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Layer-by-Layer Enzyme/Polyelectrolyte Films as a Functional Protective Barrier in Oxidizing Media

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Abstract: The influence of a catalase (Cat) layer located at different depths in the layer-by-layer hemoglobin/polystyrene sulfonate films with an $(Hb/PSS)_{20-x}/(Cat/PSS)/(Hb/PSS)_x$ ($x = 0-20$) architecture on kinetics of hemoglobin degradation under treatment with hydrogen peroxide solutions of different concentrations and features of H_2O_2 decay in surrounding solutions has been studied. While assembled on the top of the multilayers, the catalase layer shows the highest activity in hydrogen peroxide decomposition. Hemoglobin in such films retains its nativity for a longer period of time. The effect of catalase layers is compared with that of protamine, horseradish peroxidase, and inactivated catalase. Positioning an active layer with catalytic properties as an outer layer is the best protection strategy for layer-by-layer assembled films in aggressive media.

Introduction

Multilayered films and microcapsules containing enzymes and polyelectrolytes with specific properties are under investigation as chemical sensor devices,[1] ultrathin separation membranes,[2] high-performance enzymatic reactors,[3] and drug delivery systems.[4] However, pollutants, impurities, interfering substances, or aggressive media can result in a lack of desirable properties and system integrity and may cause serious problems in the usage of such devices. Therefore, developing approaches, which may protect substances encased in the films and capsules, are of special interest. The addition of low-molecular weight inhibitors into the systems or creation of an impermeable barrier has limited applications. A protection based on targeted decomposition of pollutants is preferable. A layer which is permeable for the molecules of interest and acts as a barrier for pollutants can be formed by different methods. Recently, the possibility to eliminate ascorbate interference on glucose and lactate sensors by means of an ascorbate oxidase layer was shown. The layers were obtained by sequential deposition of avidin and biotin-labeled enzymes.[1b] The layer-by-layer (LbL) assembly gives an option to design nanoscale coverage with desirable properties.[3-7] Different enzymes, polyelectrolytes, and charged nanoparticles can be assembled into one film without preliminary modification and loss of quality. Permeable for low-molecular weight substances, the films possess selective permeability for high molecular weight polymers and enzymes, which can be controlled by the film multilayer composition, pH, and ionic strength of the media.[2-7] Recently it was shown that a layer of catalase or Fe_3O_4 nanoparticles deposited via LbL assembly on the outermost surface of PAS/PAH multilayered microcapsules can significantly inhibit oxidation of an encapsulated protein in hydrogen peroxide solutions.[8a] These approaches are based on consecutive reactions of substrate with sequentially deposited layers. If the order of catalytically active layers in the film architecture corresponds to the multiple steps of a sequential reaction, a high conversion of a substrate can be achieved. For example, the high catalytic activity for an assembly with layer sequence $(urease/PDDA/arginase/PDDA)_n$ in reaction L-arginine→ urea → ammonia was shown.[8b] However, there is no general theory which can explain reactions of low molecular weight substances passing though ultrathin organized multilayer containing biocatalytic agents.

