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Long Distance Electron Transfer Across >100 nm Thick Au Nanoparticle/Polyion Films to a Surface Redox Protein

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Abstract

Glutathione-decorated 5 nm gold nanoparticles (AuNPs) and oppositely charged poly(allylamine hydrochloride) (PAH) were assembled into {PAH/AuNP}_n films fabricated layer-by-layer (LbL) on pyrolytic graphite (PG) electrodes. These AuNP/polyion films utilized the AuNPs as electron hopping relays to achieve direct electron transfer between underlying electrodes and redox proteins on the outer film surface across unprecedented distances >100 nm for the first time. As film thickness increased, voltammetric peak currents for surface myoglobin (Mb) on these films decreased but the electron transfer rate was relatively constant, consistent with a AuNP-mediated electron hopping mechanism.

Keywords

Gold nanoparticles; Layer-by-layer assembly; Electron transfer; Electron hopping; Myoglobin

1 Introduction

Dense coatings of gold nanoparticles (AuNPs), carbon nanotubes and other conductive nanomaterials have been layered on conductive surfaces as nanostructured electrodes for many applications in bioelectrochemistry. These nanostructured electrodes have large surface areas and can achieve high surface concentrations of adsorbed or covalently linked redox proteins, and provide novel ways to achieve electrical connectivity addressing redox cofactors in proteins [1–3]. Electrodes coated with small AuNPs have distinctive size-dependent electronic and optical properties [4,5], and have emerged as effective platforms for direct protein voltammetry.

Direct protein film voltammetry provides fundamental insights into biological electron transfer, and can be used to fabricate electrochemical biosensors without mediators [6–8]. Approaches have been developed to incorporate proteins into appropriate films to facilitate direct electron exchange between proteins and electrodes [1,9–12]. Films have included

polyions, insoluble surfactants, chemically attached self-assembled monolayers (SAMs), biomacromolecules, polyamines, and nanoparticles. Such approaches immobilize a high surface coverage of proteins on electrode surfaces while using a small total amount of enzymes. They facilitate direct electron transfer, avoid slow diffusion of large proteins that degrades sensitivity in solution, and largely eliminate the denaturation of proteins that often occurs at bare electrodes [10,11].

In 1996, Natan et al. reported reversible electron transfer between cytochrome (cyt) c and a SnO_2 electrode coated with 12 nm AuNPs, but found no electron exchange between cyt c and bare SnO_2 [13]. Subsequently, direct electron exchange with various AuNP electrodes was reported for cyt c [14–16], myoglobin (Mb) [17,18], hemoglobin (Hb) [19], horseradish peroxidase (HRP) [20,21], cyt P450 [22], and other proteins [23,24]. Surfaces packed with glutathione-decorated 5 nm AuNPs on a thin polyion undercoating were also used as immunosensor platforms for ultrasensitive electrochemical detection of proteins [21,25].

Clearly, AuNP electrodes facilitate direct electron transfer with proteins in films, but details of charge conduction to and from the proteins within such films are not completely clear. It is believed that the conductivity of AuNPs plays a key role in this process, even though surface organic molecules on the AuNPs keep them slightly separated. Thin organic polyion layers used in layer-by-layer (LbL) [26–28] AuNP films separate these particles even more [21,25], and the conductivity of these films is typically less than half that of bulk gold [29]. Charge transfer mechanisms to external electrochemically active probes in solution are thought to involve fast electron hopping between AuNPs through the films and possibly localized electron tunnelling [30,31]. In the case of proteins in these films, it is possible that very small AuNPs can approach electroactive prosthetic groups more closely than a flat electrode surface [5] lowering the electron transfer distance between the AuNPs and the redox sites and increasing charge transfer rates [10]. The high conductivity of AuNPs may allow them to act as “electron antennae” in layers [13] or “electron bridges” in multilayers [32] to efficiently deliver electrons between proteins and electrodes.

Enhancing electron exchange between small electroactive probes and AuNP electrodes has been the subject of several reports [33–38]. In a number of studies, a single layer of AuNPs was immobilized on an electrode via SAMs of alkylthiols. The SAMs alone on bare electrodes were electron transfer barriers, and greatly suppressed reversible responses of soluble redox probes. However, after a AuNP monolayer was attached to the SAMs, nearly reversible voltammetry returned, and the electron transfer rate of the probes was greatly enhanced. Similar observations were reported for multilayer assemblies of alkyl dithiols and 12 nm AuNPs [37]. Different explanations have been forwarded for the mechanism of rate enhancement. Diao and co-workers [36] suggested that the single layer of 13 nm AuNPs provided an electron relay and greatly facilitated electron tunnelling through the SAMs. Gooding and co-workers [37] found that charge transfer resistance to the Faradaic probes was insensitive to the length of the intervening SAMs for multiple layers of AuNPs adsorbed on SAMs when AuNPs constituted the outer layer, but electron transfer was suppressed when a SAM was the outer layer. The authors suggested that electron transfer between the redox species and the AuNPs, and not tunnelling across the SAMs, is rate-limiting. Fermin and co-workers [35] hypothesized that charge transfer occurred via a resonant “hot electron transfer” process involving 19 nm AuNP monolayers.

