemoglobin microspheres by the porous template strategy and Layer-by-Layer assembly technique and its adsorption for UO_2^{2+}. **b** The coordinating interaction of UO_2^{2+} with iron ion of heme. **c** UV-vis absorption spectra of UO_2^{2+} in aqueous solution before and after adsorbed on the magnetic Hb microspheres. **d** The sorption percentage of UO_2^{2+} with pure $CaCO_3$ particles and the magnetic Hb microspheres with different adsorption cycles of Hb/PEG as outer shell. Reproduced from Ref. [59] by permission of John Wiley & Sons Ltd

5.5 Conclusions and Perspectives

As an important protein in vital activities, hemoglobin-based molecular assembly has been extensively explored and applied. In this chapter, hemoglobin-based multifunctionalized microcapsules fabricated by covalent layer-by-layer assembly combined with template technique were introduced. The fabrication of Hb capsules could imitate in some sense its structural function in the living system, and may help us to understand its properties and can be expected to have better applications in drug delivery and controlled release, biosensors, biocatalysis and bioreactors due to the enhancement of biological availability. In particular, in view of the carrying-oxygen function of Hb protein in blood, the covalently LbL-assembled Hb microcapsules may have great potential to be developed as useful artificial blood substitutes in future. The integration of modern nanobiotechnology and self-assembly technique has greatly facilitated the development of Hb-based oxygen carriers. The as-prepared Hb spheres with highly loaded content are much more suitable for artificial oxygen

carriers, particularly for an urgent need of large amounts. By using magnetic Hb microspheres, the damage of radioactive metal toxins on the biofunction and physical chemistry mechanism of the blood cells were detailedly studied in vitro. Furthermore, the as-assembled Hb microspheres may be considered as a highly effective adsorbent for removing the metal toxins from the radioactive-contaminated body.

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