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Protecting Peroxidase Activity of Multilayer Enzyme-Polyion Films Using Outer Catalase Layers

Haiyun Lu^a, James F. Rusling^{b,c}, and Naifei Hu^{a,*}

^a Department of Chemistry, Beijing Normal University, Beijing 100875, P. R. China

^b Department of Chemistry, University of Connecticut, U-60, Storrs, CT 06269-3060, USA

^c Department of Pharmacology, University of Connecticut Health Center, Farmington, CT 06032, USA

Abstract

Films constructed layer-by-layer on electrodes with architecture {protein/hyaluronic acid (HA)}_n containing myoglobin (Mb) or horseradish peroxidase (HRP) were protected against protein damage by H₂O₂ by using outer catalase layers. Peroxidase activity for substrate oxidation requires activation by H₂O₂, but {protein/HA}_n films without outer catalase layers are damaged slowly and irreversibly by H₂O₂. The rate and extent of damage were decreased dramatically by adding outer catalase layers to decompose H₂O₂. Comparative studies suggest that protection results from catalase decomposing a fraction of the H₂O₂ as it enters the film, rather than by an in-film diffusion barrier. The outer catalase layers controlled the rate of H₂O₂ entry into inner regions of the film, and biased the system to favor electrocatalytic peroxide reduction over enzyme damage. Catalase-protected {protein/HA}_n films had an increased linear concentration range for H₂O₂ detection. This approach offers an effective way to protect biosensors from damage by H₂O₂.

Introduction

Since the 1990s, layer-by-layer assembly by alternate adsorption of oppositely charged polyions on solid surfaces has developed very rapidly,¹ and has been used to assemble a wide variety of ultrathin films of proteins and enzymes.² The layer-by-layer assembly technique shows advantages over other film-assembly methods in versatility of layer components, precise control of thickness on the nanometer scale, and simplicity of preparation. The protein multilayer films with oppositely charged polyelectrolytes or nanoparticles can thus be fabricated one layer at one time by electrostatic interaction according to a predesigned architecture. Particularly, this technique is well suited to construction of multi-enzyme systems, in which different types of enzymes with different functions are integrated into one film with precise control of the deposition sequence and film thickness.^{3,4}

Layer-by-layer assembly has also been used to fabricate enzyme electrodes and develop electrochemical biosensors.⁵ For example, Calvo and co-workers investigated the multilayer films of glucose oxidase (GOx) assembled layer-by-layer with ferrocene- or osmium complex-modified polymers on electrodes, in which GOx retained its biological activity while the redox polyelectrolyte acted as a “molecular wire” enabling the electrical communication between the enzyme and the underlying electrodes.⁶ The direct electrochemistry of redox proteins in various multilayer films on electrodes has also been realized.^{7–9} Lvov, Rusling, and co-workers assembled layer-by-layer films of myoglobin (Mb) or cytochrome P450 (cyt P450) with oppositely charged DNA or polyelectrolytes on gold electrodes, and the reversible

*Corresponding author. Tel.: +86 10 5880 5498; fax: +86 10 5880 2075. E-mail address: hunaifei@bnu.edu.cn (N. Hu).

voltammetry of the protein $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ redox couples in these films was achieved and used to drive enzyme-catalyzed epoxidation of styrene.^{7a} Films containing DNA, enzymes, and catalytic redox polyions assembled layer-by-layer on electrodes have also provided active elements for sensors for screening the toxicity of chemicals and their metabolites, and for detecting oxidative stress.¹⁰

Heme proteins or enzymes, such as Mb, horseradish peroxidase (HRP) and cyt P450s, contain iron heme prosthetic groups in their polypeptide pockets and can catalyze the oxidation of substrates when activated by hydrogen peroxide or other peroxides.^{7a,11,12} In particular, HRP and cyt P450_{cam} activated by peroxide have provided excellent product yields with high stereo- and enantio-selectivities.^{13–15} At the same time, Mb or HRP immobilized on electrodes demonstrates good electrocatalytic activity toward H_2O_2 , and can be used to detect H_2O_2 .¹⁶ Determination of H_2O_2 is of great significance in pharmaceutical, clinical, industrial and environmental analyses, in food processing industries, and in bioanalytical chemistry.¹⁷ Numerous H_2O_2 biosensors based on the direct electrochemistry of heme enzymes immobilized in films have been developed and studied.^{18,19} However, peroxidase enzymes are prone to inactivation from slow attack of hydrogen peroxide in the absence of other reductant substrates or when the ratio of H_2O_2 /enzyme is large.^{20,21} There are several irreversible damage pathways for heme proteins, including the oxidation of the heme ring and release of free iron,^{22–26} the intramolecular cross-linking of amino acid residues, and dimerization or oligomerization of proteins resulting from intra- and inter-molecular radical reactions,^{27–32} formation of amino acid peroxy radicals,²⁹ or cleavage of the polypeptide backbone.²⁹ For HRP, another possibility is reaction of ferryl intermediate with additional H_2O_2 , yielding the nonfunctional verdohemoprotein P-670.²¹

Two strategies have been used to protect enzymes from damage by peroxide: (1) an impermeable barrier on the biosensor surface to prevent H_2O_2 from diffusing into the films;³³ (2) a functional protection layer on the biosensor surface to react with H_2O_2 to protect enzymes from damage.^{34–36} The latter approach shows obvious advantages in selectivity and efficiency, since with the former method the barrier may also completely isolate the device from substrates. In this aspect, Shchukin and co-workers³⁵ assembled layer-by-layer films containing catalase on the outermost surface of polyelectrolyte capsule microcontainers that encapsulated bovine serum albumin (BSA). The protection layers of catalase effectively decomposed H_2O_2 into H_2O and O_2 ,^{37–39} and prevented BSA from oxidation by H_2O_2 in solution. Using similar approach, Shutava and co-workers³⁶ recently investigated the protective effect of a catalase layer located at different depths in hemoglobin (Hb)/polystyrene sulfonate (PSS) multilayer films on the damage of Hb by H_2O_2 using UV-vis spectroscopy.

