Hemoglobin-CdTe-CaCO₃@Polyelectrolytes 3D Architecture: Fabrication, Characterization, and Application in Biosensing**

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A Hemoglobin-CdTe-CaCO₃@polyelectrolyte 3D architecture is synthesized by a stepwise layer-by-layer method and is further used to fabricate an electrochemistry biosensor. While the calcium carbonate (CaCO₃) microsphere acts as an effective host for the loading of cadmium telluride (CdTe) quantum dots due to its channel-like structure, the polyelectrolyte layers further increase the loading amount and help in the formation of a thick and uniform quantum-dot “shell”, which not only improves the stability of the spheres in water, but also contributes to the fast and effective direct electron transfer between the protein redox center and the macroscopic electrode. The materials are characterized and compared, and the possible mechanism for the direct electrochemistry phenomenon is hypothesized. Our work not only provides a facile and effective route for the preparation of quantum-dot-loaded spheres, but also sets an example of how the structure of functional materials can be tuned and related to their applications. In addition, it is one of the few examples of using CaCO₃ microspheres in quantum-dot loading and biosensing.

1. Introduction

The convergence of nanotechnology and biotechnology has led to a great development of the design and fabrication of functional architectures from building blocks like nanomaterials and biomolecules.[1] These assemblies often exhibit unique synergistic properties and functions and are widely used in biosensing,[2,3] drug and gene delivery,[4] biocatalysis[5] and other related areas.[6,7] Nanoparticles, especially metal and semiconductor particles, are widely used as building blocks for these assemblies due to their special electronic, photonic and catalytic properties.[8] Among them, quantum dots (QDs) have attracted intensive attention ever since their first milestone applications as luminescence labels in biological detection.[9] Due to their interesting optical and electronic properties, QDs have been widely used today in both biosensing and biodiagnostic areas as versatile materials.[7,10] When QDs are conjugated with biomolecules like proteins, which are fascinating macromolecular structures in terms of their unique recognition, transport, and catalytic properties, not only can they act like as alternative labels to molecular fluorophores as fluorescence biological probes,[11] they can also function in electrochemical biosensors[2,12] or bioassays.[10] However, due to their small size, these QD-protein bioconjugates suffer from the problem of formation of QD-protein aggregates, which greatly limits their applications.[13]

An alternative and promising strategy is to utilize a biofunctionalized “host” labeled with QDs. Currently, three methods have been tested for the incorporation of QDs into a “host”: [14] a) QDs are incorporated in the beads during the bead synthesis; b) QDs are trapped or affinity-partitioned directly into a preformed bead; or c) coating of the bead surface with QDs. While the problem of the first approach lies in the drawback of phase separation between the beads and QDs during the synthesis, the second method suffers from the drawback of a non-uniform distribution of QDs inside the beads.[15] The third method offers a facile and controllable means for the loading of QDs by using the layer-by-layer method. A uniform distribution of QDs was obtained when SiO₂ spheres were used as templates.[15] In another piece of work, biologically active polystyrene (PS) beads labeled with semiconductor quantum dots (QDs) with tailored QD loading were prepared via the layer-by-layer (LBL) assembly approach and applied in immunoassays.[13] However, the smooth surface and nonporous structure of the PS and the SiO₂ beads limited the loading of the QDs to a monolayer,[16] and an increase in the loading of the QDs could only be achieved by repetitive and tedious work of multilayer assembly. Recently, Nie and coworkers developed a simple strategy for the rapid and precise doping of QDs by using organically functionalized, hydrophobic, mesoporous silica beads,[17,18] yielding QD-doped beads that are 30 times brighter than nonporous PS beads of a similar size. CdTe quantum dots were also assembled on mesoporous cellular foams silicate (MCFs) and further used...
for protein loading and biosensing.\textsuperscript{[2]} A good “host” is of paramount importance for the QD loading and following applications. Porous materials seem to be promising “hosts” for this application.

Calcium carbonate, a natural mineral with great biocompatibility, has been proven to intensify enzyme performance and has been used in industry, medicine, microcapsule fabrication, and biosensing.\textsuperscript{[3]} It has three anhydrous crystalline forms: calcite, aragonite and vaterite. Vaterite, the spherical polymorph of calcium carbonate, often composed of nanoparticles with diameters of 10–30 nm, has drawn more and more attention today because of its unique structure and specific features like high surface area and small specific gravity. Compared to its silica counterpart that has much-wider application today, vaterite has the advantages of easier preparation, low cost and better dispersibility in water. However, although vaterite is stable under dry conditions, when in contact with water, it transforms easily and irreversibly into a thermodynamically stable polymorph of calcite\textsuperscript{[19]} which greatly hinders its application. Another limitation lies in the lack of a sophisticated method to functionalize its surface and make it as flexible as the silica surface.

