

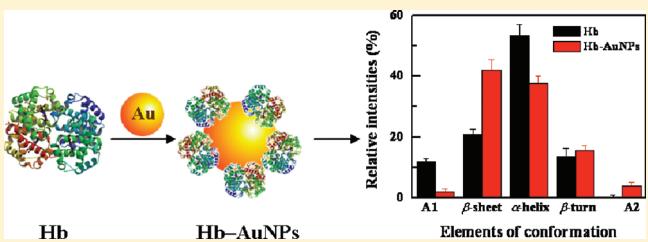
Electrochemical and Spectroscopic Studies on the Conformational Structure of Hemoglobin Assembled on Gold Nanoparticles

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ABSTRACT: Protein conformational changes may be associated with particular properties such as function, transportation, assembly, tendency to aggregate, and potential cytotoxicity. Protein misfolding, in particular, has been intimately related to protein-mediated diseases. In this study, the conformational structure changes of hemoglobin (Hb) induced by the assembly on gold nanoparticles (AuNPs) surface were studied in detail by a combination of electrochemical method and various spectroscopic techniques including UV-vis absorption, fluorescence, circular dichroism (CD), and Fourier transform infrared (FTIR) spectroscopy. The results indicated that Hb in the Hb–AuNPs bioconjugate system that was prepared by the assembly of Hb on the surface of AuNPs underwent substantial conformational changes both at secondary and tertiary structure level. The assembly of Hb on the boundary surface of AuNPs could result a disturbance of the structure of Hb and induce the exposure of the heme group and tryptophan (Trp) residues to the solvent, leading to the enhancement in the electron transfer rate of the protein. The calculation from quantitative second-derivative infrared and CD spectra of the Hb–AuNPs bioconjugate system showed that AuNPs could induce the conversion of α -helix to β -sheet structures and unfolding of the protein. Moreover, the effects of the concentration and the size of AuNPs on the conformational structure changes of Hb in the bioconjugate system were also demonstrated. The results obtained here not only provide the detailed conformational behavior of Hb molecules on nanoparticles, but also create a framework for analyzing the biosafety of nanoparticles in terms of the biological behavior of biomacromolecules.



1. INTRODUCTION

The functions of most proteins depend upon precise three-dimensional structures,^{1–7} which are affected by a number of noncovalent interactions such as hydrogen bonds, van der Waals interactions, electrostatic interactions, hydrophilic/hydrophobic effects,^{8–12} and so on. Protein conformational changes may be associated with particular properties such as function, transportation, assembly, tendency to aggregate, and potential cytotoxicity.⁵ In past decades, the thermodynamics and kinetics of protein folding/unfolding have received considerable attention because of their great importance in the manifestation of biological functions ranging from genetic information to molecular diagnostics.^{6,13–20} Protein misfolding, in particular, has been intimately related to protein-mediated diseases such as Alzheimer's disease, Parkinson's disease, amyloid disease, and many other abnormalities.^{3,21,22} Therefore, detailed elucidation of secondary and tertiary formation, stability, and their structural and dynamic properties has been one of the main topics studied in chemistry and biology.

The various specific and nonspecific interactions of nanoparticles with the residues of the proteins have important consequences to the biological function of the protein. The conjugation of protein with nanoparticles not only affords stabilization to the system, but more importantly, it also introduces biocompatible functionalities into these nanoparticles for further biological interactions or coupling. The use of protein–nanoparticle bioconjugates,

which are usually prepared by assembly of proteins on the surface of the nanoparticles via covalently linking or electrostatic interactions,^{23–27} for applications in biosensing, assembly, imaging, and control has substantially advanced.^{28–33} The success of these applications significantly depends on the activity of the assembled protein being similar to that in its native state, whereas the key feature that determines the activity of the assembled protein is the conformational structure. However, when protein is assembled on a nanoparticle surface, its conformation will undergo more or less changes at the boundary surface of the nanoparticles due to the electrostatic and hydrophobic interactions between protein and solid surface, whose physicochemical properties affect strongly the structure rearrangements of the assembled protein.^{7,34} This changes in the structure of protein can have significant effects on the function in related the applications of the bioconjugates. Although many interesting studies have been focused on this subject, there are few reports about how the conformation of the protein is affected by a solid boundary surface of nanoparticles.^{35–39} However, the fundamental understanding of the conformational behavior of proteins in a protein–nanoparticle conjugate system is of critical importance

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for the integration of biology with nanomaterial. This can directly help in the development of bioconjugate systems, molecular diagnostics, therapeutics, molecular biology, material science, as well as bioengineering.^{37,40–42} Moreover, detailed mapping of protein conformational changes can help identify optimal conditions to preserve functionality following the bioconjugation and direct the further applications. With this objective, the conformational structure changes in hemoglobin (Hb) before and after the assembly on gold nanoparticles (AuNPs) surface were studied in this work with the purpose of revealing the mechanism of unfolding of proteins by assembling on nanoparticles.

Hb and AuNPs have been chosen as the model system because Hb is one of the major biomacromolecules in lives and the major target of many kinds of viruses,⁴³ artificial drugs,⁴⁴ medicines,⁴⁵ metal ions,⁴⁶ and so on. Hb is a representative hemeoprotein, whose structure and property are well-characterized. This kind of protein has long been used as the paradigm for understanding the structure–function relationships of proteins.^{47–50} Moreover, the unfolding of this kind of protein is closely associated with physiological abnormalities. For example, under very acidic conditions, the cooperative oxygen binding property of Hb is decreased and the pro-oxidative activity is dramatically increased mainly due to the significant conformational changes in its structure and heme crevice.^{51,52} Therefore, Hb is a convenient model for the fundamental studies of protein–nanoparticles interaction.^{53,54} In addition, Hb can undergo structural changes very easily and hence can be used as a good model for conformational change studies. AuNPs has been chosen because of their unique chemical, supramolecular and recognition properties,^{55–57} their potential applications in biology including anticancer drug delivery systems and photothermal cancer treatment agents.^{58–60} Furthermore, AuNPs are the most commonly used nanoparticles in the bioconjugation study. Several groups have investigated the cellular toxicity of AuNPs with regard to particle size, shape, and surface group.^{61–66} Maiti et al.⁶⁴ reported that citrate-capped AuNPs (18 nm) can decrease mitochondrial activity of HeLa cells by 20% after the cells were exposed to the AuNPs solution (2 nM) for 6 h. However, the AuNPs were not found to cause changes of gene expression of the HeLa cells in those conditions. Rotello et al.⁶¹ investigated the toxicity of AuNPs (2 nm) on the cells by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), hemolysis, and bacterial viability assays and concluded AuNPs functionalized with cationic surface groups were moderately toxic, whereas AuNPs functionalized with anionic surface groups were quite nontoxic. Protein binding to AuNPs, as a means to form AuNPs-related bioconjugates for bioassay and protein therapy, has been addressed from the point view of maintaining the protein functionality and activity, avoiding structural changes on the protein.^{67,68} This approach has led to the development of novel biosensors, diagnostic agents, and nanostructured materials.^{69–71} Despite wide interest in the protein–AuNPs related bioconjugates, the information about the effects of AuNPs on the conformational changes of assembled protein is very limited. The conformational behavior of protein on a solid boundary surface of AuNPs is still unclear at present. This work studies the conformational changes of Hb that resulted from the assembly on the surface of AuNPs by a combination of electrochemical method and various spectroscopic techniques including UV–vis, fluorescence, circular dichroism (CD), and FTIR. The effects of the conformational structure of Hb on its electron transfer characteristics were also studied in this work. Moreover, the effects of AuNPs concentration

and size on the conformational structure of Hb were also presented. The results obtained here not only provide the detailed conformational behavior of Hb molecules on nanoparticles but also create a framework for analyzing the biosafety of nanoparticles in terms of the biological behavior of biomacromolecules.