Willner et al. [39] reconstituted glucose oxidase (GOD) at the ends of conductive, cofactor-decorated, vertically aligned single-walled carbon nanotube (SWCNT) forests to achieve electron transport over 150 nm. We hypothesized that if an electron hopping pathway was operative, thick AuNP/polyion films might also relay electrons to surface-adsorbed redox proteins over very long distances, even though there was no clear conductive path to the

protein as in SWCNTs. In the present paper, AuNP films were fabricated by alternately adsorbing oppositely charged poly(allylamine hydrochloride) (PAH) and 5 nm diameter glutathione-decorated AuNPs LbL on pyrolytic graphite (PG) electrodes. Iron heme proteins such as Mb were adsorbed onto the outer film surface to give {PAH/AuNP}_{*n*}-Mb films of different nm-scale thicknesses, and the direct electrochemistry of Mb was investigated by protein film voltammetry. Also, Mb-AuNP bioconjugates were synthesized and placed onto the outer surface of {PAH/AuNP}_{*n*} films. Mb was chosen as the main model protein because of its near reversible voltammetry in LbL films, including those containing AuNPs [32,40–42]. The influence of the number of bilayers (*n*) of the films or the thickness of AuNP layers on the voltammetric responses of {PAH/AuNP}_{*n*}-Mb films revealed that electrons were transferred across AuNP/polyion films over distances of more than 100 nm. To the best of our knowledge, this paper reports the first direct electron transfer of redox proteins across such a long distance through AuNP/polyion LbL films.

2 Experimental

Myoglobin (Mb, *MW* 17800), poly(allylamine hydrochloride) (PAH, *MW* ≈ 56000), poly(styrenesulfonate) (PSS, *MW* ≈ 70000), hydrogen tetrachloroaurate(III) trihydrate (HAuCl₄·3H₂O, 99.9%), L-glutathione reduced (GSH), ferrocenemethanol (FcOH), and silica nanoparticles (SiO₂, diameter 7 nm) were from Sigma. Gold nanoparticles (AuNPs, diameter 5 ± 2 nm) protected by glutathione were prepared by the reduction of Au(III) salt using borohydrides reported previously (see Supporting Information) [21].

For film assembly, rough PG electrodes were alternately immersed in 1 mgmL⁻¹ PAH solution at pH 4.0 and an aqueous suspension of AuNPs at pH 8.0 for 20 min with intermediate water washing. Assembled {PAH/AuNP}_{*n*} LbL films were then immersed in pH 5.0 1.0 mgmL⁻¹ Mb solutions for 30 min to adsorb Mb on the film surface, forming {PAH/AuNP}_{*n*}-Mb films. {PAH/SiO₂}_{*n*} LbL and the corresponding {PAH/SiO₂}_{*n*}-Mb films were assembled similarly.

The fabrication of {PAH/AuNP}₁₀-(AuNP-Mb) films on PG electrodes is described in detail in Supporting Information. In brief, glutaraldehyde was first added into the AuNPs dispersion, followed by the addition of Mb solution. After 30 min of reaction, the formed AuNP-Mb nanocomposites were collected with several filtration and washing steps. The {PAH/AuNP}₁₀ films assembled on PG electrodes were then immersed into the dispersion of AuNP-Mb nanocomposites at pH 8.1 for 30 min to adsorb AuNP-Mb on the surface, forming {PAH/AuNP}₁₀-(AuNP-Mb) films.

To increase the amount of AuNPs in {PAH/AuNP}_{*n*} films, Au(III) loading and reduction were done as previously described (see Supporting Information) [43]. The {PAH/AuNP}_{*n*} films were first immersed in 10 mM HAuCl₄ solution to allow the Au(III) ions to diffuse into the films, and then were placed in 0.05 M NaBH₄ solution to convert Au(III) ions loaded into the films to Au(0). This loading and reduction were repeated for 8 cycles, forming {PAH/AuNP}_{*n*}-8Au films. {PAH/AuNP}_{*n*}-8Au films were then immersed in pH 5.0 Mb solution for 30 min to adsorb Mb on the film surface, forming {PAH/AuNP}_{*n*}-8Au-Mb films.

A CHI 660A electrochemical workstation was used for electrochemical measurements with a saturated calomel electrode (SCE) as reference, a Pt wire as counter, and the PG disk with films as the working electrode. SEM images and the EDX spectra were obtained with an S-4800 scanning electron microscope equipped with an EMAX-350 energy dispersive X-ray analyzer. AFM imaging of films was obtained with an Asylum Research MFP-3D atomic force microscope operated in intermittent contact mode using Olympus model AC-160 cantilevers at a scan rate of 1 Hz.

Other instruments and the full details of sample preparations are described in Supporting Information.

3 Results

3.1 Assembly and Characterization of {PAH/AuNP}_n Films

TEM images of the synthesized glutathione-protected AuNPs (GSH-AuNPs) showed that the nanoparticles were monodisperse (Supporting Information Figure S1A) and had average particle diameter of 5 ± 2 nm (Figure S1B). The UV-vis spectrum of the AuNP dispersion showed a surface plasma resonance peak at 508 nm (Supporting Information Figure S2, curve b), predicting average size of ~ 4 nm [44], consistent with TEM.

GSH-AuNPs feature surface carboxylates and have negative surface charge at pH 8.0 [3], while PAH is cationic at pH 4.0 [45–47]. The oppositely charged AuNPs and PAH were assembled LbL into {PAH/AuNP}_n films utilizing electrostatic interactions. The assembly of {PAH/AuNP}_n films on the surface of Au/MPS/{PAH/PSS}₂ films was monitored and confirmed by quartz crystal microbalance (QCM) (Supporting Information Figure S3, curve a).