In our previous work, we reported the fabrication of enzyme-gold nanoparticle-CaCO\textsubscript{3} bioconjugates using a two-step self-assembly process and further applied the assemblies into biosensing. The unique structure of vaterite was stabilized by the gold nanoparticles and the assembly showed improved properties due to the synergic effect of its parent materials\textsuperscript{[3]} In this work, using the stepwise layer-by-layer self-assembly process shown in Scheme 1, the vaterite structure can be stabilized with polyelectrolyte layers (PEs) (Scheme 1: step a) and further used for the loading of CdTe QDs (Scheme 1: step b). While the CaCO\textsubscript{3} microspheres offered a promising template for the loading of QDs, the polyelectrolyte layers stabilized the structure and contributed to the uniform distribution of QDs in the particles. A thick and bright QD ring pattern was observed for the CdTe-CaCO\textsubscript{3}@PEs spheres in their confocal fluorescence images, indicating the presence of a QD “shell”. This structure also helps in improving the stability of the hybrid material in water. The hybrid material was still porous and can be further used for the loading of hemoglobin (HB), to fabricate the HB-CdTe-CaCO\textsubscript{3}@PEs 3D architecture (Scheme 1: step c). Once the bioconjugates were incorporated into a biosensor, direct electrochemistry of HB was observed and a fast and effective electron transfer between the heme center of HB and the macroscopic electrode was confirmed by electrochemistry studies. The biosensor showed good electrocatalytic performance to H\textsubscript{2}O\textsubscript{2} with a linear range of \( \sim 5 \times 10^{-6}–4.5 \times 10^{-5} \text{ mol L}^{-1} \) and a detection limit of \( 2.5 \times 10^{-7} \text{ mol L}^{-1} \). The small \( K_m \) of 0.77 mM indicates a high biological affinity of immobilized HB to H\textsubscript{2}O\textsubscript{2}. The good performance of the biosensor, as well as the effective direct electrochemistry transfer phenomenon, is the result of the synergic effect of each building block in the HB-CdTe-CaCO\textsubscript{3}@PEs 3D architecture. The QD “shell” acted as a conductive “shell” tethering into the redox center of the protein and facilitated the electron-transfer process. The polyelectrolytes that help to form the “shell” indirectly contribute to the direct electrochemistry process. The CaCO\textsubscript{3} microspheres, at the same time acted like a suitable matrix that accommodated both the QDs and the HB molecules, and also offered an optimized orientation and microenvironment for the immobilized protein. Our work, as a whole, not only demonstrates that a multifunctional architecture can be assembled through a simple method, but also sets an example of the application of CaCO\textsubscript{3} microspheres as a versatile and promising material for QD loading and biosensing.

2. Results and Discussion

2.1. Assembly of Polyelectrolyte Layers onto the Surfaces of CaCO\textsubscript{3} Microspheres

Porous CaCO\textsubscript{3} microspheres, the vaterite polymorph of CaCO\textsubscript{3}, is often composed of nanoparticles 10–30 nm in size and has a porous structure and channel-like interior. It has been widely used today in drug delivery,\textsuperscript{[20]} protein immobilization and encapsulation,\textsuperscript{[19]} biosensing,\textsuperscript{[3]} and polymeric-microcapsule fabrication,\textsuperscript{[21]} due to this unique structure. However, the vaterite structure suffers from the problem of recrystallization when in contact with water, which could turn it into rhombohedral calcite microcrystals.

The electrostatic layer-by-layer method is widely used today to introduce polyelectrolyte layers to colloidal micro-and nanoparticle surfaces due to its simplicity and flexibility.\textsuperscript{[22]} Recently, it has been found that the vaterite spheres could be stabilized by polyelectrolyte layers. In one report, poly(sodium 4-styrenesulfonate) (PSS)-stabilized CaCO\textsubscript{3} microspheres

![Scheme 1. Schematic illustration of the fabrication process of the HB-CdTe-CaCO\textsubscript{3}@PEs 3D architecture. a) CaCO\textsubscript{3} microspheres were firstly coated with polyelectrolyte layers to produce CaCO\textsubscript{3}@PEs microspheres. b) The polyelectrolyte-protected spheres were then used for the loading of CdTe quantum dots to fabricate the CdTe-CaCO\textsubscript{3}@PEs hybrid material. c) In the final step, the hybrid material was used for the loading of hemoglobin (HB).](image-url)
were stored in water for six months and no obvious change in shape was observed.[20] In our experiment, the same method was used to introduce PSS/poly(allylamine hydrochloride) (PAH) thin layers onto the surfaces of CaCO3 microspheres.

The layer-by-layer assembly process was monitored by microelectrophoresis measurements (expressed as a ζ-potential), which is an effective method of characterizing the surface charge of colloid particles.[23] The values of the ζ-potential of CaCO3, CaCO3/PSS and CaCO3/PSS/PAH microspheres are shown in Figure 1. The CaCO3 microspheres were positively charged at neutral pH with a ζ-potential of +11.2 mV (a in Fig. 1), which can be explained by an excess of Ca2⁺. The carbonate ion reacts with additional H⁺, whereas Ca2⁺ remains in excess with the solution absorbing on the particle surface.[19] The subsequent alternate absorption of PSS and PAH polyelectrolyte layers onto the surface yielded ζ-potentials of −14 mV (b in Fig. 1) and +16.1 mV (c in Fig. 1), respectively. In order to minimize the effect of the PEs layers on the morphology of the CaCO3 spheres, only one bilayer of PEs was chosen. The positively charged CaCO3/PEs spheres could be further used in the loading of CdTe QDs.