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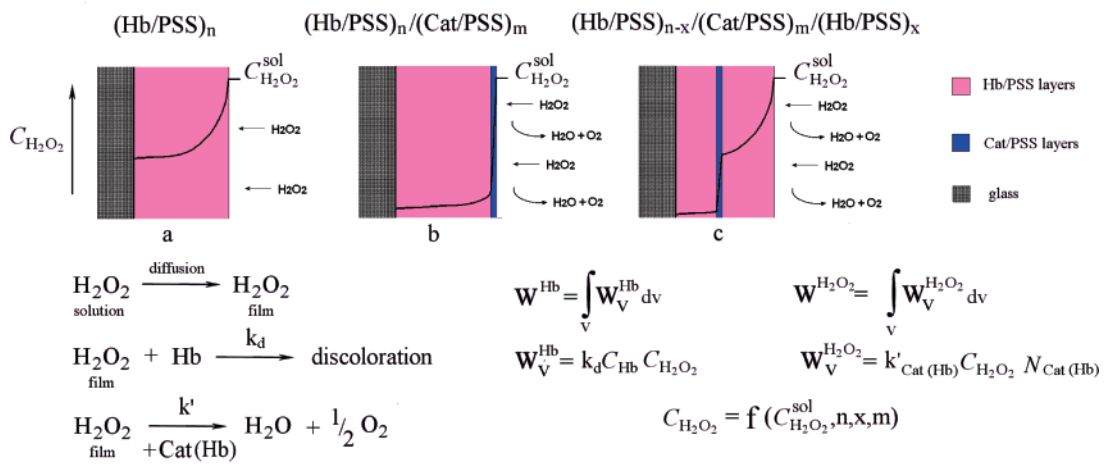

Figure 1. Scheme of H_2O_2 concentration change in $(Hb/PSS)_{n-x}/(Cat/PSS)_m/(HB/PSS)_x$ films of different architectures (upper) and two-stage kinetic reaction scheme of hemoglobin decay in the films (lower).

Oxidation is one of the serious problems for the devices based on substances originated from living organelles. Reactive oxygen species such as a superoxide anion, hydrogen peroxide, and a hydroxyl radical generated under numerous conditions have the ability to damage biomolecules, including proteins, enzymes, lipids, carbohydrates, and DNA.[9] The oxidation leads to inhibition of their activity and changing properties and can be extremely harmful for some drug formulations. A protective layer with target properties strongly attaches to the drug microparticle surface and is capable of scavenging or otherwise eliminating reactive oxygen species, while those appearing in the vicinity of the drug or protein microparticles and inactive all other times seem to be a logical development of antioxidant systems. The total amount of the layered protective substance can be very low as compared with the mass of the drug core; however, its local concentration on the surface is extremely high. It creates the conditions favorable to the catalytic decomposition of the oxidant thus decreasing the probability of oxidant−core interaction.

The purpose of this work is to compare stability and the catalytic action of proteins included in LbL multilayers of different compositions in response to hydrogen peroxide. Among the enzymes under investigation are those active toward H_2O_2, such as catalase and peroxidase,[9,10] as well as one protein which does not show any activity in peroxide decomposition (protamine sulfate). In the model proposed here, a layer of enzyme with high activity toward H_2O_2 can sufficiently decompose H_2O_2 decreasing its diffusion into the film and protect inner film layers (Figure 1). Hemoglobin/polyelectrolyte films prepared by the LbL technique were chosen as a model system due to the convenience of the hemoglobin nativity control with the Soret band absorbency pick. Hemoglobin as a heme-containing protein can be identified in biological systems because of its color;[11] it allows direct spectroscopic determination of the protein in Hb/polyelectrolyte films. It has been shown that hemoglobin (Hb) can be alternated with positively or negatively charged polyelectrolytes in LbL film without changing its nativity.[7] Moreover, hemoglobin has some biocatalytical properties and can be irreversibly destroyed by hydrogen peroxide. The detailed mechanism of the reaction of hemoglobin with hydrogen peroxide can be found elsewhere.[12] The chosen model system allows us to characterize the kinetics of the reaction of a low molecular weight reagent (hydrogen peroxide) with a substance included into LbL multilayers (hemoglobin) with respect to internal (film thickness and architecture) and external (hydrogen peroxide concentration) conditions. According to our knowledge, only few attempts have been made to study kinetic specifics of reactions in LbL assembled films and LbL microcapsules.[8]

Experimental Section

Materials. Sodium poly(styrene sulfonate) (PSS, MW 70 000, Aldrich), hemoglobin from bovine (Hb), catalase from bovine liver (Cat, Sigma C3155, 45 000−47 900 unit/mg), peroxidase from horseradish (HRP), protamine sulfate (Prot), hydrogen peroxide (H_2O_2), hydrochloric acid, and sodium chloride were purchased from Sigma-Aldrich and used without additional purification.