The {PAH/AuNP}_n films were characterized by EDX (Supporting Information Figure S4), showing the characteristic peak of Au at about 2.2 keV [48]. Under the same experimental conditions, the EDX peak height and area for Au increased with *n*, suggesting that the AuNPs are incorporated into the films and more bilayers lead to more AuNPs in the films.

SEM cross-sectional views of films assembled on QCM Au disk resonators were used to estimate the thickness of the films (Figure 1A). The average thickness of the {PAH/AuNP}₂₀ films was 200 ± 20 nm, suggesting ~ 10 nm thickness of each PAH/AuNP bilayer.

Insulating {PAH/SiO₂}_n films with 7 nm SiO₂ nanoparticles were prepared as controls for electrochemical studies, and reproducible film growth was also confirmed by QCM (Figure S3, curve b). The thickness of {PAH/SiO₂}₂₀ films estimated by SEM was 250 ± 60 nm (Figure 1B), so that the thickness of a PAH/SiO₂ bilayer was ~ 12.5 nm. In addition, the thickness of {PAH/AuNP}₂₀-8Au films was 200 ± 20 nm (Figure 1C), also suggesting ~ 10 nm thickness of each PAH/AuNP bilayer.

3.2 Voltammetry of {PAH/AuNP}₁₀-Mb Films

When {PAH/AuNP}₁₀ films assembled on PG electrodes were immersed in pH 5.0 Mb solution for 30 min, Mb was adsorbed on the film surface, forming {PAH/AuNP}₁₀-Mb films. The {PAH/AuNP}₁₀-Mb films were transferred to pH 7.0 buffers containing no Mb for cyclic voltammetry (CV). A small CV reduction–oxidation peak pair was observed at about -0.34 V vs SCE (Figure 2A curve a), characteristic of Mb Fe(III)/Fe(II) redox couple [10,11]. By extending the charging currents in the range out of the Faraday response from the same CV, the background currents in the range of -0.1 to -0.6 V were estimated and then subtracted. The background-subtracted CV of {PAH/AuNP}₁₀-Mb films demonstrated a pair of well-defined redox peaks with nearly equal heights and small peak separation (Figure 2B, curve a), indicating the good electrochemical reversibility of Mb on these 100 nm thick films at least at 0.2 Vs⁻¹. In contrast, no peak was observed in the same potential range for {PAH/AuNP}₁₀ films in the pH 7.0 buffers (Figure 2A and B, curve b).

The analysis of the small CV peaks of {PAH/AuNP}₁₀-Mb films required subtraction of a large background current, which could be the source of considerable error. Thus, the more sensitive square wave voltammetry (SWV) was employed, which gave well-defined SWV “difference” peaks at -0.36 V for Mb, and also for hemoglobin (Hb) and catalase (Cat)

without background subtraction (Figure 3A). SWV “forward” and “reverse” peaks for the {PAH/AuNP}₁₀-Mb films were also clearly observed (Figure 3B), confirming electrochemical reversibility of the system with 10 Hz frequency and 15 mV amplitude.

3.3 Comparison of {PAH/AuNP}_n and {PAH/SiO₂}_n Films

We wished to have control films made from insulating particles, so 7 nm SiO₂ nanoparticles were assembled with PAH into {PAH/SiO₂}₁₀ LbL films, and the films were immersed into Mb solutions at pH 5.0. The loading behavior of {PAH/SiO₂}₁₀ films toward Mb (Figure 4A, curve b) was quite different from that of {PAH/AuNP}₁₀ films (curve a). The SWV difference peak current for the {PAH/AuNP}₁₀-Mb films (*I_p*) increased with the immersion time (*t*) when *t* was less than 20 min, and then tended to level off. This time to the steady state was in good agreement with the results for adsorption of a single layer of a wide variety of proteins on LbL films from solutions of comparable concentrations [27]. However, the SWV response of {PAH/SiO₂}₁₀-Mb films was much larger than that of {PAH/AuNP}₁₀-Mb films and increased with the immersion time of the {PAH/SiO₂}₁₀ films in Mb solution for >40 min. These results suggest that Mb is loaded into the interior of {PAH/SiO₂}₁₀ films since more time to the steady state would be necessary for the protein to diffuse inside the films and this time has been previously measured in hours [49–52]. Thus, the fact that the steady state was reached within only 20 min for the {PAH/AuNP}₁₀-Mb films strongly suggests that Mb is most probably confined to the outer layer of the films.

This view was further supported by the treatment of the Mb-loaded films with 4 M urea, which denatured proteins [53–56]. For {PAH/SiO₂}₁₀-Mb films, a urea treatment of only 20 s caused a very small decrease in the SWV peak (Figure 5 A). This is because most of Mb molecules are located in the interior of the SiO₂ films, and the urea treatment with very short time (20 s) cannot denature the interior Mb. For {PAH/AuNP}₁₀-Mb films, however, the SWV peak was greatly suppressed by urea treatment of only 2 s, and completely disappeared in 20 s (Figure 5B). This is because Mb resides mainly on the outer surface of the AuNP films, and the same 20 s urea treatment leads to the complete denaturation of the accessible surface Mb.