2.2. Assembly of CdTe QDs onto CaCO3/PEs Microspheres

The absorption process of CdTe QDs onto the surface of CaCO3/PEs was monitored by fluorescence spectroscopy. The insert in Figure 2 shows the fluorescence spectra of the as-prepared CdTe QDs (curve a) and the supernatant after CdTe absorption and centrifugation (curve b). The sharp decrease in the intensity of the emission peak at 584 nm indicates the absorption of CdTe QDs onto the surface of the CaCO3/PEs microspheres. The fluorescence spectra of the CaCO3 microspheres (curve c) and the CdTe-CaCO3/PEs spheres (curve d) are shown in Figure 2. The salmon-pink-colored CdTe-CaCO3/PEs deposit shows a strong emission at 579 nm compared to the pure CaCO3 microspheres, indicating loading of the CdTe QDs. The loading amount of CdTe onto CaCO3 can be calculated by the difference in intensity of the emission peak at 584 nm of the initial CdTe solution before and after loading. A rough estimate indicated that 1 mg of CaCO3/PEs microspheres can accommodate 1.3 × 10¹⁸ CdTe QDs. In a control experiment, conventional mesoporous silica (MPS), CaCO3 and MPS@PAH spheres were used for the loading of CdTe QDs. The loading amounts were calculated in the same way and are compared in Table 1. The loading amount of CaCO3/PEs is the highest of the four samples. This indicates that the unique structure of the CaCO3 microspheres and the presence of the polyelectrolyte layers both played an important part in the loading of the QDs.

The structure and surface features of the CaCO3 microspheres are vital for the loading of CdTe QDs. Figure 3A shows a scanning electron microscopy (SEM) image of a polyelectrolyte-coated CaCO3 microsphere. The ultrathin polyelectrolyte layers, with a thickness of around 4 nm,[24] do not affect the morphology of the CaCO3 microspheres, but help to retain their spherical shape. The surfaces of the CaCO3/PEs microspheres are very rough and are suitable for the loading

Table 1. Comparison of the loading amounts of CdTe QDs on CaCO3/PEs, CaCO3, MPS/PAH and MPS microspheres. The absorption amount was calculated by the difference of the emission intensity at 584 nm between the as-prepared CdTe and the supernatant after CdTe QDs assembly.

<table>
<thead>
<tr>
<th>Template</th>
<th>Loading amount [10¹⁸ CdTe per mg]</th>
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<tbody>
<tr>
<td>CaCO3/PEs[a]</td>
<td>131</td>
</tr>
<tr>
<td>CaCO3</td>
<td>88</td>
</tr>
<tr>
<td>MPS/PAH</td>
<td>42</td>
</tr>
<tr>
<td>MPS</td>
<td>8</td>
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[a] Compared with the pure CaCO3 microspheres, the polyelectrolyte layers could increase the loading amount of CdTe QDs by ~49%. The loading amount on CaCO3/PEs microspheres is three times that on conventional mesoporous silica spheres with PAH as the outer-most layer.
The presence of the element Cd, which is further evidence for the presence of CdTe QDs. The spheres are still porous and this structure can be visualized at a higher magnification by field-emission scanning electron microscopy. Figure 3D shows the SEM image of a broken sphere of CdTe-CaCO₃@PEs, which reveals the channel-like interior structure of the spheres. The channels that go directly from the outer-most surface to the center of the CaCO₃ microspheres are suitable for the loading of the CdTe QDs, not only onto the outside surface, but also into the interior.

The polyelectrolyte layers on the CaCO₃ surface are also important to the loading of QDs. First of all, they stabilize the structure of the CaCO₃ microspheres, so that they can be stable templates for the loading of QDs in water. They also prevent the particles from aggregating by introducing more positive charges onto the vaterite surface. CaCO₃@PEs microspheres were kept in water for a week and the vaterite shape was not affected. However, when CaCO₃ microspheres were kept in water overnight, more than 80% of the vaterite spheres were turned into the calcite polymorph.