In the present report, films of Mb and HRP were firstly assembled layer-by-layer with hyaluronic acid (HA) respectively to form {protein/HA}_n multilayer films on pyrolytic graphite (PG) electrodes. The assembly of {Mb/HA}_n layer-by-layer films and the direct electrochemistry of Mb in the films were studied by our group recently.^{40,41} Herein, the electroactive Mb or HRP in these films was used as a probe to monitor and characterize the extent of protein damage by H_2O_2 and the protective effect of outside catalase layers. At the same time, the electrocatalytic reduction of H_2O_2 by Mb or HRP at {protein/HA}_n film electrodes and the effect of catalase protection layers on the electrocatalysis of H_2O_2 were also investigated. Since H_2O_2 can act as both the damage reagent and the substrate in enzyme electrocatalysis, we investigated interactions between these two functions in the above films. While the protection of inner proteins by outer catalase layers was studied previously,³⁶ to the best of our knowledge, the interplay between catalase-initiated peroxide decomposition and catalytic peroxidase activation in enzyme films has not been addressed up to now. Results described provide a promising and effective strategy to protect biosensors from damage by

H₂O₂, while at the same time allow the sensitive detection of H₂O₂ by electrocatalysis based on the direct electrochemistry of enzymes.

Experimental Section

1. Chemicals

Equine skeletal muscle myoglobin (Mb, MW 17 800), bovine liver catalase (2150 units mg⁻¹ protein, MW 240 000), and chitosan (the degree of acetylation is less than 15%, MW ~ 200 000) were purchased from Sigma. Horseradish peroxidase (HRP, MW 42 000) was purchased from Shanghai Xueman Biotechnology. Hyaluronic acid (HA, MW ~ 400 000) was purchased from Fluka. Hydrogen peroxide (H₂O₂, 30%) was obtained from Beijing Chemical Plant. All other chemicals were reagent grade. All solutions were prepared with twice-distilled water. Buffers were 0.1 M sodium acetate (pH 5.0, containing 0.3 M NaCl) and potassium dihydrogen phosphate (pH 7.0, containing 0.1 M KCl), the pH of which was adjusted with HCl or NaOH solutions.

2. Film assembly

Multilayer films were grown on a basal plane pyrolytic graphite disk (PG, Advanced Ceramics, geometric area 0.16 cm²) electrode. Prior to use, PG electrodes were abraded with metallographic sandpaper (320 grit) while flushing with pure water. The electrodes were then ultrasonicated in pure water for 30 s. For assembly of {Mb/HA}_n layer-by-layer films, the PG electrodes were first immersed successively in 1 mg mL⁻¹ solutions of chitosan and HA (both at pH 5.0 containing 0.3 M NaCl) for 20 min with intermediate water washing, forming a chitosan/HA precursor bilayer on the PG surface. Chitosan is a weak polybase and its conjugated acid has pK_a of ~ 6,⁴² and HA is a weak polyacid with pK_a = 2.9.⁴³ Thus, at pH 5.0, positively charged chitosan and negatively charged HA can be adsorbed on the electrode surface via electrostatic interactions between them. The PG/chitosan/HA electrodes were then alternately immersed in Mb and HA solutions (both at 1 mg mL⁻¹ and pH 5.0 containing 0.3 M NaCl) for 20 min with intermediate water washing and nitrogen stream drying until the desired number of bilayers (n) was reached. {Catalase/HA}_n and {HRP/HA}_n films were assembled with the similar procedure but using 5 mg mL⁻¹ catalase solutions and 3 mg mL⁻¹ HRP solutions at pH 5.0 containing 0.3 M NaCl, respectively. For assembly of the protection layers, layers of catalase and HA were adsorbed alternately on the surface of {Mb/HA}_n or {HRP/HA}_n films with the same method. The protection layers were always terminated with catalase, designated as {Mb/HA}_n/ {catalase/HA}_{m-1}/catalase or {HRP/HA}_n/ {catalase/HA}_{m-1}/catalase. Inactivated catalase (inact-catalase) was prepared by heating a 5 mg mL⁻¹ catalase solution to 70 °C for 5 h,³⁶ and the inactivation of catalase was confirmed by UV-vis spectroscopy.

3. Procedures

The protein film electrodes were characterized by cyclic voltammetry (CV) in pH 7.0 buffers containing no proteins. For damage/protection experiments, the {protein/HA}_n or {protein/HA}_n/ {catalase/HA}_{m-1}/catalase films were first incubated in H₂O₂ solutions for a certain time. After being washed with water and dried in air, the film electrodes were transferred in pH 7.0 buffers for CV scans. The CV reduction peak current ratio I_t/I₀ was usually used to reflect the extent of protein damage in the films, where I₀ and I_t represented the CV reduction peak currents of the Mb or HRP films before and after incubation in H₂O₂ solutions for t min, respectively.

For electrocatalytic experiments, H₂O₂ was added in pH 7.0 buffer solutions, and CVs at protein film electrodes were performed immediately. For a certain film electrode, only the first cycle of CV was recorded, and all the electrocatalytic experiments were finished within < 4

min to minimize incubation time in H_2O_2 solutions. In order to decrease the variation between electrode-to-electrode or film-to-film induced by the difference in electrode area and roughness, catalytic efficiency, expressed as the ratio of CV reduction peak current in the presence (I_c) and absence (I_d) of H_2O_2 , I_c/I_d , was used to characterize the films.

4. Apparatus

A CHI 660A electrochemical workstation (CH Instruments) was used for electrochemical measurements. A saturated calomel electrode (SCE) was used as the reference, a platinum wire as the counter electrode, and the PG disk electrode coated with films as the working electrode. Buffers were purged with high-purity nitrogen for at least 15 min prior to electrochemical measurements, and a nitrogen environment was maintained during the CV scans. All experiments were performed at ambient temperature of 20 ± 2 °C.