The different loading behavior of {PAH/AuNP}₁₀ and {PAH/SiO₂}₁₀ films toward Mb is most probably attributed to different porosities of the two films. Thus, amperometry using neutral electroactive probe ferrocenemethanol (FcOH) was done in pH 7.0 buffers to probe the film porosities (Figure 4B). Keeping potential at 0.2 V, FcOH was injected, and the change of oxidation current of FcOH with time was monitored. For {PAH/SiO₂}₁₀ films, about 3 s was needed to detect a rise in current (curve b), while for {PAH/AuNP}₁₀ films, more than 10 s were required (curve a). Moreover, the maximum current for {PAH/SiO₂}₁₀ films was much larger than that for {PAH/AuNP}₁₀ films. These results suggest that {PAH/SiO₂}₁₀ films are much more porous than {PAH/AuNP}₁₀ films. The much larger Mb with *MW* ≈ 17 000 is expected to have great difficulty entering the less porous, more compact {PAH/AuNP}₁₀ films.

The surface morphologies of {PAH/AuNP}₁₂ and {PAH/SiO₂}₁₂ films were further investigated by AFM. The SiO₂ films (Figure 6B) exhibited surfaces that were much rougher than the AuNP films (Figure 6A). The RMS roughness of the SiO₂ films (88.2 nm) was more than 5 times larger than that of AuNP films (16.8 nm) (Supporting Information Figure S5). Surface roughness thus correlates with porosity measured by the probe amperometry. The 2-D AFM surface images with higher magnification also reflect the presence of diffusional pathways in the SiO₂ films (Figure 6E) that are much more prominent than in the AuNP films (Figure 6D). The AFM images of {PAH/AuNP}₁₂-8Au films, described in more detail below, are also presented in Figure 6 for comparison.

All these results (Figure 4–6) support the conclusion that for {PAH/AuNP}_n-Mb films, Mb is mainly adsorbed on the outer film surface, but for {PAH/SiO₂}_n-Mb films, most Mb molecules are located in the interior of the films.

3.4 {PAH/AuNP}₁₀-(AuNP-Mb) Films

To support the conclusion that Mb is mainly adsorbed on the outer surface of {PAH/AuNP}_n-Mb films, and not located in the interior, a method was used to ensure that Mb was present only on the film surface. Mb was chemically linked to 5 nm AuNPs in a dispersion by amidization of GSH-AuNPs to Mb with bifunctional cross-linker glutaraldehyde. Unbound Mb was removed from the dispersion, and the AuNP-Mb nanocomposite particles were adsorbed onto the outer surface of {PAH/AuNP}₁₀ films on PG to give {PAH/AuNP}₁₀-(AuNP-Mb) films. To ensure that there was no free Mb in the AuNP-Mb dispersion, the Soret band of Mb in filtrates after each filtration/washing step was detected (Supporting Information Figure S6, inset). After the 4th step in filtration/washing procedure, the Soret band was negligible (Figure S6), indicating that nearly all free Mb is removed from the AuNP-Mb dispersion.

The formation of the AuNP-Mb nanocomposites was confirmed by UV-vis spectroscopy, and the dispersion at pH 8.1 showed a Soret band at 409 nm (Figure S2, curve c), consistent with that of Mb in solution (curve a). The AuNP-Mb dispersion also showed an absorption peak at 516 nm (curve c), attributed to the surface plasma resonance peak of AuNPs. Compared with the AuNP dispersion, for which the surface plasma resonance peak was observed at 508 nm (curve b), the red shift of 8 nm for the AuNP-Mb dispersion is attributed to the interaction between AuNPs and Mb [40,57].

To verify that Mb in the AuNP-Mb nanocomposites retains its bioactivity, oxidation of 2,2'-azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) by H₂O₂ catalyzed by Mb was monitored by observation of the growth of an absorption peak at 740 nm for oxidized ABTS [58–60]. When Mb was added to an ABTS+H₂O₂ solution, an absorption peak appeared immediately at 740 nm (Supporting Information Figure S7, curve b), and increased with reaction time (curve c), which was not observed in Mb-free control (curve a). These results confirm that Mb acts as a peroxidase in this reaction [58–60]. When the same volume of AuNP-Mb dispersion was added to the ABTS+H₂O₂ solution, the absorption peak at 740 nm was also observed and grew with reaction time, demonstrating very similar behavior to free Mb (Figure 7A, curves a and b). In the control experiment with bare AuNPs without Mb, the AuNPs+ABTS+H₂O₂ system showed little increase at 740 nm (curve c). These results indicate that Mb on AuNP-Mb nanocomposites retains its natural catalytic activity.

{PAH/AuNP}₁₀ films on PG electrodes were then immersed into the dispersion of AuNP-Mb at pH 8.1 for 30 min to adsorb AuNP-Mb on the surface, forming {PAH/AuNP}₁₀-(AuNP-Mb) films. These films were then placed into pH 7.0 buffers for CV and SWV. The background-subtracted CV of the films (Figure 7B curve b) showed a pair of redox peaks at about -0.34 V, characteristic of Mb Fe(III)/Fe(II) couple [10,11]. This result confirms that Mb in the AuNP-Mb on the surface of {PAH/AuNP}₁₀ films can exchange electrons with underlying electrodes over long distances through the AuNP films. This was further confirmed by SWV, in which a well-defined difference SWV peak was found at about -0.36 V (Figure 7C, curve b). CV and SWV of {PAH/AuNP}₁₀ films with no Mb are shown in Figure 7B and C for comparison (curve a).