2. EXPERIMENTAL SECTION

2.1. Chemicals. Hemoglobin (Hb, MW ~ 64500, lyophilized powder, from bovine blood, Sigma) and HAuCl₄·3H₂O (Shanghai Chemical Reagent, Shanghai) were used as received. All other chemicals were of analytical grade or better. All solutions were prepared with double distilled water. Phosphate buffer solution (PBS, 0.1 M) was made up from K₂HPO₄ and NaH₂PO₄. The solution of Hb was prepared in 0.1 M PBS (pH 7.0) just before each experiment.

2.2. Preparation of Gold Nanoparticles (AuNPs). AuNPs were prepared using the procedures reported previously.⁷² Briefly, all glassware used in the preparation was thoroughly cleaned in aqua regia (3 parts concentrated HCl, 1 part concentrated HNO₃), rinsed in double-distilled water, and oven-dried prior to use. *Caution: aqua regia is extremely dangerous and should be handled with extreme caution. Gloves and eye protection are required for handling.* In a 250 mL round-bottom flask equipped with a condenser, 100 mL of HAuCl₄ (1 mM) was brought to a rolling boil with vigorous stirring. Rapid addition of 10 mL of sodium citrate solution (38.8 mM) to the vortex of the solution resulted in a color changing from pale yellow to burgundy. Boiling was continued for 10 min; the heating mantle was then removed, and stirring was continued for an additional 15 min. After the solution reached room temperature, it was filtered through a 0.2 μm Nylon membrane filter. Transmission electron microscopic (TEM) images indicated a particle size of about 15 nm.

To study the effects of AuNPs size on the conformation changes of Hb in the Hb–AuNPs bioconjugate system, the big size of AuNPs with diameter of about 40 nm were prepared using the similar procedures depicted above except changing the volume of sodium citrate solution (38.8 mM) from 10 to 5 mL. The small size AuNPs with diameter of about 4 nm were also prepared using published procedures.⁷³ A 20 mL aqueous solution containing 0.25 mM HAuCl₄ and 0.25 mM sodium citrate was prepared in a conical flask. Then, 0.6 mL of ice-cold, freshly prepared NaBH₄ (0.1 M) solution was added to the solution under stirring. The solution turned pink immediately after addition of NaBH₄, indicating the formation of AuNPs. The solution was stirred for 5 min and then stored at 4 °C. The size of the prepared AuNPs was also checked by TEM.

2.3. Preparation of the Hb–AuNPs Bioconjugates. The bioconjugates were prepared by mixing Hb and AuNPs in PBS and then incubated at 4 °C for at least 24 h before each measurement. A series of the bioconjugates with different molar ratios of Hb/AuNPs were prepared by keeping the concentration of Hb constant (0.1 mg/mL), while varying the concentration of AuNPs (from 0.1 to 0.5 nM). To retain their original conformational states in the solution, and also to prevent shear forces from disrupting the structure of the bioconjugates, no separation techniques (i.e., centrifugation or filtration) were employed in the experiment. All the measurements in the following discussion were performed at ambient temperature (22 ± 2 °C).

2.4. Apparatus and Procedures. TEM images of AuNPs were obtained on a JEOL-2010 microscope with an accelerating

voltage of 200 kV. The sample was prepared by diluting AuNPs stock solutions with double distilled water and evaporating one drop of the suspension onto a carbon-coated film supported on a copper grid for TEM measurements. The distribution and the mean sizes of the prepared AuNPs and Hb–AuNPs bioconjugates were determined by a BI-200SM dynamic light scattering instrument (DLS; Brookhaven Instruments). The value of the ζ potential of the prepared AuNPs, Hb, and Hb–AuNPs bioconjugates were measured with a Zeta potential analyzer (Nano Z, Malvern).

Cyclic voltammetry (CV) was used to study the electron transfer characteristics of Hb in Hb–AuNPs bioconjugates. The experiments were performed with a CHI 660B electrochemical workstation (CH Instruments). A two-compartment three-electrode cell with a sample volume of 5 mL was employed. A coiled Pt wire and a saturated calomel electrode (SCE) were used as the counter and the reference electrode, respectively. The buffer (0.1 M PBS, pH 7.0) was purged with high-purity nitrogen for at least 30 min prior to each electrochemical measurement and the nitrogen environment was then kept over the solution to prevent oxygen from reaching the solution.

Prior to CV experiments, the glassy carbon (GC, 3 mm in diameter, CH Instruments) electrode was polished sequentially with metallographic abrasive paper (No. 6) and slurries of 0.3 and 0.05 μm alumina to create a mirror finish; the electrode was then sonicated with absolute ethanol and double distilled water for about 1 min, respectively. It was rinsed thoroughly with double distilled water and then dried under ambient temperature. Hb–AuNPs bioconjugates (4 μL ; prepared by mixing Hb and AuNPs with final concentration of 0.1 mg/mL and 0.5 nM, respectively) were cast onto the surface of the pretreated GC electrode with a microsyringe, and solvent was allowed to be evaporated at ambient temperature before use. The electrode (denoted as the Hb–AuNPs/GC electrode) was stored at 4 °C under 80% humidity when not in use.

For comparison, the CV characteristics of AuNPs and free Hb at the GC electrode were also studied. The AuNPs/GC and Hb/GC electrodes were fabricated by casting 4 μL of AuNPs (0.5 nM) and 4 μL of Hb solution (0.1 mg/mL, in PBS, pH 7.0), respectively, on the surface of GC electrode and allowing the solvent to be evaporated before use. These electrodes were also stored at 4 °C under 80% humidity when not in use.

UV-vis spectra measurements were performed in PBS (pH 7.0) on a Cary 5000 UV-vis-NIR spectrometer (Varian). A protein-free AuNPs sample (the concentration of AuNPs is the same as that in each Hb–AuNPs bioconjugate system) was used to record the baseline for the Hb–AuNPs bioconjugate system. Therefore, the obtained spectra reflect solely the UV-vis absorption of Hb. All measurements were repeated and found to be reproducible within experimental errors and performed at room temperature. Fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Varian). The spectra were recorded in the wavelength range of 300–410 nm upon excitation at 280 nm using 5 nm/5 nm slit widths. A 1.00 cm path length rectangular quartz cell was used for these studies. Each spectrum was an average of three scans. To avoid self-absorption and inner filter effects,⁷⁴ Hb concentration of 8 μM and AuNPs concentration in the range of 0.1 to 0.43 nM were used in the measurements. Appropriate blanks corresponding to the buffer were subtracted to correct the fluorescence background. The experiments were repeated until reproducible results within experimental errors were attained.