The second role of the polyelectrolyte layers lies in their function of increasing the loading amount of CdTe QDs. Although the CaCO₃ microspheres alone could absorb CdTe QDs due to electrostatic interactions, using PAH as the outmost layer on the CaCO₃ microspheres, the absorption amount could be increased by 49% (Table 1). The z-potential of CaCO₃@PEs particles shifted from +16.1 mV to −28.8 mV, while the corresponding value of the pure CaCO₃ microspheres changed from +11.2 mV to −20.8 mV after the loading of CdTe QDs. The larger shift and more-negative value of the z-potential after the loading resulted from higher absorption amount of CdTe QDs onto the outer surface when the polyelectrolyte layers were introduced. The cationic groups (−NH₃⁺) of the positively charged PAH interacts so strongly with the negatively charged carboxyl (−COO⁻) groups on the surface of the CdTe QDs[13] that the loading amount was greatly increased when polyelectrolyte layers were involved. Meanwhile, since the positively charged PAH also introduced more positive charges onto the surface of the CaCO₃ microspheres (see the value of the z-potentials), the enhanced electrostatic interaction between the QDs and the microspheres also contributes to the increased loading amount.

The most important function of the polyelectrolyte layers is related to the distribution of the CdTe QDs inside the microspheres. The distributions of the CdTe QDs in the CaCO₃@PEs and CaCO₃ microspheres were demonstrated using confocal laser-scanning fluorescence microscopy (CLSM), shown in Figure 4. Without the polyelectrolyte layers, the CdTe QDs distribute randomly in the CaCO₃ microspheres (Fig. 4A). The differences in the distribution of CdTe from particle to particle are not very obvious in the CdTe-CaCO₃@PEs microspheres (Fig. 4B), indicating a uniform distribution. Due to a highly effective loading of QDs, an ultrabright and thick ring pattern was observed for the CdTe-CaCO₃@PEs microspheres. The CdTe QD “ring” was directly visualized in the transmission electron microscopy.
The rough surface features and the presence of open channels in the CaCO₃ microspheres offer a great opportunity for QDs to load onto both the outside surface and into the open channels, resulting in a "thick ring". QDs could also penetrate deeply into the center of the CaCO₃ spheres (Fig. 4B), but most of the loaded CdTe QDs were distributed onto the outside surfaces or infiltrated into the outer-most pores and channels of the CaCO₃@PEs microspheres. Compared to a previous result for loading of dextran of different molecular weights into CaCO₃ microspheres,[19] it is very likely that diffusion limitation is one of the reasons that cause this kind of distribution. The pores and channels of the CaCO₃ microspheres could become blocked and thus prevent further infiltration of more CdTe QDs into the central area. However, an increased loading amount of QDs into the interior of the spheres can be observed for the CaCO₃@PEs compared to the CaCO₃ spheres, as shown in Figure 4. It is very likely that the infiltration and further absorption of PAH molecules into the inner surface of the microspheres increased the loading amount of QDs in the interior surface. To further investigate the distribution of the CdTe QDs in the three-dimensional CdTe-CaCO₃@PEs microspheres, a series of confocal images along the z-axis were taken and are shown in Figure 6. A ring pattern is observed for most of the optical sections collected at different levels, (Fig. 6A-I) perpendicular to the optical axis (the z-axis). However, from the center (Fig. 6A) of the microsphere to the top (Fig. 6I), the dark circle inside the "ring" becomes smaller and brighter, and finally disappears. This indicates that the QD "ring" observed in the 2D confocal image is actually a thick QD "shell" in the whole CdTe-CaCO₃@PEs microsphere. It is interesting to find that the thick QD "shell" contributes to enhanced stability of the structure of the composite spheres. When the CaCO₃@PEs particles were stored in water for more than two weeks, calcite began to form. It is probably because one bilayer of polyelectrolyte is not enough to fully cover the whole vaterite surface, thus resulting in slow recrystallization. The spherical shape of the CdTe-CaCO₃@PEs, in contrast, is stable in water for more than a month. This result is similar to our previous work using gold nanoparticles to stabilize the vaterite structure. The closely packed QD "shell" prevented the leakage of Ca²⁺ and CO₃²⁻/C₀²⁻, thus greatly reducing the chance of recrystallization.[19] In a word, the polyelectrolyte layers help to form a uniform QD "shell" in the CdTe-CaCO₃@PEs spheres which helps in enhancing the stability of the hybrid material in water.

To further investigate the efficiency of the quantum-dos doping, conventional mesoporous silica spheres (MPS) were used for control experiments. A SEM image of the MPS beads used is shown in Figure 7A. Brunauer–Emmett–Teller (BET) data revealed surface areas of 10.4 m² g⁻¹ and 60 m² g⁻¹ and pore volumes of 0.093 cm³ g⁻¹ and 0.36 cm³ g⁻¹ for the CaCO₃ microspheres and the MPS beads, respectively. The average pore size was 33 nm for the CaCO₃ microspheres and 30 nm for the MPS beads. The average size of the CaCO₃ and MPS spheres was 5 μm as determined by SEM. As shown in Table 1, without the presence of polyelectrolyte layers, the positively charged CaCO₃ surface can accommodate ten times more QDs than the negatively charged MPS surface. It is obvious that QDs might have more affinity with the CaCO₃ surface than the silica surface. Even using PAH as the outer-most layer, the loading amount can be tripled by using CaCO₃ microspheres instead of MPS particles. It is quite interesting to find that the CaCO₃ microspheres, with a much lower surface area and pore

Figure 4. Confocal images of A) CdTe-CaCO₃ microspheres and B) CdTe-CaCO₃@PEs microspheres. The inserts show the overviews of the spheres.