Results

1. Assembly of {protein/HA}_n layer-by-layer films

With the isoelectric point at 6.8 for Mb,⁴⁴ 5.8 for catalase,⁴⁵ and 8.9 for HRP,⁴⁶ all these proteins have net positive surface charges at pH 5.0. HA is negatively charged at pH 5.0 since its pK_a is 2.9.⁴³ Thus, oppositely charged proteins and HA can be assembled into {protein/HA}_n layer-by-layer films mainly by electrostatic interactions. The successful assembly of {Mb/HA}_n films and direct electrochemistry of Mb in the films were reported in our previous work.^{40,41} This report focused on evaluating the protective effect of exterior catalase layers on the peroxidase activity of {protein/HA}_n films. First, {protein/HA}_n films were assembled on PG electrodes. When the films were immersed in pH 7.0 buffers containing no proteins, and CVs were performed, a well-defined and quasi-reversible reduction-oxidation peak pair was observed with midpoint potentials at about -0.34 V vs SCE for {Mb/HA}_n and {HRP/HA}_n films, respectively, and at -0.47 V for {catalase/HA}_n (Supporting Information Figure S1). These redox peak pairs are characteristic of protein heme $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ redox couples in films.^{7,40,41,47}

In general, CV peak potentials of redox proteins in thin films depend on the interactions of the proteins with the film material, the electrode material, and the identity of the protein.^{7c,47} In the present case, the different midpoint potentials for different {protein/HA}_n films do not reflect redox potentials of proteins in solution,^{7,47} as also observed in the previous work.⁴⁸ The peak currents increased with the number of protein/HA bilayers (n) up to a certain saturation value (n_{max}), confirming the successful assembly of the layer-by-layer films. The value of n_{max} was 10 for {Mb/HA}_n, 5 for {catalase/HA}_n, and 7 for {HRP/HA}_n films, respectively. Beyond n_{max} , peak currents reached a steady state and did not increase further. These results indicate that while the assembly of {protein/HA}_n films can be extended to a much larger number of bilayers with a regular or exponential growth mode,⁴⁰ the electroactive proteins are restricted to the 5 – 10 bilayers closest to the electrode surface.^{40,41} Similar results were observed in other heme protein layer-by-layer films.^{7,8}

2. Protection of peroxidases from damage by H_2O_2 by using outer catalase layers

Reduction and oxidation peak currents for the {Mb/HA}₁₀ films decreased significantly after incubation in 1 mM H_2O_2 for 60 min (Figure 1A), suggesting that Mb in the films was partially damaged by H_2O_2 as has been observed in similar films previously.⁴⁹ However, when five layers of catalase were assembled on the top of {Mb/HA}₁₀ films, incubation in 1 mM H_2O_2 for the same time gave CV peaks that decreased only slightly. This result suggests that catalase layers protect Mb in the inner layers from oxidative damage by H_2O_2 . Figure 1B shows the CV reduction peak current ratio (I_t/I_0) for {Mb/HA}₁₀ and {Mb/HA}₁₀{catalase/HA}₄/catalase films with incubation time, where I_0 and I_t are the reduction peak currents before and

after incubation in 1 mM H_2O_2 solutions for t min, respectively. For $\{\text{Mb}/\text{HA}\}_{10}$ films, I_t/I_0 decreased drastically with incubation time in the initial 30 min followed by a slower decay. After 60 min of incubation, the decrease of I_t/I_0 continued at a smaller rate. The exponential decay was also observed for damage of hemoglobin by H_2O_2 in similar films using UV-vis spectroscopy.³⁶ For $\{\text{Mb}/\text{HA}\}_{10}/\{\text{catalase}/\text{HA}\}_4/\text{catalase}$ films, however, the decrease of I_t/I_0 with incubation time was barely observed.

In these experiments, $\{\text{Mb}/\text{HA}\}_{10}$ films with $n = 10$ were used because at $n = 10$ CV gave the largest response with the maximum amount of electroactive Mb (Γ_{max}^*). In addition, optimization studies showed that $n = 10$ films gave the largest difference in Mb damage between $\{\text{Mb}/\text{HA}\}_n$ and $\{\text{Mb}/\text{HA}\}_n/\{\text{catalase}/\text{HA}\}_4/\text{catalase}$ films.

Damage of $\{\text{HRP}/\text{HA}\}_n$ films by H_2O_2 and the effect of catalase protection layers were also investigated. Since the CV response of $\{\text{HRP}/\text{HA}\}_n$ films was much smaller than that of their Mb counterpart, background-subtracted CVs were used. The results showed that $\{\text{HRP}/\text{HA}\}_{10}$ films could also be damaged by H_2O_2 (Figure 2A), and the I_t/I_0 ratio decreased with incubation time in H_2O_2 solutions (Figure 2B). After incubation in 1 mM H_2O_2 solutions for 60 min, the reduction and oxidation peak currents for HRP $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ redox couple in the films decreased by about 33%. When five catalase layers were deposited on the top of the $\{\text{HRP}/\text{HA}\}_{10}$ films, both CV responses for catalase $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ couple at about -0.47 V and HRP $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ couple at about -0.35 V could be observed. After incubation in 1 mM H_2O_2 solutions for 60 min, the reduction peak current of HRP $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ couple for $\{\text{HRP}/\text{HA}\}_{10}/\{\text{catalase}/\text{HA}\}_4/\text{catalase}$ films was almost unchanged (Figure 2A), and the I_t/I_0 ratio barely decreased with the incubation time (Figure 2B), all indicating that catalase layers can protect HRP in the inner layers from oxidative damage by H_2O_2 .