FTIR spectra were recorded on a Nexus 670 FTIR spectrophotometer (Nicolet Instruments) equipped with an attenuated total reflection (ATR) accessory, a DTGS (deuterated triglycine sulfate) detector, and a standard KBr beam splitter. The IR spectra were recorded for Hb solution (0.1 mg/mL) and Hb–AuNPs bioconjugate system (formed with 0.1 mg/mL Hb and AuNPs concentrations ranging from 0.1 to 0.5 nM), respectively, in 0.1 M PBS (pH 7.0). The samples for IR measurements were prepared by casting the Hb or Hb–AuNPs bioconjugates solution (100 μL) on the surface of horizontal placed ZnSe crystal to form a liquid thin film. The spectra were taken via an ATR mode with a resolution of 4 cm^{-1} . A total of 256 interferograms were coadded in the experiment to ensure a good signal-to-noise ratio. To minimize the absorption of water vapor, the ATR equipment was purged by dry air during the measurements. After each measurement, the crystal was cleaned by double distilled water before loading the next solution to ensure that all Hb molecules were removed from the crystal surface. The protein IR spectra were obtained after subtraction of the solvent absorption, strictly collected under the same conditions, by adjusting the subtraction factor until a flat baseline was obtained at 2000–1750 cm^{-1} .⁷⁵ Furthermore, subtraction of residual vapor absorption was also performed if necessary. Second-derivative spectra were obtained using OMNIC software (version 7.0) provided by Thermo Fisher Scientific (Waltham) following the Savitsky-Golay method. Curve fitting of the amide I band from 1700 to 1600 cm^{-1} was performed by OMNIC software as a linear combination of Gaussian components. In the fitting, the number of components and initial values of their peak positions were taken from the second-derivative spectra.

Circular dichroic (CD) spectra were measured with a Chirascan circular dichroism spectrometer (Applied Photophysics) at ambient temperature. The measurements were performed in 0.1 M PBS (pH 7.0) for native Hb (0.1 mg/mL) and Hb–AuNPs bioconjugate system, respectively. The bioconjugate system contains 0.1 mg/mL Hb and various concentrations of AuNPs (from 0.1 to 0.5 nM). CD spectra were recorded over the range 200–260 nm at a scan rate of 120 nm/min with a 0.1 cm path length rectangular quartz cell. The final spectrum was the average of three scans after correction for the unspecific dichroic absorbance of the medium by computer manipulation. The data were expressed in term of the molar ellipticity, $[\theta]$, in deg $\text{M}^{-1} \text{m}^{-1}$. The secondary structures of Hb such as α -helix, β -sheet, β -turn, unordered, and so on, were calculated from the CD data using the CDNN program (version 2.0).⁷⁶

3. RESULTS AND DISCUSSION

3.1. Characterization of AuNPs and the Hb–AuNPs Bioconjugates. The prepared AuNPs with various sizes exhibited color from wine red (4 nm) to dark red (40 nm) due to the collective oscillation of electrons in the conduction band known as surface plasmon resonance (SPR).⁷⁷ This band presents an exceptionally high absorption coefficient, centered at about 522 nm (not shown here). The wavelength of the SPR depends weakly on the size of AuNPs.

The shape and size of the synthesized AuNPs were examined by TEM (Figure 1). The prepared AuNPs are almost spherical with a diameter about 4 nm (Figure 1A), 15 nm (Figure 1B), and 40 nm (Figure 1C), respectively. The TEM results also indicate that the prepared AuNPs are well-dispersed. This feature is important for the AuNPs being used as a substrate to fabricate

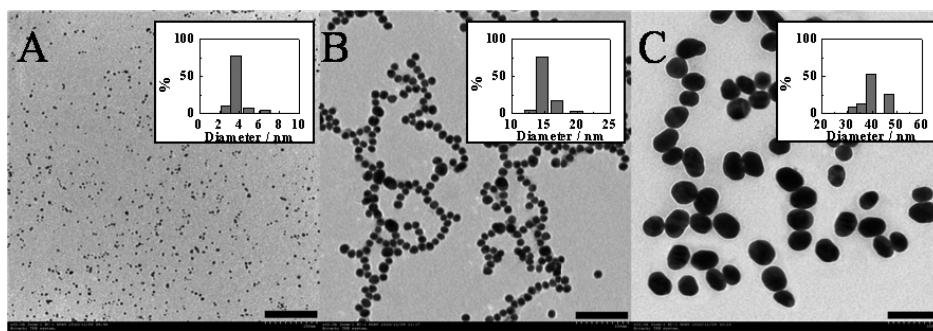


Figure 1. Typical TEM images of the prepared AuNPs with diameter of about 4 (A), 15 (B), and 40 nm (C), respectively. The inset shows the DLS size-distribution of the prepared AuNPs. The scale bar is 100 nm.

protein–AuNPs bioconjugates because the high surface area can be obtained and is favorable for the assembly of proteins. The narrow size distribution of the prepared nanoparticles is demonstrated by DLS measurement (the inset of Figure 1). The average hydrodynamic diameters of the AuNPs obtained from DLS analysis are about 3.8, 14.9, and 40.1 nm, respectively, which are in good agreement with these values obtained from the TEM results.

The concentration of prepared AuNPs at various sizes can be calculated by assuming that all AuCl_4^- ions in the solution can be reduced in each preparation.⁷⁸ This assumption, together with the average volume of an AuNP, which can be estimated from the size of the nanoparticle, gives the concentration of AuNPs of approximately 150, 10, and 0.5 nM for 3.8, 14.9, and 40.1 nm AuNPs, respectively.

After the assembly of Hb, the diameter of AuNPs has a significant increase. The diameter of 3.8, 14.9, and 40.1 nm AuNPs increases to about 5.1, 26.9, and 55.3 nm, respectively, indicative of the formation of the Hb–AuNPs bioconjugate. Moreover, the size distribution of the Hb–AuNPs bioconjugates becomes broad comparing with that of AuNPs (not shown here), which may be due to the different amount of Hb assembling onto surface of each AuNP. Furthermore, DLS analysis was also applied to measure the size of Hb molecules and to evaluate the aggregate degree of the molecule in solution. DLS results indicated that the size of Hb in solution is 6.4 ± 1.2 nm, suggesting that Hb molecules do not undergo significant aggregation because Hb in its native state is approximately spherical in shape with dimensions of $6.5 \times 5.5 \times 5.0$ nm.⁷⁹

The formation of the Hb–AuNPs bioconjugates can be further confirmed by the ζ potential measurements. The value of ζ potential is a measure of the electrical potential at the surface of the bioconjugates. The magnitude of this surface potential is in turn related to the surface charge and the thickness of the double layer, and so it depends on the amount of protein (considered as a charged polymer) assembled at the bioconjugates surface. AuNPs in solution with the size of 3.8, 14.9, and 40.1 nm present a ζ potential of -48.0 ± 1.2 , -42.2 ± 3.2 , and -30.7 ± 2.3 mV (the \pm value represents standard deviation), respectively. These values agree well with previous reports on citrate-stabilized AuNPs (-49.0 ± 4.2 mV for 11.5 nm AuNPs).³⁵ After assembly of Hb, these values of ζ potential increase to -43.0 ± 1.4 , -34.0 ± 3.1 , and -20.0 ± 1.3 mV, respectively. Hb alone presents a ζ potential of 5.0 ± 1.6 mV at the concentration of 0.1 mg/mL, indicating that Hb is slightly positive charged in presented experimental conditions because measurement was

taken at pH 7.0, close to its isoelectric point (pI of Hb is 6.8–7.0).⁸⁰ These values indicate that the ζ potentials of AuNPs have 5–10 mV positive shift after the assembly of Hb, suggesting the formation of the Hb–AuNPs bioconjugates. These values also indicate that Hb imparts some of its more positive surface charge characteristics to the AuNPs.