Figure 5. TEM image of CdTe-CaCO₃@PEs microspheres, illustrating the presence of CdTe QDs.

Figure 5. TEM image of CdTe-CaCO₃@PEs microspheres, illustrating the presence of CdTe QDs.
volume, seem to be superior templates to conventional mesoporous silica beads for the loading of CdTe QDs. Compared to mesoporous silica beads, the channel-like structure of the CaCO₃ spheres might be more suitable for the loading of CdTe QDs, thus increasing the loading amount. Meanwhile, since it has been demonstrated that the penetration depth of QDs into PS beads does not exceed 5% of the bead radius even under extensive swelling conditions, our method also demonstrates that an increased loading amount can be achieved by simply changing the template, rather than tedious and repeated work of multilayer assembly on a smooth surface.

2.3. Assembly of Protein onto CdTe-CaCO₃@PEs Hybrid Material for Biosensing

As shown in the SEM image in Figure 3D, the CdTe-CaCO₃@PEs hybrid material is still porous and can be a suitable template for protein loading. With an isoelectric point (IEP) of pH 6.8, HB was positively charged at pH 6.0 in phosphate buffered saline (PBS) and can be easily assembled onto the CdTe-CaCO₃@PEs spheres. The bioconjugates are stable at neutral pH due to the strong interactions between the amino group of the protein and the carboxylic group of the CdTe QDs surface. The \( \zeta \)-potential value changed from \(-28.8\) mV (d in Fig. 1) to \(-8.57\) mV (e in Fig. 1) after the loading of HB. When the negatively charged QD outer-most layer was replaced by almost-neutral protein molecules, the \( \zeta \)-potential had a positive shift. A predominant change in the whole assembly process is the color change that can be observed in Figure 8A. CaCO₃@PEs microspheres (Fig. 8A: a) exhibit a white color, while the color of the CdTe-CaCO₃@PEs microspheres (Fig. 8A: b) is salmon pink. After the loading of...
protein, the spheres (Fig. 8A: c) turned into dark brown. Under UV illumination (Fig. 8B), the CaCO₃@PEs particles (Fig. 8B: a) showed no fluorescence while the fluorescence emitted by the CdTe-CaCO₃@PEs (Fig. 8B: b) can be clearly observed. The fluorescence was quenched somehow after the loading of the HB and only weak fluorescence can be observed for the bioconjugates (Fig. 8B: c). This is in accordance with the results of the fluorescence spectra in Figure 2. According to a previously reported result, it is the heme center of HB that acts like an effective quencher for the fluorescence of CdTe QDs. The SEM image in Figure 3C shows the surface structure of a protein-loaded sphere. It can be clearly observed that protein filled into the pores and formed aggregations on some parts of the spheres. EDX analysis (data not shown) shows an additional peak of S, compared to CdTe-CaCO₃@PEs spheres, and further confirmed the presence of protein. The loading amount of HB on the microspheres was calculated from UV-vis spectra before and after protein loading. It was found that around 283 µg of HB was absorbed for 1 mg of the CaCO₃ microspheres that were used for the preparation of the CdTe-CaCO₃@PEs hybrid materials.

2.4. Direct Electrochemistry of HB-CdTe-CaCO₃@PEs-Modified Electrodes

The quenching of the fluorescence of CdTe QDs is a very interesting phenomenon that is not only evidence for the interaction between the heme center of HB and CdTe QDs, but also renders the possibility of using the bioconjugates for biosensing. When the bioconjugates were incorporated into a biosensor, direct electron transfer of HB was observed and the biosensing. When the bioconjugates were incorporated into a biosensor, direct electron transfer of HB was observed and the feasibility of using this sensor in biosensing was evaluated.

Figure 9 shows the cyclic voltammograms (CVs) of: a) HB-CdTe-CaCO₃@PEs/PVA, b) HB-CaCO₃@PEs/PVA, c) CdTe-CaCO₃@PEs/PVA, and d) HB-CdTe-MPS@PAH/PVA-modified electrodes in 0.1 M PBS (pH = 7.0) solution at 200 mV s⁻¹. CdTe-CaCO₃@PEs/PVA and HB-CdTe-MPS@PAH/PVA-modified electrodes exhibit no redox peaks in the potential window, indicating electro-inactivity. HB-CaCO₃@PEs/PVA-modified electrodes exhibit one reduction peak that corresponds to the irreversible reduction of HB. Only when HB-CdTe-CaCO₃@PEs was used could a pair of stable redox peaks be observed. The anodic peak potential (Epa) and cathodic peak potential (Epce) are located at −0.342 V and −0.370 V (vs. saturated calomel electrode (SCE)), respectively. The small potential difference of the cathodic and anodic peak potentials of 28 mV at the scan rate of 200 mV s⁻¹ indicated a fast electron-transfer process obtained for the electroactive center of the immobilized HB.[12] To further study the electron-transfer process, the effect of scan rate on the electrochemical response of immobilized HB was studied and the results are shown in Figure 10. The redox peak currents increased linearly with the increase of scan rate from 20 mV s⁻¹ to 500 mV s⁻¹ as can be observed in the inset, indicating a surface-controlled electrode process. According to the slope of the Ip−ν curve and following Equation (1) below, where Ip stands for the peak current n is the number of electrons, F is the Faraday constant, A is the surface area, v is the scan rate, and T is the temperature, the average surface coverage (I) of HB on the surface of the modified glassy carbon electrode (GCE) was estimated to be 1.5 × 10⁻¹⁰ mol cm⁻², which was eight times that of a monolayer coverage (1.89 × 10⁻¹¹ mol cm⁻² for HB).[25]