The number of catalase layers on the top of the $\{\text{Mb}/\text{HA}\}_n$ films had a significant influence on the protection of Mb (Figure 3). Generally, the more catalase layers, the less the peak current ratio (I_t/I_0) decreased. This is understandable since more catalase in the outermost layers would have higher capability to decompose H_2O_2 and provide more protection for Mb in the inner layers. For example, when $\{\text{Mb}/\text{HA}\}_{10}$ films were incubated in 1 mM H_2O_2 solutions for 60 min, the reduction peak current of Mb decreased by about 37%, while for $\{\text{Mb}/\text{HA}\}_{10}/\{\text{catalase}/\text{HA}\}_4/\text{catalase}$ films, only 10% decrease was observed. If the number of catalase layers was increased to 5, the decrease in Mb peak current was only 3%.

The Mb damage by H_2O_2 was also influenced by H_2O_2 concentration ($C_{\text{H}_2\text{O}_2}$) in incubation solutions (Supporting Information Figure S2). With the same incubation time (60 min) in H_2O_2 solutions, the reduction peak current ratio (I_{60}/I_0) for $\{\text{Mb}/\text{HA}\}_{10}$ films decreased with $C_{\text{H}_2\text{O}_2}$, suggesting that higher concentration of H_2O_2 leads to a higher damage extent of Mb. With five additional catalase layers on the $\{\text{Mb}/\text{HA}\}_{10}$ film surface, the protection effect made the I_{60}/I_0 ratio become much larger, as expected. However, the general decreasing trend of I_{60}/I_0 ratio with $C_{\text{H}_2\text{O}_2}$ was also observed for the $\{\text{Mb}/\text{HA}\}_{10}/\{\text{catalase}/\text{HA}\}_4/\text{catalase}$ films. After incubation in H_2O_2 solution for 60 min, the I_{60}/I_0 ratio remained 97% of its initial value in 1 mM H_2O_2 , but decreased to 81% in 10 mM H_2O_2 and 72% in 100 mM H_2O_2 . This indicates that while 5 catalase layers can essentially protect the inner Mb from damage and maintain the electroactivity of Mb for at least 60 min in 1 mM H_2O_2 , they are not as effective for the protection in H_2O_2 solutions with higher concentration. When the concentration of H_2O_2 solution was lower than 1 mM, while the inactivation of Mb in the films could still be observed, the Mb damage rate became much smaller, and the protection effect of outer catalase layers became more pronounced.

3. Comparison of catalase and inactivated catalase layers in protection of Mb

Two factors should be considered in protection of proteins in {protein/HA}_n films from damage by H₂O₂. On the one hand, the catalase in outer layers on the surface of {Mb/HA}_n films may decompose H₂O₂ before H₂O₂ can reach the inner layers of Mb. On the other hand, the additional catalase layers may constitute a diffusion barrier to H₂O₂ entering the inner {Mb/HA}_n layers. In order to distinguish which factor plays the key role in the protection, inactivated catalase (inact-catalase) was prepared and deposited on the top of the {Mb/HA}₁₀ films. The influence of incubation time in H₂O₂ solutions on Mb decay was investigated and compared with that of {Mb/HA}₁₀/ {catalase/HA}₄/catalase films (Figure 4). Inactivation of catalase resulted in the release of heme prosthetic groups from the protein, which was confirmed by the disappearance of the Soret band at about 403 nm after the catalase solution was heated to 70 °C for 5 h.³⁶ Thus, inact-catalase lost its enzymatic activity and could not decompose hydrogen peroxide. The I_t/I₀ ratio for {Mb/HA}₁₀/ {inact-catalase/HA}₄/inact-catalase films showed a pronounced decrease with incubation time, with a similar trend to that of the unprotected Mb films, but much different from that of {Mb/HA}₁₀/ {catalase/HA}₄/catalase films. The results indicate that for the small molecule H₂O₂, the diffusion barrier from additional {catalase/HA}₄/catalase layers is almost negligible. The protection effect of catalase layers is mainly related to the enzyme activity of catalase for H₂O₂ decomposition.

4. Influence of catalase layers on electrocatalytic reduction of H₂O₂ by {protein/HA}_n films

Hydrogen peroxide can be electrocatalytically reduced by Mb or HRP in films, and this process activates the enzymes for substrate oxidation.^{11,12} This activation is manifested electrochemically by a catalytic reduction peak increase when H₂O₂ is added.⁴⁹ For example, in pH 7.0 buffers containing 0.1 mM H₂O₂, the {Mb/HA}₂₅ film electrode showed a significant increase of reduction peak at about -0.35 V compared with that in the absence of H₂O₂, accompanied by the disappearance of the MbFe^{II} oxidation peak (Figure 5A). The reduction peak current increased with the concentration of H₂O₂. Catalytic efficiency, expressed as the ratio of reduction peak current in the presence (I_c) and absence of H₂O₂ (I_d), I_c/I_d, decreased with the increase of scan rate (Supporting Information Figure S3). These are typical characteristics of electrochemical catalysis,⁵⁰ suggesting that Mb catalyzes the reduction of H₂O₂ electrochemically as is well known.^{7,18} To make sure that the reaction time of H₂O₂ with proteins in the films was identical and the results were reproducible, CVs were performed immediately after the injection of H₂O₂. For films with one outer catalase layer, the catalytic reduction peak of H₂O₂ at the same position was observed but it was slightly smaller than that for the {Mb/HA}₂₅ films. When two catalase layers were assembled on the surface of {Mb/HA}₂₅ films, a further decrease of the catalytic reduction peak was seen. This is understandable since the actual amount of H₂O₂ that enters into the films decreases because it is decomposed by the catalase layers.