3.2. Electron Transfer Characteristics of Hb Assembled on AuNPs. In this work, the conformational structure changes of Hb assembled on AuNPs were first evaluated by measuring its electron transfer (ET) characteristic because this characteristic can be regarded as the most sensitive probe for studying the changes in the protein conformation during various treatments. ET characteristics of protein also reflect subtle readjustments at the active site, allowing very small conformation variations of protein structure to be detected.

Figure 2A displays time-dependent voltammetric responses of the Hb–AuNPs/GC electrode in N_2 -saturated PBS (pH 7.0). When the cyclic voltammogram (CV) was recorded immediately after the electrode was immersed in PBS, the CV curve did not show any observable redox peaks (curve a, Figure 2A) in the potential range of interest, indicating that Hb cannot undergo the redox reaction in the experimental potential ranges. However, after 10 min immersion of the electrode in PBS, a pair of redox peaks resulted from ET reaction of Hb²⁷ is clearly recorded for the electrode with a formal potential (E°') of about -0.280 V (curve b, Figure 2A), calculated by averaging the cathodic and anodic peak potentials. The ratio of cathodic-to-anodic peak current is nearly unity. With increasing time of the electrode in PBS, the redox peak becomes more pronounced (curves b–i, Figure 2A) and reaches a relatively stable value at 24 h (curves h–i, Figure 2A), suggesting that the ET rate of Hb is enhanced with increasing the time of the electrode in PBS.

To further clarify the effects of AuNPs on the ET characteristics of Hb, CV features of the AuNPs/GC and Hb/GC electrodes were recorded with time of these electrodes in PBS (Figure 2B,C). The results demonstrate that neither of the two electrodes can display the detectable CV peaks even these electrodes were in PBS for 48 h, suggesting the AuNPs and Hb cannot undergo the effective ET reaction at the GC surface at the present conditions. These results indicate that the changes on the conformational structure of Hb on the AuNPs have occurred.

Hb consists of a four-polypeptide chain, each with one heme group.⁸¹ In the native state, the heme group of Hb is deeply buried in the central cavity (hydrophobic pocket) of each subunit. The long distance between the heme groups and electrode and the large steric hindrance of native Hb essentially result in the poor

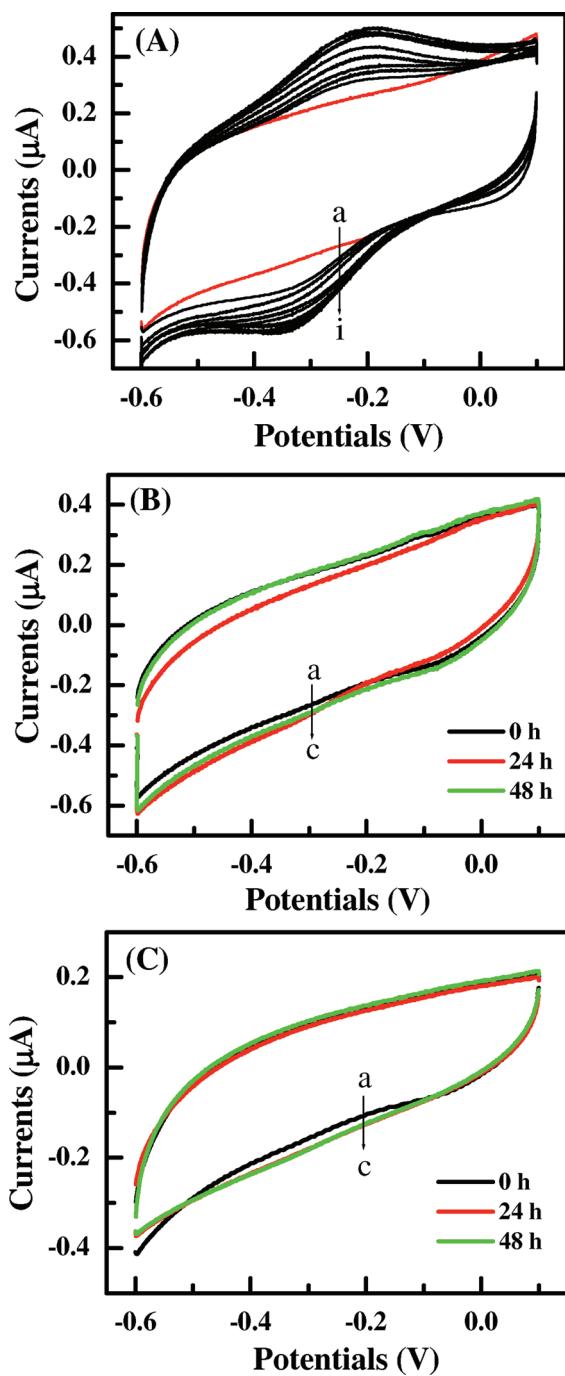


Figure 2. (A) Typical cyclic voltammograms of the Hb–AuNPs/GC electrode in N_2 -saturated PBS (pH 7.0) after the electrode immersing in PBS for (a) 0 min, (b) 10 min, (c) 30 min, (d) 1 h, (e) 3 h, (f) 5 h, (g) 18 h, (h) 24 h, and (i) 48 h. (B, C) Typical cyclic voltammograms of the AuNPs/GC (B) and Hb/GC electrode (C) in N_2 -saturated PBS (pH 7.0) after the electrodes immersing in PBS for (a) 0, (b) 24, and (c) 48 h. The scan rate is 100 mV/s.

electrochemical response of Hb as depicted in curve (a) in Figure 2A. It has been reported that^{38,39,82} nanostructures, such as carbon nanotubes, CdTe quantum dots, and so on, can denaturalize the proteins probably through destroying their quaternary, tertiary, and secondary structures, enhancing the water solubility of hydrophobic side chains. Similarly, the

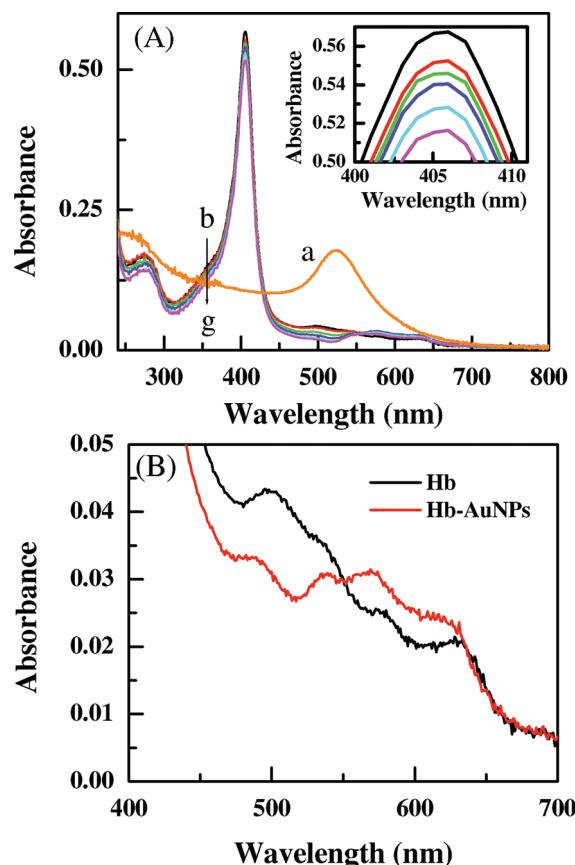


Figure 3. (A) UV–vis absorption spectra of (a) AuNPs (14.9 nm at the concentration of 0.2 nM), (b) native Hb (0.1 mg/mL), and (c–g) Hb–AuNP bioconjugate system with AuNP concentrations of (c) 0.1, (d) 0.2, (e) 0.3, (f) 0.4, and (g) 0.5 nM in 0.1 M PBS (pH 7.0). The spectra of Hb–AuNPs bioconjugate system have been corrected with the spectrum of AuNPs sample as background. (B) Q-band (400–700 nm) of Hb in native (0.1 mg/mL, black line) and in the Hb–AuNPs bioconjugates (red line, 0.1 mg/mL Hb and 0.5 nM AuNPs) in 0.1 M PBS (pH 7.0).

conformational structure of Hb may also be destroyed by AuNPs and the water solubility of the hydrophobic side chains of the protein is increased. These effects can result in the dissociation of the heme groups from the hydrophobic pockets and thus lead to the enhancement in ET rate. Therefore, a pair of well-defined redox peaks can be observed as those presented in curves (b)–(i) in Figure 2A.