\[ I_p = \frac{n^2F^2\nu A T}{4RT} \]

It is obvious that the 3D architecture increased the protein coverage on the surface, thus increasing the electrochemistry signal. The electron-transfer rate constant \( K_s \) could be calculated according to Equation (2) when the peak-to-peak separation was less than 200 mV.

\[ K_s = \frac{nnFv}{RT} \]
In Equation (2), \( m \) is a parameter related to the peak-to-peak separation, \( T \) is the temperature, \( n \) is the number of electrons, and \( v \) is the scan rate. The \( K_m \) value was estimated to be 10.36 s\(^{-1}\). It was much larger than that for HB immobilized on CaCO\(_3\) nanoparticles,\( ^{26} \) indicating a much-faster electron-transfer process.

In order to investigate the electrocatalytic activity of HB of the HB-CdTe-CaCO\(_3\)@PES/PVA-modified electrode, the electrocatalytic reduction of H\(_2\)O\(_2\) was tested by CV, as shown in Figure 11. With the increase of the concentration of the H\(_2\)O\(_2\) in the solution, the reduction peak at about -0.37 V was enhanced (curves b–m) corresponding to the decrease of the oxidation peak. The increase of the reduction current in the reaction is because HB-Fe(II) can be oxidized with H\(_2\)O\(_2\) and transferred to HB-Fe(III) quickly when HB-Fe(III) is directly electrochemically reduced to HB-Fe(II).\(^{25}\) The calibration curve is shown in the insert of Figure 11. The biosensor’s response is linear with the concentration of H\(_2\)O\(_2\) from 6 \times 10^{-6} to 4.5 \times 10^{-5} mol L\(^{-1}\), and the detection limit is 205 \( \mu \)M H\(_2\)O\(_2\) at a scan rate of 200 mV s\(^{-1}\). The insert shows the calibration curve.

\[
\frac{1}{I_{\text{max}}} = \frac{K_m}{I_{\text{max}}} \times \frac{1}{C} + \frac{1}{I_{\text{max}}} \quad \text{(3)}
\]

The apparent \( K_m \) is 0.77 mM which is even smaller than previously reported results for HB/gold-nanoparticle-doped SBA\(^{27}\) which is a type of mesoporous silica, indicating a high biological affinity of the immobilized HB to H\(_2\)O\(_2\). Six protein electrodes made at the same electrode independently showed an acceptable reproducibility with a relative standard deviation of 3% for the current determined at 20 \( \mu \)M H\(_2\)O\(_2\), indicating reproducibility of the biosensor. The electrode was stored at 4°C when not in use. No obvious decrease in the response of H\(_2\)O\(_2\) was observed after one month of storage, indicating the stability of the biosensor.
When CdTe QDs are optically excited (Scheme 2C: A), static electrons (electrons located in the valence band) become mobile (electrons located in the conduction band) within the semiconductor matrix, leaving behind them holes in the valence band. The electron-hole recombination (Scheme 2C: B) causes the CdTe QDs to emit photons, and fluorescence can be observed. However, after the loading of the HB, due to electron transfer (the interaction between the HB and the CdTe QDs might also come from energy transfer but we take electron transfer to better illustrate the whole process) between the HB and the CdTe QDs (Scheme 2C: C), the electron-hole recombination was halted, resulting in the quenching of the fluorescence of the CdTe QDs. When an electrode was involved, electrons could firstly be transferred from the electrode to the CdTe QDs and then to the heme center of the HB as shown in Scheme 2B, resulting in the direct electrochemistry phenomenon of the HB on the bioconjugates-modified electrode. The CdTe QDs act like electronic wires or nanoelectrodes between the macroscopic electrode and the redox center of the protein and help to produce the direct electrochemistry phenomenon. Meanwhile, since the thick QD “shell” of the CdTe-CaCO3@PEs sphere (Scheme 2D) renders the possibility of electron transfer from one CdTe QD to the other (Scheme 2C: D), it is also likely that the electron can be transferred among the CdTe QDs and finally reach the redox center of the protein (Scheme 2B). In fact, the QD “shell” can be treated as a conductive shell with mobile electrons, and the HB-CdTe-CaCO3@PEs bioconjugates can be treated as a model of a group of protein molecules immobilized on a shell that tethers into their redox center and connects the electrode at the same time (Scheme 2A). Due to the effective electron transfer between the conductive shell and the redox center of the protein, a fast and effective direct electrochemistry phenomenon was achieved for the bioconjugates-modified electrode. In this case, the polyelectrolyte layers, which help to form the QD “shells”, indirectly contribute to the performance of the biosensor. Finally, the CaCO3 microspheres, which act like a matrix that accommodates both the CdTe QDs and the HB molecules, are an indispensable element for the direct electrochemistry of the 3D architecture. When the CaCO3 microspheres were replaced with mesoporous silica templates, no direct electrochemistry phenomenon was observed. This is strong evidence for the important function of the CaCO3 microspheres in the direct electrochemistry transfer process. On one hand, an increased loading amount of the CdTe QDs can be achieved due to their channel-like structure, thus increasing the efficiency of the electron-transfer process. On the other hand, it is very likely that the microspheres help to provide an optimal orientation of the HB, thus promoting the direct electrochemistry of the HB on the electrode. In a word, it is the synergetic effect of the HB molecules, CdTe quantum dots, polyelectrolyte layers and CaCO3 microspheres that results in the fast and effective direct electron transfer between the redox center of the HB and the macroscopic electrode, as well as the good performance of the biosensor.