Herein, {Mb/HA}₂₅ films with n = 25 instead of n = 10 were used in order to minimize the influence of electroactive catalase in electrocatalysis. In the n = 25 films, catalase in the protection layers was confirmed to be inactive in both electrochemistry and electrocatalytic reduction of H₂O₂. The nominal thickness of {Mb/HA}₂₅ films was estimated to be about 390 nm.⁴⁰ The distance between catalase molecules in the outermost layers and the underlying PG electrodes was thus too large for catalase to accept electrons. To further confirm this viewpoint, {catalase/HA}₆ films were assembled on PG surface, and electrocatalysis of H₂O₂ at {catalase/HA}₆ films was tested by CV and compared with that at {Mb/HA}₂₅/ {catalase/HA}_{m-1}/catalase films. Catalase in the {catalase/HA}₆ films showed good electrocatalytic property toward reduction of H₂O₂ (Figure 5B). However, the peak potential of {catalase/HA}₆ films was at about -0.50 V, more negative than that of {Mb/HA}₂₅ and {Mb/HA}₂₅/ {catalase/HA}_{m-1}/catalase films (-0.35 V). This suggests that in the {Mb/HA}₂₅/ {catalase/HA}_{m-1}/catalase films, it is Mb in the inner layers closest to PG surface, and not the catalase in the

outermost layers, that electrocatalyzes the reduction of H_2O_2 . The rather thick Mb films with $n = 25$ would also be helpful to rule out the interference of oxygen produced by reaction of H_2O_2 with catalase in outer layers, which will be discussed in more detail in Discussion section.

Figure 6 shows the effect of number of catalase layers (m) on catalytic efficiency (I_c/I_d) for $\{\text{Mb}/\text{HA}\}_{25}/\{\text{catalase}/\text{HA}\}_{m-1}/\text{catalase}$ films in electrocatalysis of H_2O_2 . When the concentration of H_2O_2 was 0.1 mM, the I_c/I_d ratio decreased initially and then increased with m , passing through a minimum value at $m = 2$, indicating that the influence of m on the ratio of I_c/I_d has a complicated character. There are two opposite factors that influence the I_c/I_d ratio in the electrocatalysis. On the one hand, the decomposition of H_2O_2 by outermost catalase layers reduces the actual concentration of H_2O_2 that can enter the films, and the decrease in substrate concentration leads to the decrease of catalytic reduction peak current. On the other hand, the decrease of H_2O_2 concentration in the films caused by catalase layers reduces the extent of Mb damage, and the smaller decrease in catalyst concentration in the films would result in an increase of catalytic reduction peak current. When $m \leq 2$, the substrate concentration factor is considered to be the predominant force, while at $m > 2$, the protection factor may become dominant. The minimum value of I_c/I_d can thus be rationalized by involving these two opposing factors. It is also understandable that with different concentration of H_2O_2 in catalysis, the minimum ratio of I_c/I_d might occur at different m value.

Similar to the $\{\text{Mb}/\text{HA}\}_n$ films, the $\{\text{HRP}/\text{HA}\}_n$ films also electrochemically catalyzed the reduction of H_2O_2 at a suitable H_2O_2 concentration, showing a typical catalytic behavior,⁵⁰ i.e. an increase of reduction peak current accompanied by the disappearance of the oxidation peak (Supporting Information Figure S4). The catalase protection layers assembled on the top of $\{\text{HRP}/\text{HA}\}_n$ films also affected the electrocatalytic performance, but demonstrated a different trend from that of Mb films under the same condition. For example, in the same 0.1 mM H_2O_2 solution, the additional catalase layers resulted in the increase instead of decrease of catalytic reduction peak currents (Figure S4). This suggests that the influence of H_2O_2 concentration on competition between catalysis and damage may be different for Mb and HRP films. For $\{\text{HRP}/\text{HA}\}_{25}$ films, when the H_2O_2 concentration was 0.1 mM, the decomposition of H_2O_2 by outermost catalase layers reduced the actual concentration of H_2O_2 that entered the films, and decreased HRP damage. When this trend became the predominant force, the smaller decrease in HRP surface concentration in the films would result in the increase of catalytic reduction peak currents.

5. Interplay of catalase protection layers and H_2O_2 concentration on electrocatalysis

Generally, when the concentration of H_2O_2 was ≤ 0.1 mM, the extent of Mb damage by H_2O_2 in $\{\text{Mb}/\text{HA}\}_n$ films is limited, and the electrocatalytic reduction of H_2O_2 by Mb in the $\{\text{Mb}/\text{HA}\}_n$ films was observed, as in Figures 5A and 6. On the contrary, when the concentration of H_2O_2 was relatively high, such as at 1 mM, the Mb damage by H_2O_2 in $\{\text{Mb}/\text{HA}\}_n$ films is more extensive. The damaged Mb in the films lost its enzyme-like and electrochemical activity to a great extent, and demonstrated little electrocatalytic response for reduction of H_2O_2 (Figure 7). With catalase layers assembled on the surface of $\{\text{Mb}/\text{HA}\}_{25}$ films, the electrocatalytic responses to H_2O_2 were greatly improved. The catalytic reduction peak current of $\{\text{Mb}/\text{HA}\}_{25}/\{\text{catalase}/\text{HA}\}_4/\text{catalase}$ films became much higher than that of $\{\text{Mb}/\text{HA}\}_{25}$ films, and the peak shape turned into more like a typical electrocatalytic process. It is because of the protection of catalase layers that the considerable amount of Mb in the films could still function as the catalyst in electrocatalysis even at relatively high H_2O_2 concentration.

Further studies demonstrated that the dependence of catalytic efficiency (I_c/I_d) on H_2O_2 concentration was distinctly different between $\{\text{Mb}/\text{HA}\}_{25}$ and $\{\text{Mb}/\text{HA}\}_{25}/\{\text{catalase}/\text{HA}\}_4/\text{catalase}$ films (Figure 8A). For $\{\text{Mb}/\text{HA}\}_{25}$ films, the I_c/I_d ratio initially increased with $C_{\text{H}_2\text{O}_2}$ up to 0.3 mM, and then declined with H_2O_2 concentration at $C_{\text{H}_2\text{O}_2} > 0.4$ mM. However,

for {Mb/HA}₂₅/catalase films, the I_c/I_d ratio could increase with $C_{H_2O_2}$ up to 1 mM, and then tended to decline. This is a very interesting result, especially from the view of biosensing or analytical chemistry. In determination of H_2O_2 by electrocatalysis with {Mb/HA}_n film electrodes, the deposition of additional catalase protection layers on the surface of {Mb/HA}_n films would significantly increase the concentration range of H_2O_2 to be determined.