To ensure the ascription of the observed redox peak presented in Figure 2A to the heme groups dissociated from the hydrophobic pockets in Hb, the control experiments with free heme were conducted because this compound is structurally identical with the heme groups in Hb. The CV of the free heme at the GC electrode shows a pair of well-defined, reversible redox peaks with an $E^{\circ\prime}$ of -0.30 V (not shown here). This feature is actually similar to that for Hb denatured by AuNPs (Figure 2A), implying the assembly of Hb on AuNPs may lead to the conformational structure changes and dissociation of the heme groups from the hydrophobic pockets.

3.3. UV–Vis Absorption Spectroscopy Studies. The AuNP-induced conformational change of Hb was also studied with UV–vis absorption spectroscopy. UV–vis spectrum of AuNPs shows the maximum wavelength of the SPR at 522 nm (curve a in

Figure 3A). The absorption spectra of native Hb (curve b in Figure 3A) show several electronic bands located at 280 (corresponding to the phenyl group of tryptophan (Trp) and tyrosine (Tyr) residues), 349 (ϵ band), 406 (heme or Soret band), 539, and 576 nm (oxy-band or Q-band).^{36,83,84} After addition of AuNPs into the Hb solution, an alteration in the intensity of the Trp/tyrosine band, ϵ band, and Soret band, as well as the Q-band, can be clearly observed, implying that AuNPs can access both the heme and the Trp residues (curves b–g, Figure 3A). Talapara et al. reported the similar observation in the case of Hb and Ag⁺ ions.³⁶

It has been reported that the Soret band is originated from the heme group, which is buried in a hydrophobic pocket formed by the protein's backbone through appropriate folding.³⁶ The appearance of a strong Soret band (strongly allowed $\pi \rightarrow \pi^*$ electronic transition) at 406 nm in UV-vis absorption spectra suggests that Hb is in its native form (Figure 3A). The shape and position of the Soret band are associated with the porphyrin ring and therefore can provide insight into the environmental influence on the native structural configuration of Hb, especially around the heme group. Moreover, important information on the possible unfolding/folding and denaturation of the protein during conjugation can also be acquired by studying the shape and position of the Soret band.^{83–87} AuNPs concentration dependent absorption spectra demonstrate a significant spectral change of the Soret band in intensity (curves b–g, Figure 3A), which indicates that AuNPs are directly involved in producing a disturbance of the structure and the exposure of the heme group to the aqueous medium. With the increase of the concentration of AuNPs, the intensity of Hb in Soret band decreases (the inset in Figure 3A). These results indicate that the coverage of Hb on the AuNPs surface also affects the conformation of the Hb in the Hb–AuNPs bioconjugate system. The position of the Q–band reflects the changes of heme microenvironment in Hb. The Q–band appears at 576 nm for the native Hb, however, the band shows a blue-shift to 571 nm after addition of 0.5 nM AuNPs (14.9 nm; Figure 3B), reflecting the microenvironment surrounding heme in Hb–AuNPs bioconjugates is different from that of native Hb. These results further suggest that the heme group experiences a perturbation due to the interaction of the protein with AuNPs.

The intensity of the Soret band depends on the interaction time of Hb with AuNPs. The intensity decreases with the increase in the interaction time between Hb and AuNPs, and it reaches a stable value after 24 h interaction between Hb and AuNPs (not shown here), indicating that the environment around heme has tended to be stable. This length of time is in good agreement with that for electrochemical analysis (Figure 2). Therefore, the interaction time between Hb and AuNPs of 24 h is chosen in the following studies.

3.4. Fluorescence Studies. Fluorescence spectroscopy is a useful tool to obtain information on the conformational changes of protein, degree of exposure of the fluorophore to the solvent, and the extent of its local mobility.⁸⁸ Most proteins contain amino acid residues that are intrinsically fluorescent, such as Trp, Tyr, and phenylalanine (Phe). For proteins with intrinsic fluorescence, more specific local information can be obtained by selectively exciting the Trp residues. Although large changes in protein conformation can alter the fluorescence intensity of Tyr and Phe, as well as the local mobility of these side chains, their low extinction coefficients and quantum yields, coupled with the

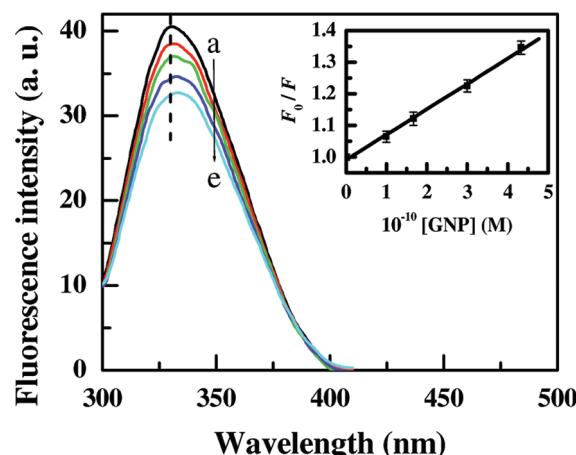


Figure 4. Fluorescence emission spectra of Hb (8 μ M) in the absence (curve a) and presence of (b) 0.10, (c) 0.17, (d) 0.30, and (e) 0.43 nM AuNPs (14.9 nm) upon excitation at 280 nm in 0.1 M PBS (pH 7.0). Inset shows the Stern–Volmer plots of Hb with the increasing concentration of AuNPs.

relative lack of environmental sensitivity of their emission energy, render them significantly less useful than Trp.⁸⁹

Hb has strong fluorescence because it contains three Trp units (α 214Trp, β 215Trp, and β 217Trp) in each α and β chain.⁹⁰ Therefore, on observing the fluorescence emission of Trp in the Hb–AuNPs bioconjugate system, information on the protein conformational behavior around the Trp residues can be obtained. The emission spectra of Hb (0.1 mg/mL) in the Hb–AuNPs bioconjugate system with increasing the concentration of AuNPs at pH 7.0 upon excitation at 280 nm were recorded (Figure 4). The results demonstrate that the Trp fluorescence is quenched drastically upon conjugating with AuNPs. Moreover, there is a slight red-shift of the maximum wavelength of Trp fluorescence spectrum when the concentration of AuNPs is increased (from 330 to 334 nm), revealing the interaction with AuNPs. The measurement of the emission maximum and the shift in its position are useful to understand the microenvironment of amino acid residues. The red-shift of the emission maximum observed here indicates the occurrence of conformational changes for Hb at tertiary structure levels because the shift in the position of emission maximum reflects the changes in the polarity around the Trp residues. Generally, the blue shift of the emission maximum is a consequence of transferring Trp residues into a more hydrophobic environment, whereas the red shift indicates that Trp residues are, on average, more exposed to the solvent.⁹¹ Thus, the red shift here indicates that Trp residues are more exposed to the solvent due to the tertiary structural change of Hb after conjugating at the boundary surface of AuNPs. The present data may be indicative of the feature that Trp is at or near the possible binding site.