The AuNP-Mb nanocomposite is much larger than Mb in size. Thus, AuNP-Mb is not able to enter into the interior of the non-porous {PAH/AuNP}₁₀ films. The good CV and SWV responses of {PAH/AuNP}₁₀-(AuNP-Mb) films (Figure 7B and C) indicate that the AuNP-Mb nanocomposites on the surface of {PAH/AuNP}₁₀ films can exchange electrons with

underlying PG electrodes. These results are consistent with those for electrodes with Mb adsorbed on the outer surface of AuNP/polyion films, and further confirm that electron transfer can occur over distances of 100 nm or more through these films.

3.5 Influence of Film Thickness

The number of bilayers (n) was modulated to change thickness of {PAH/AuNP} $_n$ films, which had an influence on the electrochemical response of the Mb on the outer surface. The SWV difference peak current (I_p) of {PAH/AuNP} $_n$ -Mb films decreased with increase of n , and after $n > 13$, no electrochemical response could be detected (Figure 8 A and B). The thickness of 13 PAH/AuNP bilayers was about 130 nm according to the SEM results (Figure 1A). These results suggest that the AuNPs in the films act as “charge transfer relays”, and with the aid of AuNPs, the direct electron transfer of Mb with underlying electrodes can be realized across over 100 nm. If assuming that the Mb molecules adsorbed on the surface of {PAH/AuNP} $_4$ films with $n = 4$ were all electroactive, the corresponding SWV I_p could represent the expected peak current for one full close-packed monolayer of Mb on the surface. Thus, the fraction of electroactive Mb in the {PAH/AuNP} $_{10}$ -Mb films with $n = 10$ would be about 40%.

The reduction-oxidation peak separation in CV ($\Delta E_p = E_{pa} - E_{pc}$) reflects the electrochemical reversibility of redox species. In films, it arises from both charge transfer kinetics [61] and non-kinetic factors [62]. The ΔE_p of {PAH/AuNP} $_n$ -Mb films measured by CV maintained an essentially constant value of about 50 mV at 0.2 Vs $^{-1}$ when n was between 4 and 8, and then showed a slight increasing trend up to about 62 mV when n was increased from 8 to 11 (Figure 8C). The apparent heterogeneous electron transfer rate constant (k_s) for the Mb films was estimated from ΔE_p according to an approach developed by Laviron [61], and the average was in the narrow range of 3.6–5.3 s $^{-1}$ with different n values (Figure 8D). Similarly, forward and reverse SWV peak separations (ΔE_p) for the {PAH/AuNP} $_n$ -Mb films remained at ~10 mV for the films with n of 4–11 (Supporting Information Figure S8). These minimal changes in ΔE_p in CV and SWV, and the effectively constant and large k_s values reflect nearly reversible Mb voltammetry even when the film thickness becomes quite large.

As an additional control, true insulating {PAH/PAA} $_n$ LbL films containing no AuNPs were assembled on PG electrodes, where PAA represents negatively charged poly(acrylic acid). The thickness of one PAH/PAA bilayer is ~9 nm under the assembly condition (PAH/PAA pH 8.0/4.0) [63,64]. Thus, the thickness of {PAH/PAA} $_{14}$ films with $n = 14$ is ~126 nm. These films were immersed in Mb solution at pH 5.0 for 30 min to adsorb Mb on the film surface. Under these conditions, the positively charged Mb is known to bind only to the outer anionic PAA surface, and will not intermix with more than the two top layers [27]. {PAH/PAA} $_{14}$ -Mb films gave no observable signal either by CV or SWV (Supporting Information Figure S9). As there is no possibility here of Mb entering the bulk of the films, these results suggest that over 100 nm of insulating layers between the adsorbed Mb and underlying PG completely blocks direct electron transfer.

Mb can catalyze the electrochemical reduction of H $_2$ O $_2$ [10,11], and this was confirmed by an increase in the CV reduction peak at -0.4 V for {PAH/AuNP} $_{10}$ -Mb when H $_2$ O $_2$ was added (Supporting Information Figure S10A). As expected for this catalytic process, the reduction peak current increased with concentration of H $_2$ O $_2$ in solution. No direct reduction of H $_2$ O $_2$ at {PAH/AuNP} $_{10}$ film electrodes was observed between -0.1 and -1.2 V (Figure S10B). All these results are characteristic of electro-catalytic reduction of H $_2$ O $_2$ by the Mb films [10,11]. The catalytic behavior of Mb in the {PAH/AuNP} $_{10}$ -Mb films toward H $_2$ O $_2$ is evidence that the denaturation of Mb does not take place and the Mb essentially keeps its native state. Also, the extensive UV-vis spectroscopy studies of Mb/AuNP LbL films in our

previous work [40] have demonstrated clearly that Mb essentially retains a near-native conformation when it is adsorbed on the surface of citrate-decorated AuNPs. The effect of number of film bilayers (n) on the reduction peak current (I_{pc}) in the presence of H_2O_2 was also monitored. With the same amount of H_2O_2 injected, the I_{pc} of the $\{PAH/AuNP\}_n$ -Mb films decreased dramatically with n (Figure S10C), showing the same trend as in Figure 8B. It was observed that when $n > 14$, no electrocatalytic response could be detected for the films, consistent with the lack of Mb electroactivity in these systems.