Comparing curves a and b in Figure 8A, we found that the I_c/I_d values of {Mb/HA}₂₅/catalase films were always smaller than those of {Mb/HA}₂₅ films at $C_{H_2O_2} \leq 0.4$ mM, but when $C_{H_2O_2} > 0.4$ mM, the I_c/I_d ratios were always larger than those of {Mb/HA}₂₅ films. A possible explanation is that at lower $C_{H_2O_2}$, the damage of Mb in the films by H_2O_2 was very limited within the time scale of CV scans, and the {Mb/HA}₂₅ films demonstrated good electrocatalysis for H_2O_2 reduction. However, the decomposition of H_2O_2 by additional catalase layers would decrease the actual concentration of H_2O_2 that entered the films and thus decreased the catalytic efficiency. In the higher H_2O_2 concentration range, the unprotected {Mb/HA}₂₅ films were damaged to a great extent, leading to a drastic decrease in the amount of electroactive Mb in the films and lower values of I_c/I_d . However, the additional catalase layers on the {Mb/HA}₂₅ film surface protected the inner Mb molecules from damage by H_2O_2 , leading to the increase of catalyst concentration compared with that of unprotected {Mb/HA}₂₅ films, and resulting in higher ratios of I_c/I_d .

For {HRP/HA}₂₅ and {HRP/HA}₂₅/catalase films, the general trend of dependence of I_c/I_d on $C_{H_2O_2}$ was similar to that of their Mb counterparts (Figure 8B). For {HRP/HA}₂₅ films, the catalytic efficiency initially increased with $C_{H_2O_2}$ up to 0.2 mM and then declined, showing a bell-like shape. In Saveant's work on mediated HRP reduction, a bell-shaped calibration curve in I_{cat} vs $[H_2O_2]$ relationship was also observed.⁵¹ The deposition of additional catalase layers on the surface of {HRP/HA}₂₅ films extended the maximum of I_c/I_d to ~0.3 mM. However, the $C_{H_2O_2}$ at maximum I_c/I_d for HRP films was smaller than that for the Mb films, which is probably related to the smaller amount of electroactive proteins in {HRP/HA}₂₅ films than that in {Mb/HA}₂₅ films. For example, integration of CV reduction peak of the {Mb/HA}₂₅ films gave the surface concentration of electroactive Mb (Γ^*) of $(2.44 \pm 0.38) \times 10^{-10}$ mol cm⁻², about 12 times larger than that of {HRP/HA}₂₅ films $((0.21 \pm 0.03) \times 10^{-10}$ mol cm⁻²). However, the catalytic efficiency for reduction of H_2O_2 by HRP in the films was much larger than that in Mb films, which was also observed in the previous work.⁵² This suggests that the hydrogen peroxide that enters the HRP films is reduced more efficiently and will give larger currents per nmol of enzyme than that for the corresponding Mb films. Different amounts of electroactive protein in the films and different catalytic efficiencies of Mb and HRP are responsible for differences between Figure 8A and 8B, although the diminished H_2O_2 concentration resulting from the H_2O_2 decomposition by catalase should be identical for the two protein films with the same number of outer catalase layers.

Discussion

Results above demonstrate that outer layers of catalase on layer-by-layer films of peroxidases effectively protect the enzymes from damage by H_2O_2 while allowing efficient electrochemical catalysis to proceed. This approach has advantages over a previous method of crosslinking the enzymes in a poly(L-lysine) matrix, since crosslinking lowered the current density of the coated electrode,⁴⁹ while the catalase approach actually increased current density at higher peroxide concentrations (Figure 8).

Mb and HRP in the {protein/HA}_n films can be damaged by H_2O_2 at relatively high concentration (Figures 1 and 2) and the damage extent increases with H_2O_2 concentration and

incubation time (Figures S2 and 3). At the same time, the {protein/HA}_n films also demonstrate good electrocatalytic activity toward reduction of H₂O₂ at lower concentration (Figures 5A and S4). The reactions between proteins and hydrogen peroxide demonstrate two different pathways: (1) Mb or HRP can be damaged irreversibly by H₂O₂ and lose its bioactivity and electroactivity; (2) Mb or HRP can electrochemically catalyze the reduction of H₂O₂ at electrodes.

The reaction of ferric myoglobin (MbFe^{III}) with H₂O₂ yields an intermediate similar to a peroxidase Compound I (*MbFe^{IV}=O), a two-equivalent ferryl-oxo radical form compared with the resting state MbFe^{III}.^{53–57} For the electrocatalysis pathway, in the absence of other reductants, H₂O₂ may act as a reductant and be oxidized by Compound I, giving O₂, H₂O and MbFe^{III} as products.^{7a,18a,e,58} When MbFe^{III} is electrochemically reduced to MbFe^{II} at electrode, a fast reaction of MbFe^{II} with oxygen occurs, producing MbFe^{II}-O₂. MbFe^{II} is an oxygen carrier in biological systems and has a strong affinity to O₂ with a large rate constant of $2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for the formation of MbFe^{II}-O₂ at neutral pH.⁵⁹ MbFe^{II}-O₂ can then undergo electrochemical reduction at the potential of MbFe^{III} reduction, producing H₂O₂ and MbFe^{II}.^{58a}

The electrocatalytic reduction of O₂ was also observed at {Mb/HA}_n film electrodes at the same peak potential as that of H₂O₂ with very similar peak shape (Supporting Information Figure S5), as has been reported for other Mb films.^{18a,e,58} The electrocatalytic mechanism of HRP film electrodes toward hydrogen peroxide is similar to that of Mb films,^{18a,e,58d} which was confirmed by the similarity of catalytic CV behaviors of {HRP/HA}_n and {Mb/HA}_n films (Figures 5A and S4).