Quenching of the fluorophore fluorescence by AuNPs that results in energy transfer to metallic nanoparticles has been reported,^{92,93} and AuNPs are known to exhibit efficient energy transfer behavior as excited state quenchers. In the Hb–AuNPs bioconjugate system, where Hb is situated at the vicinity of AuNPs, efficient energy transfer will occur between Hb and AuNPs. As a result, the emission of Trp residues in Hb is quenched. Fluorescence quenching mechanism can be divided into dynamic and static quenching mechanisms. Both static and

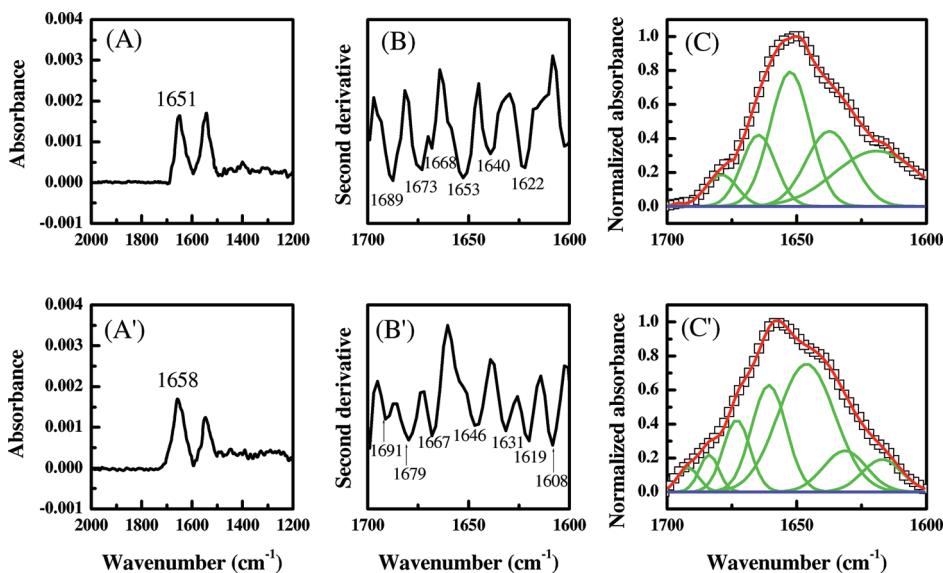


Figure 5. IR spectra of amide bands for native Hb (A; 0.1 mg/mL) and Hb–AuNPs bioconjugate system (A'; 0.1 mg/mL Hb and 0.5 nM AuNPs, 14.9 nm) in 0.1 M PBS (pH 7.0). (B and B') Corresponding second-derivative IR spectra for amide I of native Hb (B) and Hb–AuNPs bioconjugates (B'), respectively. (C and C') Curves fitting into Gaussian components for amide I of native Hb (C) and Hb–AuNPs bioconjugate system (C'), respectively. Squares are the measured data and the red lines are the curve-fitted data.

dynamic quenching requires molecular contact between the fluorophore and quencher. Dynamic quenching process follows the Stern–Volmer equation:⁹⁴

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \quad (1)$$

where F_0 and F are the maximum fluorescence intensities in the absence and presence of AuNPs, respectively, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of quencher, K_{SV} is the Stern–Volmer fluorescence quenching constant, which is a measure of the efficiency of quenching, and $[Q]$ is the quencher concentration. A typical Stern–Volmer plots (F_0/F versus $[AuNPs]$) for Hb–AuNPs bioconjugate system is presented in the inset of Figure 4, and the value of K_{SV} can be calculated to be $(8.5 \pm 0.6) \times 10^8 \text{ M}^{-1}$ (an average of three measurements) by linear regression of the plots, indicative of the quite strong quenching ability of AuNPs.⁹⁴ The bimolecular quenching constant (k_q) can be estimated to be $(3.3 \pm 0.23) \times 10^{17} \text{ M}^{-1} \text{ s}^{-1}$ by using the relation of $K_{SV} = k_q \tau_0$ (from eq 1) and assuming the lifetime (τ_0) of approximately $2.58 \times 10^{-9} \text{ s}$ for Hb.⁹⁵ It has been reported that the maximum value of k_q for a diffusion-controlled quenching process is about $10^{10} \text{ M}^{-1} \text{ s}^{-1}$,⁹⁶ the higher value obtained here may suggest that the quenching of Trp fluorescence occurs by a specific interaction between Hb and AuNPs. This also implies that static quenching mechanism (formation of the bioconjugates) is the dominating quenching mechanism.

3.5. FTIR Analysis. To further analyze the conformational structures of Hb, FTIR spectra of the protein in native and in Hb–AuNPs bioconjugate system were recorded because this spectroscopy is sensitive to vibration transitions of protein structure and has been a powerful technique for determination of the secondary structure of proteins with no restriction on their molecular mass.^{97,98} Quantitative information on the secondary structure elements of the proteins can be obtained by the analysis of the amide I band, appearing in the $1600\text{--}1700 \text{ cm}^{-1}$ region,

which results mainly from carbonyl stretching vibrations of the peptide backbone that depends on the strength of the hydrogen bond and the interactions between the amide units.⁹⁹ Various secondary structural motifs including helices, sheets, turns, coils, and intra- and intermolecular aggregates can be identified by the decomposition of the band into its components through curve-fitting procedures. An important and critical step in the FTIR study of proteins is the assignment of the different components of secondary structure in the amide I, and a rough assignment as suggested in most protein studies is as follows: $1651\text{--}1658 \text{ cm}^{-1}$ (α -helix), $1618\text{--}1642 \text{ cm}^{-1}$ (β -sheet), $1666\text{--}1688 \text{ cm}^{-1}$ (turn), $1618\text{--}1623 \text{ cm}^{-1}$ (intermolecular aggregate, A1), and $1683\text{--}1689 \text{ cm}^{-1}$ band (intramolecular aggregate, A2), respectively.^{37,38}

Figure 5 shows the original IR spectra of native Hb (panel A) and its bioconjugate system (panel A') in pH 7.0, where an intense band in the amide I region centered at 1651 cm^{-1} could be observed, indicating that native Hb is in an α -helix-rich conformation. As compared with that for the native Hb, the amide I band in the Hb–AuNPs bioconjugate system shows obvious differences in both shape and peak position ($1651\text{--}1658 \text{ cm}^{-1}$), which suggests the occurrence of the changes in secondary structure of the Hb in the bioconjugate system. To obtain more detailed information on the conformational changes in the secondary structures of Hb, the second-derivative spectrum of amide I was acquired and analyzed (panels B and B'). A large band centered at 1653 cm^{-1} relative to the other bands in the amide I region indicates a higher content of α -helix structure of native Hb at pH 7.0 (panel B). The intensities of the band assigned to β -sheet (1640 cm^{-1}) structure for the native Hb are found to increase in the bioconjugate system, while that assigned to α -helix structure (1653 cm^{-1}) and intermolecular aggregates (A1, 1622 cm^{-1}) decrease relatively. Moreover, almost all the bands shift to lower frequencies for the conjugates as shown in panel B' (Figure 5). These changes in the IR spectra of Hb after the conjugation with AuNPs show that the secondary structure of