3.6 $\{PAH/AuNP\}_n$ -8Au-Mb Films

In our previous work [43], a cyclic Au(III) loading-reduction procedure was developed to enhance the density of AuNPs in $\{PAH/SiO_2\}_n$ LbL films. The films were first placed in $HAuCl_4$ solutions to load Au(III) ions into the films, followed by chemical reduction of Au(III) into AuNPs with $NaBH_4$ solutions. This in situ deposition of AuNPs inside the films could be repeated for several cycles to increase the amount of AuNPs in the films, and the formation and growth of AuNPs were confirmed by SEM and EDX.

In the present work, to further elucidate the role of AuNPs in electron transfer of Mb in the $\{PAH/AuNP\}_n$ -Mb film system, the Au(III) loading-reduction process was used to increase the density of AuNPs in the $\{PAH/AuNP\}_n$ films. We expected that such increased particle density would enhance the electrochemical response of the surface Mb. The Au(III) loading-reduction cycle was repeated 8 times to give films denoted $\{PAH/AuNP\}_n$ -8Au. This resulted in $\{PAH/AuNP\}_{16}$ -8Au films that had about 4-fold more Au than that in $\{PAH/AuNP\}_{16}$ films as shown by EDX (Figure 9 A). The SEM cross-section view of $\{PAH/AuNP\}_{20}$ -8Au films showed that the thickness of the films was about 200 ± 20 nm (Figure 1C). Thus, one PAH/AuNP bilayer was ~ 10 nm, same as that for the corresponding bilayer of $\{PAH/AuNP\}_{20}$ films (Figure 1A). AFM images demonstrated that compared to $\{PAH/AuNP\}_{12}$ films (Figure 6D), the number of AuNPs and their aggregates in $\{PAH/AuNP\}_{12}$ -8Au films increased significantly and the distribution of the particles became more uniform (Figure 6F), while the size increase of AuNPs in $\{PAH/AuNP\}_{12}$ -8Au films could not be excluded. When the $\{PAH/AuNP\}_{16}$ -8Au films with $n = 16$ were immersed in Mb solutions for 30 min to adsorb Mb onto the surface, the as-prepared $\{PAH/AuNP\}_{16}$ -8Au-Mb films in protein-free buffers showed a SWV peak at -0.33 V (Figure 9B, curve a), while no peak was detected for the corresponding $\{PAH/AuNP\}_{16}$ -Mb films (curve b). Similar results were found by CV (Supporting Information Figure S11).

The number of bilayers (n) of $\{PAH/AuNP\}_n$ -8Au-Mb films also had a large influence on voltammetry of Mb. SWV difference peak current (I_p) of $\{PAH/AuNP\}_n$ -8Au-Mb films in pH 7.0 buffers decreased with n until about $n = 18$ (Supporting Information Figure S12). When $n > 18$, the SWV peaks of the $\{PAH/AuNP\}_n$ -8Au-Mb films could no longer be detected. This result indicates that the electron transfer of adsorbed Mb on the film surface with underlying electrodes can occur across 180 nm when the density of AuNPs in the films is high enough.

4 Discussion

Results presented above demonstrate that electron exchange between a redox protein and an electrode can be realized over remarkable distances of AuNP (5 nm)/polyion films exceeding 100 nm (Figures 2, 3, 7 and 8), much larger than previously reported [14,65,66]. In contrast, Chi and co-workers reported a reversible CV response for cyt c at AuNP-monolayer modified gold electrodes across 5 nm [65]. Leopold et al. found direct electron transfer of cyt c attached on the surface of AuNP multilayers with underlying electrodes over 13 nm [14] and reported direct CV response for the redox protein azurin across AuNP multilayer films for distances up to 20 nm [66].

In the present work, immersion of $\{\text{PAH/AuNP}\}_n$ films into Mb solution for 30 min clearly provided a system with nearly all Mb molecules adsorbed on the film surface as demonstrated by adsorption time (Figure 4A), and very rapid decomposition by urea treatment (Figure 5) compared to the porous silica control films. We had initially hoped that Mb would also be adsorbed on the outer layer of the insulating $\{\text{PAH/SiO}_2\}_n$ films to provide a control in which no electron transfer was observed. However, these films provided a different type of control that had higher porosity than the $\{\text{PAH/AuNP}\}_{10}$ films, into which Mb could diffuse. Effect of urea treatment (Figure 5), influence of immersion time on Mb signals (Figure 4A), relative film porosities (Figure 4B), the 2-D and 3-D AFM images (Figure 6), and surface roughness (Figure S5) of these two types of films are consistent with the fact that the $\{\text{PAH/AuNP}\}_n$ films are densely packed and internal density inhibits entry of Mb (diameter ≈ 4 nm [27]), while the much more porous $\{\text{PAH/SiO}_2\}_n$ films admit Mb into the interior.

The $\{\text{PAH/AuNP}\}_{10}$ -(AuNP-Mb) films made with a preformed AuNP-Mb outer layer provided a system in which it was not possible for Mb to diffuse into the interior of $\{\text{PAH/AuNP}\}_{10}$ films. Results for these films again demonstrated realization of long distance electron transfer between Mb on the outer film surface and electrodes (Figure 7B and C). Results with these two films prepared in different ways unequivocally confirm that electrons can be transferred across a distance of 100 nm through the AuNP/polyion films to an outer layer of a redox protein.