Inactivation of heme proteins by hydrogen peroxide in the absence of other substrates, or in the presence of large H₂O₂/substrate concentration ratio, has been studied extensively, and the mechanisms are complicated. No matter what type of inactivation pathway for Mb or HRP is, the inactivation of these two proteins by excessive H₂O₂ leads to the loss of the electroactivity of the proteins, as confirmed by the decrease in CV peak currents of protein heme Fe^{III}/Fe^{II} couple and the poor electrocatalytic behaviors at high concentration of H₂O₂ (Figures 1, 2 and 7). At the {protein/HA}_n film electrodes, the electrocatalytic reduction of H₂O₂ and the protein damage reaction compete with each other. In the relatively low H₂O₂ concentration range, electrocatalysis may be predominant, while at higher H₂O₂ concentration, the inactivation of enzymes by H₂O₂ may become more serious when catalytic activity of the enzyme becomes saturated.

Like HRP and Mb, catalase is also a heme enzyme, and is present in almost all-aerobic organisms.⁶⁰ Catalase has four identical subunits, each containing a single heme prosthetic group as its active center. As a catalyst, catalase effectively decomposes H₂O₂ into O₂ and H₂O.^{37–39} Thus, under the normal physiological conditions in living systems, catalase controls the H₂O₂ concentration so that it does not reach the high and toxic level that can bring about oxidative damage to cells. The mechanism of H₂O₂ decomposition by catalase is similar to the first two steps of reaction between Mb and H₂O₂, in which H₂O₂ first serves as an oxidant to oxidize catalase from its ferric or resting state to a Compound I form, and H₂O₂ then acts as a reductant to reduce catalase from its Compound I form to its ferric form.^{37–39,61–63} In this way, catalase in {catalase/HA}_{m-1}/catalase layers on the top of {protein/HA}_n films decomposes H₂O₂ and protects the proteins in the inner layers from damage by H₂O₂, while still lets in just enough H₂O₂ to facilitate electrocatalytic activation. Herein, the interference of oxygen produced by reaction of catalase with H₂O₂ should not be very serious in electrocatalysis of H₂O₂ with inner {protein/HA}_n layers, since the continuous bubbling of nitrogen during the whole experiments and rather thick inner layers (n = 25) would greatly limit the contact of the oxygen with electroactive Mb or HRP. Nevertheless, the interference

from the oxygen generated by reaction of catalase and H_2O_2 could not be ruled out completely, especially when H_2O_2 concentration was relatively high. This could provide an alternative explanation of why the catalytic efficiency (I_c/I_d) toward H_2O_2 increased with the number of catalase layers (m) at relatively large m for $\{\text{Mb}/\text{HA}\}_{25}/\{\text{catalase}/\text{HA}\}_{m-1}/\text{catalase}$ films in 0.1 mM H_2O_2 solutions (Figure 6).

The damage and electrocatalysis of Mb or HRP in $\{\text{protein}/\text{HA}\}_n$ films with H_2O_2 and the effect of catalase protection layers are illustrated schematically in Scheme 1. In addition, according to the literature,^{64–68} catalase can also be inactivated by hydrogen peroxide, especially at rather high H_2O_2 concentration.

During the incubation of $\{\text{protein}/\text{HA}\}_n$ films in H_2O_2 solutions, the protein inactivation or damage was a relatively slow and time-dependent process (Figures 1–3). However, under the condition of CV potential scan, the inactivation of proteins was greatly accelerated (Figure 7, curve b). In order to confirm the accelerating effect of potential scan on the damage of proteins in $\{\text{protein}/\text{HA}\}_n$ films by H_2O_2 , the following comparison experiments were designed and performed. 20 cycles of continuous CV scans at 0.2 V s^{-1} between 0.1 and -0.8 V for $\{\text{Mb}/\text{HA}\}_{10}$ films in 0.1 mM H_2O_2 demonstrated the successive decrease of the catalytic reduction peak with scan cycle (Supporting Information Figure S6A). After the CV scan in H_2O_2 solution was finished, the $\{\text{Mb}/\text{HA}\}_{10}$ film electrode was transferred into pH 7.0 blank buffers and examined by CV again. While the peak pair of Mb $\text{Fe}^{\text{III}}/\text{Fe}^{\text{II}}$ couple was still observed, the peak heights were obviously lower than those before the test (Figure S6B). As a control, an $\{\text{Mb}/\text{HA}\}_{10}$ film was incubated in 0.1 mM H_2O_2 solution for 3 min, the same period of time as the 20 cycles of CV scan at 0.2 V s^{-1} , and then transferred into blank buffers for CV test (Figure S6B). The CV response of the control was almost unchanged compared with that of untreated Mb films. These results confirm that the potential scan can accelerate the protein damage by H_2O_2 . It is possible that the heme Fe^{II} form of Mb may be more easily inactivated by H_2O_2 than its Fe^{III} counterpart at the resting state, as observed with Hb.²⁶ The extra layers of catalase on the surface of $\{\text{Mb}/\text{HA}\}_{10}$ films could also protect the inner Mb from damage by H_2O_2 under CV scans.