3. Conclusions

In this paper, the HB-CdTe-CaCO3@PEs 3D architecture was fabricated through a layer-by-layer method. While the rough surface features and channel-like structure of the CaCO3 microspheres offer an ideal template for quantum-dot loading, the polyelectrolyte layers contribute to the formation of a uniform distribution of CdTe QDs inside the microspheres. The thick and bright CdTe QD “shell” of the CdTe-CaCO3@PEs not only enhanced the stability of the hybrid material, but also helped to realize a fast and effective direct electron transfer between the heme center of the HB molecules and the macroscopic electrode. The biosensor showed good affinity with H2O2 due to the synergetic effect of each building block in the HB-CdTe-CaCO3@PEs 3D architecture. Our work offers a model to study how the structure of functional materials can be tuned and related to their applications. The CdTe-CaCO3@PEs hybrid material, with an interesting structure and unique features, is a promising material for both electronic and optical applications. While the electrochemistry application is demonstrated in this paper, the optical application of the CdTe-CaCO3@PEs hybrid material is currently under investigation in our lab.

4. Experimental

Materials: HB (hemoglobin) was obtained from Sigma and was used without further purification. \( \text{H}_2\text{O}_2 \) (30% w/v solution), \( \text{CaCl}_2 \cdot 2\text{H}_2\text{O} \), \( \text{Na}_2\text{CO}_3 \), \( \text{KBB}_4 \) (96%), tellurium powder (99.999%), and poly(vinyl alcohol) (PVA) (average degree of polymerization, 1800 ± 100) were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Polysodium 4-styrenesulfonate (PSS) (\( M_w \approx 70000 \)), poly(allylamine hydrochloride) (PAH) (\( M_w \approx 15000 \)), and 3-mercaptopropionic acid (MPA) were obtained from Aldrich Chemical Co. All of the other chemicals were of analytical grade. The water used in all of the experiments was prepared in a Millipore Milli-Q purification system with a resistivity higher than 18.2 MΩ cm. Mesoporous silica (MPS) spheres with an average size of 5 μm were obtained from Lanzhou Institute of Physical Chemistry. The average pore diameter of these beads was ~30 nm. The surface area was 60 m² g⁻¹ and pore volume was 0.36 cm³ g⁻¹.

Preparation of the CaCO3 Microspheres and the CdTe QDs: The CaCO3 microspheres were prepared by rapidly pouring a 0.33 M Na2CO3 solution into an equal volume of a 0.33 M solution of CaCl2 at room temperature on a magnetic stirrer. While the precipitate was centrifuged, washed with MilliQ-water, and dried. Water-soluble CdTe QDs were prepared by our previous reported method.

Assembly of Polyelectrolyte Layers onto the CaCO3 Microspheres to Prepare CaCO3@PEs: Polyelectrolyte layers were assembled onto the CaCO3 microspheres by the sequential deposition of the PSS polyelectrolyte and the PAH polyelectrolyte. Typically, 10 mg of CaCO3 particles were suspended in 1 mL of PSS solution (5 mg mL⁻¹) in 0.5 M NaCl. The suspension was sonicated for 10 min (occasionally shaking) and the excess polyelectrolyte was removed by three repeated centrifugation (4000 rpm, 5 min) washing and redispersion cycles with water. For the subsequent assembly of positively charged PAH polyelectrolyte, 1 mL of PAH-polyelectrolyte solution (5 mg mL⁻¹, containing 0.5 M NaCl) was added.