A most intriguing question concerns the mechanism of electron transport through these $\{\text{PAH/AuNP}\}_n$ films, which contain considerable insulating material in the form of the glutathione coating of the AuNPs and the polyions deposited in alternate layers. It has become clear that although LbL films are formed a layer at a time, the final result is a film with considerable interlayer mixing [26–28]. Thus, the $\{\text{PAH/AuNP}\}_n$ films can be viewed as AuNPs interspersed in an organic matrix. From the amount of AuNPs estimated by QCM (Figure S3) and the average film thickness obtained by SEM (Figure 1A) for the $\{\text{PAH/AuNP}\}_n$ films, assuming a uniform distribution of AuNPs in the 3-D space of the films, the average center-to-center distance between AuNPs is estimated to be about 10 nm and the edge-to-edge separation between two adjacent GSH-AuNPs is ~ 1.6 nm (For detailed calculation, see Supporting Information).

There seems to be some consensus that the charge transport in AuNP/polyion LbL films occurs via electron hopping between individual AuNPs, with the possible involvement of localized electron tunnelling between AuNPs in the films [29–31]. Charge transport rates can depend on particle size, but at 5 nm diameters our AuNPs have metal-like properties [30]. Our experimental data appear to be consistent with electron hopping, since the electron transfer rate constant is nearly independent of film thickness (Figure 8D). A mechanism of “electron tunnelling” across the complete film can be ruled out as this would elicit an exponential dependence of electron transfer rate on thickness [30]. Typically, such electron tunnelling can only take place across a few nanometers [67]. However, intervening conducting AuNPs (separated by an average of 1.6 nm between two adjacent GSH-AuNPs) may act as electron transfer “bridges” or “relays” to establish discrete electron hopping pathways between Mb and electrodes. If Mb molecules on the film surface can exchange electrons with the neighboring AuNPs in the films, electron transfer can be extended sequentially from the neighboring AuNPs in the films to the electrode surface by successive electron hopping among these neighboring AuNPs in the films, leading to the realization of the electron transfer of Mb with underlying electrodes. The driving force here is the oxidizing or reducing potential applied to the underlying electrodes.

The function of electron self-exchange relay involving AuNPs was further supported by CVs and SWVs for dense, insulating {PAH/PAA}₁₄ LbL films containing no AuNPs and the corresponding {PAH/PAA}₁₄-Mb films. No voltammetry was detected for these films with $n = 14$ and thickness ≈ 126 nm [63,64] (Figure S9). In the insulating films, > 100 nm between the adsorbed Mb and underlying PG surface is too long for Mb to obtain electrons by tunnelling.

The key role of AuNPs in relaying electrons between the surface Mb on {PAH/AuNP} _{n} films and the underlying PG electrodes was also demonstrated for other redox proteins. For example, both {PAH/AuNP}₁₀-Hb and {PAH/AuNP}₁₀-Cat films with $n = 10$ also showed SWV difference peaks (Figure 3A, curves b and c), indicating the generality of AuNPs in enhancing the direct electrochemistry of redox proteins.

If there are enough closely-spaced AuNPs in the films, electron hopping between neighboring AuNPs could provide conducting pathways to the surface Mb and electronic communication between the surface Mb and underlying PG electrodes could be realized across an infinite film thickness. In practice, however, the current response (Figure 8A and B) and the electrocatalytic response to H₂O₂ (Figure S10) for the {PAH/AuNP} _{n} -Mb films is restricted to a distance less than ~ 130 nm. There are two possible reasons: (a) There may be restricted compensation of charge generated by electron transfer to maintain electro-neutrality in the films, usually achieved by the transport of small counterions between the solution phase and the film phase, which may become a limiting factor [30,68]. The mobility of counterions within the film phase depends on the permeability and/or porosity of the films. Thicker films may have poorer permeability, thus limiting the mobility of counterions in the films. (b) There must be discrete conducting pathways of AuNPs in the films so that the successive electron hopping among neighboring AuNPs and the corresponding electron transfer of surface Mb with underlying electrodes can be realized. However, defects and disorder in the assembly may lead to the nonuniform or inhomogeneous distribution of AuNPs in the films. In this case, the number of effective pathways will decrease as film thickness increases. Either or both of these possibilities are consistent with the observed peak current decrease with increasing film thickness (Figures 8A and B).

The central role of AuNPs in achieving direct electron transfer of Mb across the {PAH/AuNP} _{n} films was further evidenced by increasing the density of AuNPs in the films, as achieved in the {PAH/AuNP}₁₆-8Au films, which have about 4 times higher density of AuNPs than the {PAH/AuNP}₁₆ films (Figure 9A). Thus, the {PAH/AuNP}₁₆-8Au-Mb films gave SWV peaks (Figure 9B, curve a), while no peak was detected for the corresponding {PAH/AuNP}₁₆-Mb films (curve b). This is consistent with enhancement of the electron-hopping pathway by decreasing the average distance between neighboring AuNPs. For {PAH/AuNP} _{n} -Mb films, since the density of AuNPs is relatively low, the distance between some AuNPs and their neighboring counterparts is too far for them to exchange electrons, thus leading to the limited number of routes for electron transfer. However, for the corresponding {PAH/AuNP} _{n} -8Au-Mb films, the increase in AuNP density places more AuNPs in the films closer to their neighbors, and increases routes for electron hopping. Another way of saying this is the increase in the number of AuNPs in the films increases the number of "percolation pathways" [29] resulting in the increase of electron exchange of Mb with the electrodes. According to the SEM results (Figure 1), both {PAH/AuNP}₁₆ and {PAH/AuNP}₁₆-8Au films have the same thickness of 160 nm. For {PAH/AuNP}₁₆-Mb films, this distance is too long for Mb to transfer electrons through {PAH/AuNP}₁₆ films. However, for {PAH/AuNP}₁₆-8Au-Mb films, due to the increased AuNP density and number of routes for electron hopping, the electron transfer of Mb across 160 nm can be realized. For {PAH/AuNP} _{n} -8Au-Mb films, the SWV difference peak current (I_p) also shows a decreasing trend with n until about $n = 18$ (Figure S12). This is also

consistent with electron hopping and the limiting charge compensation and decreasing conducting pathways as thickness increases.