Conclusion

Damage of Mb or HRP in $\{\text{protein}/\text{HA}\}_n$ films by H_2O_2 increased with the concentration of H_2O_2 and the incubation time in H_2O_2 solutions, and was accelerated by potential scanning. Catalase layers deposited on the surface of $\{\text{protein}/\text{HA}\}_n$ films decomposed H_2O_2 effectively and protected the inner proteins from oxidative damage by H_2O_2 , thus influencing electrocatalytic performance for reduction of H_2O_2 . Protection with catalase layers increased the linear concentration range of H_2O_2 that can be determined by electrocatalysis, an issue of practical significance in biosensing. This mode of protection, based on the selective and controllable decomposition of damage agents as they diffuse into bioactive films, offers a new and effective way to protect many biosensors from damage, particularly those that utilize H_2O_2 . This work provides a general and promising methodology to construct the third-generation enzyme biosensors based on the direct electrochemistry of enzymes that can sensitively detect H_2O_2 at relatively low concentration with extended life-span due to effective protection of the enzymes from damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) CVs at 0.2 V s^{-1} in pH 7.0 buffers for (a) untreated $\{\text{Mb/HA}\}_{10}$ and (b) untreated $\{\text{Mb/HA}\}_{10}/\{\text{catalase/HA}\}_4/\text{catalase}$ films; (c) $\{\text{Mb/HA}\}_{10}$ and (d) $\{\text{Mb/HA}\}_{10}/\{\text{catalase/HA}\}_4/\text{catalase}$ films after being incubated in 1 mM H_2O_2 solutions for 60 min. (B) Influence of the incubation time (t) in 1 mM H_2O_2 solutions on CV reduction peak current ratio (I_t/I_0) for (a) $\{\text{Mb/HA}\}_{10}$ and (b) $\{\text{Mb/HA}\}_{10}/\{\text{catalase/HA}\}_4/\text{catalase}$ films.

Figure 2.

(A) Background-subtracted CVs at 0.2 V s⁻¹ in pH 7.0 buffers for (a) untreated {HRP/HA}₁₀ and (b) untreated {HRP/HA}₁₀/catalase films; (c) {HRP/HA}₁₀ and (d) {HRP/HA}₁₀/catalase films after being incubated in 1 mM H₂O₂ solutions for 60 min. (B) Influence of the incubation time (t) in 1 mM H₂O₂ solutions on CV reduction peak current ratio (I_t/I₀) for (a) {HRP/HA}₁₀ and (b) {HRP/HA}₁₀/catalase films.

Figure 3.

Influence of incubation time (t) on CV reduction peak current ratio (I_t/I_0) for (a) {Mb/HA}₁₀, (b) {Mb/HA}₁₀/catalase, (c) {Mb/HA}₁₀/catalase/HA/catalase, (d) {Mb/HA}₁₀/catalase/HA₂/catalase, (e) {Mb/HA}₁₀/catalase/HA₃/catalase, and (f) {Mb/HA}₁₀/catalase/HA₄/catalase films in 1 mM H₂O₂ solutions. CVs were performed at 0.2 V s⁻¹ in pH 7.0 buffers after the incubation.

Figure 4.

Influence of incubation time (t) on CV reduction peak current ratio (I_t/I_0) for (a) $\{\text{Mb}/\text{HA}\}_{10}$, (b) $\{\text{Mb}/\text{HA}\}_{10}/\{\text{inact-catalase}/\text{HA}\}_4/\text{inact-catalase}$, and (c) $\{\text{Mb}/\text{HA}\}_{10}/\{\text{catalase}/\text{HA}\}_4/\text{catalase}$ films in 1 mM H_2O_2 solutions. CVs were performed at 0.2 V s^{-1} in pH 7.0 buffers after incubation.

Figure 5.

(A) CVs at 0.2 V s⁻¹ in pH 7.0 buffers for (a) {Mb/HA}₂₅ films in the absence of H₂O₂; (b) {Mb/HA}₂₅, (c) {Mb/HA}₂₅/catalase, and (d) {Mb/HA}₂₅/catalase/HA/catalase films in the presence of 0.1 mM H₂O₂. (B) CVs at 0.2 V s⁻¹ in pH 7.0 buffers for (a) {catalase/HA}₆ films in the absence of H₂O₂, and (b) {catalase/HA}₆ films in the presence of 0.1 mM H₂O₂.

Figure 6.

Influence of the number of catalase layers (m) on catalytic efficiency (I_c/I_0) for $\{\text{Mb/HA}\}_{25}/\{\text{catalase/HA}\}_{m-1}/\text{catalase}$ films in 0.1 mM H_2O_2 solutions, where I_0 and I_c represent the CV reduction peak currents of the films at 0.2 V s^{-1} in pH 7.0 buffers in the absence and presence of H_2O_2 , respectively. All the data of I_c were collected from the first cycle of CV performed immediately after the injection of H_2O_2 , and each data point in the figure was an averaged result from 3 – 5 parallel experiments.

Figure 7. CVs at 0.2 V s^{-1} in pH 7.0 buffers for (a) $\{\text{Mb/HA}\}_{25}$ films in the absence of H_2O_2 ; (b) $\{\text{Mb/HA}\}_{25}$ and (c) $\{\text{Mb/HA}\}_{25}/\{\text{catalase/HA}\}_4/\text{catalase}$ films in the presence of $1 \text{ mM H}_2\text{O}_2$.

Figure 8.

Influence of H_2O_2 concentration ($C_{H_2O_2}$) in incubation solutions on catalytic efficiency (I_c/I_a) for (A) (a) $\{Mb/HA\}_{25}$, (b) $\{Mb/HA\}_{25}/\{catalase/HA\}_4/catalase$; and (B) (a) $\{HRP/HA\}_{25}$, (b) $\{HRP/HA\}_{25}/\{catalase/HA\}_4/catalase$ films, where I_a and I_c represent the CV reduction peak currents of the films at 0.2 V s^{-1} in pH 7.0 buffers in the absence and presence of H_2O_2 , respectively. All the data of I_c were collected from the first cycle of CV performed immediately after the injection of H_2O_2 .

Scheme 1.

Schematic illustration of the damage and electrocatalysis for Mb or HRP in {protein/HA}_n films with H₂O₂ and the protection effect of {catalase/HA}_{m-1}/catalase layers on the {protein/HA}_n film surface.