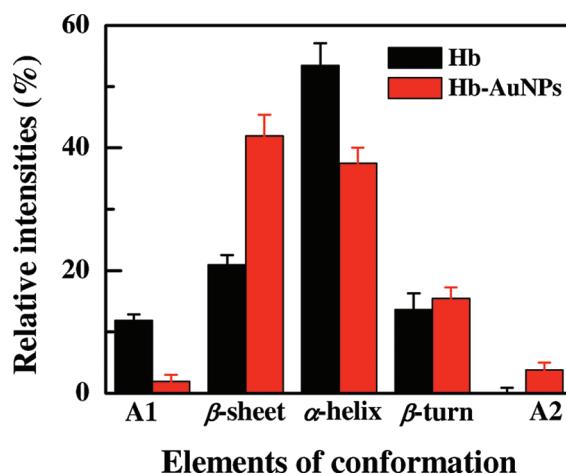


Figure 6. Relative amount of different conformations in native Hb (black bar) and in Hb–AuNPs (with 14.9 nm AuNPs) bioconjugate system (red bar) obtained from fitted data of FTIR as those presented Figure 5.

Hb undergoes obvious changes in the Hb–AuNPs bioconjugate system at pH 7.0. The second derivative spectra further indicate the increase of the sheet and turn, whereas the content of helical structures decreases. The conformational changes imply that the Hb would adopt a more incompact conformation state on the surface of AuNPs.

More quantitative information on the secondary structures of Hb can be obtained by a curve-fitting analysis, which was performed as a linear combination of the components identified in the second-derivative spectrum (panels C and C'). These components were approximated by Gaussian functions whose peak positions, widths, and heights were adjusted iteratively in the curve-fitting procedure. The outcomes of the fitting for Hb in native state and in Hb–AuNPs bioconjugate system are presented in panels C and C' (Figure 5), respectively. The band area of each component, expressed as a percentage of the total amide I area, can be taken as a measure of the secondary structure assigned to it, assuming that the peptide bond molar absorptivities were the same for the different secondary structures of the protein. The native conformation of Hb contains ~53% α -helix structures, ~21% β -sheets, ~14% turns, ~12% intermolecular aggregates (A1), and ~0.11% intramolecular aggregates (A2; Figure 6). After conjugating with AuNPs, the amount of the α -helix and A1 structure decreases rapidly to ~37 and ~2%, respectively, and the amount of β -sheet structure increases greatly to ~42%, suggesting that the main conformational structure of Hb in the bioconjugate system is β -sheet structure. The significant variations in α -helix and β -sheet imply that some α -helix structures have been converted into the β -sheet structures when Hb is conjugated with AuNPs. Moreover, the data presented in Figure 6 also demonstrate that the amount of A1 in Hb–AuNPs bioconjugate system is drastically decreasing (from ~12 to ~2%) and A2 is increasing (from ~0.11 to ~4%), indicating that intramolecular aggregates dominate over intermolecular aggregates. The enhancement of the β -sheet structure accompanied by the loss of the α -helix structure in Hb–AuNPs bioconjugate system may be the main factor accounting for the enhancement of ET rate as those presented in Figure 2 because the secondary structure relates well with the ET characteristics of proteins. The alteration of the secondary structures of Hb may

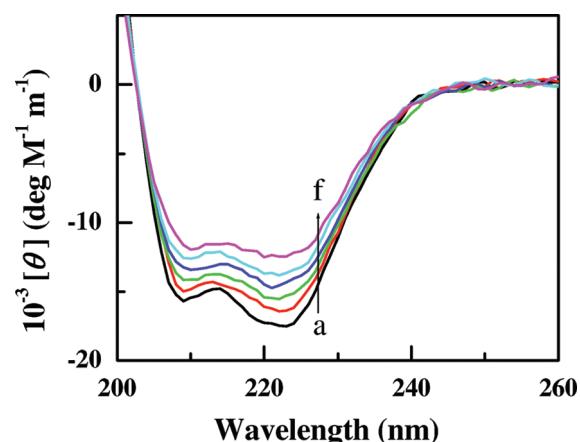


Figure 7. Far–UV CD spectra of (a) native Hb (0.1 mg/mL), and (b–f) Hb–AuNPs bioconjugate system in 0.1 M PBS (pH 7.0) with the concentrations of 14.9 nm AuNPs of (b) 0.1, (c) 0.2, (d) 0.3, (e) 0.4, and (f) 0.5 nM.

induce the change of the structure of heme group, which is the active center for Hb. Such a change in heme structure can significantly affect the ET features of the protein.

3.6. CD Studies. Circular dichroism (CD) spectroscopic measurements were performed to evaluate the extent of the conformational changes of Hb in the bioconjugate system in comparison to the native protein since CD spectroscopy is one of the most powerful methods to study protein conformations in solution or adsorbed onto solid surfaces. The CD spectra were recorded in solution (in 0.1 M PBS, pH 7.0) and not in cast film since CD spectra of thin film are very weak and may not be quantitative regarding the determination of secondary structure of protein.³⁶ The CD spectrum of native Hb at pH 7.0 exhibited two negative bands in the far–UV region (190–250 nm) at 208 and 222 nm (curve a in Figure 7), which is characteristic of an α -helix structure of protein.¹⁰⁰ The band at 208 nm corresponds to $\pi \rightarrow \pi^*$ transition of the α -helix, whereas the band at 222 nm corresponds to $\pi \rightarrow \pi^*$ transition for both the α -helix and random coil.¹⁰¹ These results suggest that the structure of native Hb is predominately α -helix conformation, which is in good agreement with that obtained by FTIR spectra. The intensities of bands at both 208 and 222 nm are found to decrease in the Hb–AuNPs bioconjugate system (curves b–f in Figure 7) in comparison with that of the native Hb (curve a), indicating the loss of the α -helix structure and the occurrence of protein unfolding after the conjugation with AuNPs. In addition, with the increasing concentration of AuNPs in the bioconjugate system, the intensities of the two bands increase further, and two bands appear to move together toward the region between 208 and 222 nm. As a result, the CD spectra evolve toward a shape more similar to that typical of β -sheet-rich structure (the CD feature of β -sheet is usually characterized with a negative band at ca. 215 nm¹⁰⁰), which is likely to be an indication of the conformational transition from α -helix to β -sheet structure in the bioconjugate system. This conclusion also agrees well with that obtained by FTIR spectra.