5 Conclusions

Direct electron exchange between underlying electrodes and surface redox proteins across distances > 100 nm of AuNP/polyion LbL films is achieved by the AuNPs acting as electron hopping relays. Increasing the AuNP density in the films greatly enhances the voltammetric response of surface Mb, further confirming the electron hopping function of the AuNPs. The present work provides the first example that AuNPs in polyion films can act as long distance electron relays to achieve direct electron exchange between surface redox proteins and underlying electrodes.

In more general terms, our results show that a composite material made up of nm-scale conducting particles surrounded by organic coatings and polyions can provide electron conduction over 100 nm or more. Most likely, this is facilitated by discrete electron hopping pathways facilitated by particle disorder in the AuNP/polyion films.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. SEM cross-sectional view of (A) $\{\text{PAH}/\text{AuNP}\}_{20}$, (B) $\{\text{PAH}/\text{SiO}_2\}_{20}$, and (C) $\{\text{PAH}/\text{AuNP}\}_{20-8\text{Au}}$ films assembled on Au/MPS surface.

Fig. 2.
(A) CVs and (B) background-subtracted CVs at 0.2 Vs^{-1} for (a) {PAH/AuNP}₁₀-Mb and (b) {PAH/AuNP}₁₀ films in pH 7.0 buffers.

Fig. 3. (A) SWV difference currents for (a) {PAH/AuNP}₁₀-Mb, (b) {PAH/AuNP}₁₀-Hb, (c) {PAH/AuNP}₁₀-Cat, and (d) {PAH/AuNP}₁₀ films in pH 7.0 buffers. (B) SWV (a) forward and (b) reverse currents for {PAH/AuNP}₁₀-Mb films in pH 7.0 buffers.

Fig. 4. (A) Effect of immersing time (t) in 1 mgmL⁻¹ Mb adsorbate solutions at pH 5.0 for (a) {PAH/AuNP}₁₀ and (b) {PAH/SiO₂}₁₀ films on SWV difference peak current (I_p) of the corresponding Mb-loaded films in pH 7.0 buffers. The data are the average of three parallel measurements. (B) Amperometric response to 10 μ M ferrocenemethanol (FcOH) injected at $t = 10$ s into pH 7.0 buffers at 0.2 V for (a) {PAH/AuNP}₁₀ and (b) {PAH/SiO₂}₁₀ films.

Fig. 5. SWV difference currents of (A) {PAH/SiO₂}₁₀-Mb and (B) {PAH/AuNP}₁₀-Mb films in pH 7.0 buffers after the films were immersed in 4 M urea solutions for (a) 0, (b) 2, (c) 10, and (d) 20 s.

Fig. 6. 3-D AFM topography of (A) {PAH/AuNP}₁₂, (B) {PAH/SiO₂}₁₂, and (C) {PAH/AuNP}₁₂-8Au films. 2-D AFM topography of (D) {PAH/AuNP}₁₂, (E) {PAH/SiO₂}₁₂, and (F) {PAH/AuNP}₁₂-8Au films.

Fig. 7.

(A) Dependence of ΔA on reaction time at 740 nm for (a) Mb + ABTS + H₂O₂, (b) AuNP-Mb + ABTS + H₂O₂, and (c) AuNPs + ABTS + H₂O₂ systems in pH 6.5 HEPES buffers.

(B) Background-subtracted CVs at 0.2 Vs⁻¹ and (C) SWV difference currents for (a) {PAH/AuNP}₁₀ and (b) {PAH/AuNP}₁₀-(AuNP-Mb) films in pH 7.0 buffers.

Fig. 8.

(A) SWV difference currents of {PAH/AuNP}_n-Mb films in pH 7.0 buffers with different number of bilayers (n): (a) 6, (b) 10, (c) 13, and (d) 16. (B) Dependence of SWV difference peak current (I_p) on the number of bilayers (n) or on the thickness (d) of {PAH/AuNP}_n-Mb films in pH 7.0 buffers. Dependence of (C) CV peak separation (ΔE_p) and (D) k_s value of {PAH/AuNP}_n-Mb films at 0.2 V s^{-1} in pH 7.0 buffers on the number of bilayers (n). Data are the average of three parallel measurements.

Fig. 9.
(A) EDX of (a) $\{\text{PAH/AuNP}\}_{16}\text{-8Au}$ and (b) $\{\text{PAH/AuNP}\}_{16}$ films fabricated on PG disks.
(B) SWV difference currents in pH 7.0 buffers for (a) $\{\text{PAH/AuNP}\}_{16}\text{-8Au-Mb}$ and (b) $\{\text{PAH/AuNP}\}_{16}\text{-Mb}$ films.