Assembly of CdTe QDs to Fabricate CdTe-CaCO3@PEs Hybrid Material: The as-prepared CaCO3@PEs particles were dispersed into 2 mL of the CdTe QDs solution and sonicated for 20 min. After centrifugation, the salmon-pink-colored CdTe-CaCO3@PEs hybrid...
material was obtained and the supernatant was used for the analysis of the loading amount. The composites were further washed with water for three times.

Preparation of HB-CdTe-CaCO$_3$@PEs Bioconjugates: The resulting hybrid material was dispersed in 1 mL of water and an aliquot of 100 µL was added to a solution of HB (1 mg mL$^{-1}$ in 10 mM PBS at pH = 6) and shaken for 1 h for protein absorption. The bioconjugates with a dark-brown color were then centrifuged and washed with Milli-Q water for three times. The UV-vis spectrum was used to monitor the concentration of the HB solutions before and after the assembly process. The decrease in the absorbance at 405 nm was applied to quantify the loading amount of HB immobilized onto the CdTe-CaCO$_3$@PEs particles. The bioconjugates were further washed with Milli-Q water for three times.

Fabrication of the Biosensor: A glassy carbon electrode (GCE) with a diameter of 3 mm was polished successively with 1.0, 0.3, and 0.05 µm alumina powder; and was rinsed thoroughly with Milli-Q water between each polishing step. Next, the polished electrode was sonicated in ethanol and Milli-Q water and was then allowed to dry at room temperature. The bioconjugates obtained were resuspended in 0.5 mL of water, and 5 µL of this suspension was deposited on the surface of the pretreated GCE. It was left to dry at room temperature. 10 µL of a 3% PVA sol (ethanol/water (v/v) = 1:1) was added for encapsulation of the bioconjugates. The electrode was then left to dry and was stored for at least 24 h at 4 °C. The biosensor was stored under the same conditions when not in use. The CdTe-CaCO$_3$@PEs/PVA-, HB-CaCO$_3$@PEs/PVA- and HB-CdTe-MPS@PAH/PVA-modified GCEs were fabricated using the same GCE by the same method except that CdTe-CaCO$_3$@PEs and HB-CaCO$_3$@PEs and HB-CdTe-MPS@PAH were used instead of the HB-CdTe-CaCO$_3$@PEs bioconjugates.

Characterization: Electrokinetic mobilities of CaCO$_3$ microparticles were measured using a Zetasizer 4 (Malvern Instruments) by taking the average of five measurements at the stationary level. The mobility $u$ was converted to a $z$-potential using the Helmholtz-Smoluchowski relation: $z = \frac{u}{\eta}$, where $\eta$ is the viscosity of the solution and $z$ is the permittivity. Confocal micrographs were taken with a Leica TCS-CL confocal scanning system mounted to a Leica Aristoplan and equipped with a 40× oil-immersion objective with a numerical aperture of 1.25. The excitation wavelength was 488 nm. UV-vis spectra were recorded on a Shimadzu UV-3600 recording spectrophotometer at room temperature. The fluorescence spectra were recorded on a RF-5301PC spectro-fluorophotometer (SHIMADZU). Brunauer–Emmett–Teller (BET) data were collected with a Micromeritics-5301 PC spectro-fluorophotometer (SHIMADZU). Energy-dispersive X-ray (EDX) analysis was performed with an EX-250 (Horiba, Japan) attachment. Scanning electron microscopy (SEM) measurements were carried out on an S-3000N (Hitachi, Japan) scanning electron microscope at 20 kV. Energy-dispersive X-ray (EDX) analysis was performed with an EX-250 (Horiba, Japan) attachment. Scanning electron microscopy (SEM) measurements were carried out on a JEOL JEM 200CX transmission electron microscope, using an accelerating voltage of 200 kV. Scanning electron microscopy (SEM) measurements were carried out on an S-3000N (Hitachi, Japan) scanning electron microscope at 20 kV. Energy-dispersive X-ray (EDX) analysis was performed with an EX-250 (Horiba, Japan) attachment. Scanning electron microscopy (SEM) measurements were carried out on a JEOL JSM-6300F field-emission scanning electron microscope at 5 kV. Before SEM measurements were made, a double-faced adhesive paper was adhered to a stainless-steel sample desk, and the samples were uniformly scattered on another side of the paper; then the samples were gilt in an ion-sputter chamber (E-1010, Hitachi), with the pressure of the chamber less than 10 Pa. The samples were gilt for 120 s with an initial discharge current of less than 20 mA.

Amperometric cyclic-voltammetry experiments were performed using a CHI660B workstation (Shanghai Chenhua, Shanghai, China). All of the experiments were carried out using a conventional three-electrode system with the protein electrode as the working electrode, a platinum wire as the auxiliary electrode, and a saturated KCl electrode as the reference electrode. The electrolysate solutions were purged with high-purity nitrogen prior to the electrochemical experiments and blanketed with nitrogen during them.

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