The approximate fraction of each secondary structure type in proteins can be estimated by analyzing their CD spectra in far–UV region by assuming the CD spectrum is a sum of fractional multiples of reference spectra for each structural type. In this work, the secondary structural elements of Hb in native and in

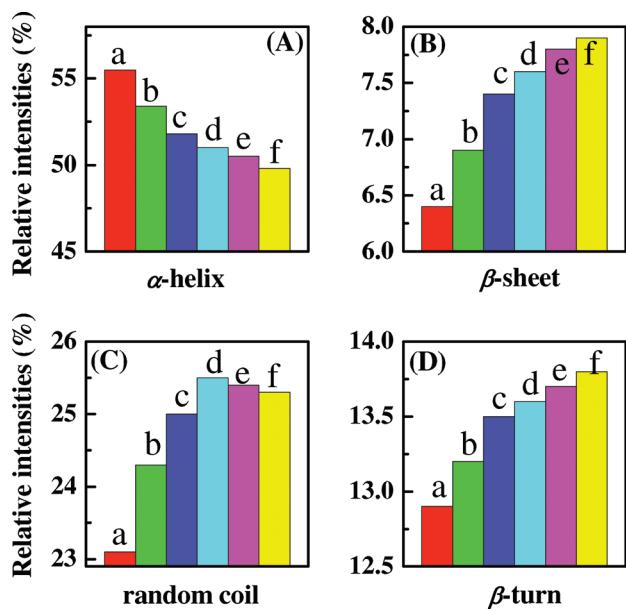


Figure 8. Relative amount of (A) α -helix, (B) β -sheet, (C) random coil, and (D) β -turn in (a) native Hb and in the (b–f) Hb–AuNPs bioconjugate system in PBS (0.1 M, pH 7.0) with the concentrations of 14.9 nm AuNPs of (b) 0.1, (c) 0.2, (d) 0.3, (e) 0.4, and (f) 0.5 nM. The data were obtained from CD spectra presented in Figure 7 using CDNN program.

Hb–AuNPs bioconjugate system are calculated from CD data at pH 7.0 using CDNN program,⁷⁶ and the results are represented in Figure 8. It is indicated that the native conformation of Hb contains \sim 55% α -helix structures, \sim 6% β -sheets, \sim 13% turns, and \sim 23% random coils. After formation of the Hb–AuNPs bioconjugate system, the amount of α -helix decreases, and β -sheet, β -turn, and random coil increase along with increasing the concentration of AuNPs in the bioconjugate system. It can be noted that there is a discrepancy in the result of secondary structure calculation from CD and FTIR spectra due to the aqueous medium in solution phase CD spectra, whereas the aqueous medium is absent in liquid thin film FTIR spectra because of the detection depth of the ATR IR mode is less than 6 μ m; rather, the hydrophobic air phase is present there. However, the change of α -helix, β -sheet, and β -turn, as obtained from the CDNN program is still in the same trend at least qualitatively with the FTIR data (as discussed in the previous section), demonstrating the results are reliable.

It should be noted that the Hb–AuNPs bioconjugate system contains both free (not bound) and bound Hb molecules, therefore, the CD spectra and the related results obtained for Hb–AuNPs bioconjugate system are the overall results of free and bound Hb molecules included in the system. The CD spectrum resulted from solely Hb–AuNPs bioconjugates cannot be obtained at the present conditions as indicated in Experimental Section (section 2.3). Therefore, the results presented in Figure 8 reflect the changes of the secondary structures of Hb in the Hb–AuNPs bioconjugate system (including free and bound Hb molecules) not only those Hb molecules in Hb–AuNPs bioconjugates (bound Hb molecules).

The CD results on the secondary structures of Hb obtained here can be applied to explain the electrochemical results as those presented in Figure 2. It is generally accepted that α -helix, which

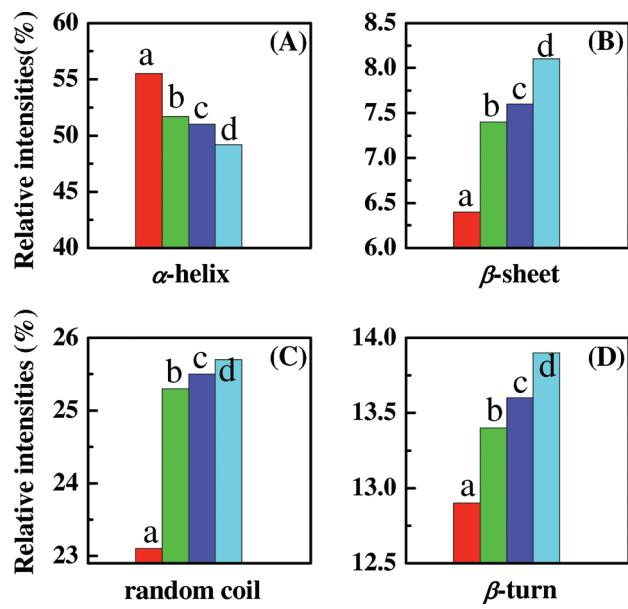


Figure 9. Relative amount of (A) α -helix, (B) β -sheet, (C) random coil, and (D) β -turn in (a) native Hb and in (b–d) Hb–AuNPs bioconjugate system (0.1 mg/mL Hb and 0.2 nM AuNPs) in PBS (0.1 M, pH 7.0) with the size of AuNPs of (b) 40.1, (c) 14.9, and (d) 3.8 nm. The data were obtained from CD spectra using the CDNN program.

forms the basic protein backbone, is more compact than other secondary structures. They are expected to protect the protein from outside disturbance and form the first barrier for the ET process.¹⁰² Therefore, the native Hb cannot undergo the direct ET reaction as depicted in Figure 2. After conjugating with AuNPs, the amount of these compact α -helix structure is reduced and the loose β -sheet structure is increased, leading to a more exposed redox active site of the Hb from the hydrophobic pockets, thereby weakening the barrier of ET between the protein and the electrode.¹⁰³ The ET rate is therefore significantly enhanced as shown by the CV in Figure 2.

3.7. Effects of AuNPs Size. It has been reported that the conformational changes of protein such as cytochrome c were greatly dependent on the size of nanoparticles, on which the protein was assembled.³⁸ This work also evaluates the effects of AuNPs size on the conformational changes of Hb in the Hb–AuNPs bioconjugate system by CD measurements. Three different sizes of AuNPs with diameter of 3.8, 14.9, and 40.1 nm were chosen to form the Hb–AuNPs bioconjugate system. The amount of α -helix, β -sheet, random coil, and β -turn structures of the protein in the Hb–AuNPs bioconjugate system with the different AuNPs size is extracted from the corresponding CD spectra (not shown here) by the CDNN program and is presented in Figure 9. For better comparison, the amount of those secondary structures in native Hb is also depicted the same Figure. It can be concluded that the amount of α -helix in the Hb–AuNPs bioconjugate system decreases with decreasing of the AuNPs size (panel A in Figure 9), however, the amount of β -sheet (panel B), β -turn (panel C), and random coil (panel D) greatly increases along with a decrease in the size of AuNPs in the bioconjugate system. These results indicate that the conformational structures of Hb in the Hb–AuNPs bioconjugate system are influenced significantly by the size of AuNPs. With increasing of the size of AuNPs, a more compact α -helix conformation is induced to the loose conformational structures of β -sheet,

random coil, and β -turn, leading to the unfolding of the protein and the exposure of redox active site (heme) of the protein from the hydrophobic pockets. Please note that the results presented in Figure 9 refer to the changes of the secondary structures of Hb in the Hb–AuNPs bioconjugate system (not only those Hb molecules in Hb–AuNPs bioconjugates) with the size of AuNPs. Therefore, the relative intensities of each secondary structure cannot be normalized by the available surface area or the size of the AuNPs.

4. CONCLUSIONS

In conclusion, this work has studied the conformational changes of Hb before and after conjugating with AuNPs by a combination of electrochemical and spectroscopic techniques. The results demonstrate that the AuNPs can induce Hb undergoing substantial conformational changes both at secondary and tertiary structure level, leading to the enhancement in the electron transfer rate of the protein. The results presented here not only provide the detailed conformational behavior of Hb molecules on nanoparticles but also create a framework for analyzing the biosafety of nanoparticles in terms of the biological behavior of biomacromolecules.

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