Multilayer Deposition. Hemoglobin/PSS films were deposited by sequential dipping of 1.0 × 2.0 × 0.1 cm^3 glass slides in 3.0 mg/mL solutions of Hb and PSS in 0.5 M NaCl (pH 5.0) for 10 min with two intermediate washings in DI water using a dipping machine (Riegler & Kirstein, Germany). For one of the slides, UV−vis spectra were taken (Agilent 8453 spectrometer, 300−1000 nm) in DI water at pH 5.0 after deposition of each Hb/PSS bilayer. Layers of catalase, HRP, or Prot were deposited on the top of the films from 3.0 mg/mL solutions in DI water at pH 5.0 by immersing the slides in corresponding solutions for 15 min followed by two rinses in water. Inactivated catalase was prepared by heating a 3 mg/mL catalase solution to 70 °C for 2 h. In a separate series of experiments, layers with an $(Hb/PSS)_{20-x}/(Cat/PSS)/(Hb/PSS)_x$ architecture ($x = 0-20$) were deposited using active or inactive catalase. After deposition of a desirable number of layers, the films were stored in water at pH 5.0 and used for kinetic experiments within 2 h after preparation.

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The thickness of the deposited films was estimated by the Quartz Crystal Microbalance (QCM) technique. The frequency changes of resonators were monitored using a USI-System, JapanQCM instrument with the accuracy ±1 Hz and converted into thickness using experimental scaling with the following formula: $\Delta D(\text{nm}) = -0.016\Delta F$ (Hz). The detailed description of the procedure can be found elsewhere.[7] The experiments were done in three parallel independent runs for all PSS/protein pairs, and the average value was taken. The divergence of thickness for films with a certain number of bilayers from the corresponding average value was less than 1 nm and dependent on the enzyme used.

Treatment of Enzyme/Polyelectrolyte Multilayers with H_2O_2. The Hb/PSS multilayers prepared without intermediate drying were used for all kinetic experiments. Glass slides with the deposited protein/polyelectrolyte multilayers were immersed into water at pH 5.0 (2 slides per cuvette), and then after 1 min, 0.025−0.5 mL of a 0.1 M H_2O_2 solution (pH 5.0) was added. The total volume of reaction mixture was 2.5 mL in all cases. The changes of film spectra over the range 335−700 nm were recorded in a kinetic mode. The Hb decay was monitored at 409 nm (the Soret band of Hb). The fraction of hemoglobin remaining in the films under treatment (C_t/C_0) was estimated as a A_t/A_0 ratio, where $A_t = A^{409} - A_b$, $A_b = {}^1/_2(A^{369} - A^{449})$, and A_0 is equal to $A_{t=0}$ at the addition of H_2O_2 into the system. A^{369}, A^{409}, and A^{449} are film absorbance values at 369, 409, and 449 nm, respectively.

Kinetics of H_2O_2 decomposition on polyelectrolyte/protein films were estimated by measuring the absorbance decrease at 230 nm for 2 mL solutions containing 0.001−0.04 M H_2O_2 which were in contact with an enzyme/polyelectrolyte film.[10] The absorbance was converted into H_2O_2 concentration using the effective extinction coefficient of 64.6 M^{-1} cm^{-1}. The effective constant of hydrogen peroxide decomposition k_{eff} was calculated using a first-order reaction equation.

Atomic Force Microscopy (AFM). AFM images of dried films on glass slides were taken on a Q-Scope 250 Quesant instrument in intermittent-contact modes.

Results

Layer-by-Layer Assembly of Hemoglobin/PSS Multilayers. The films were prepared by sequential adsorption of hemoglobin (Hb) and PSS in a 0.5 M NaCl solution at pH 5.0. It has been shown previously[7] that, while being LbL-assembled from solutions with high ionic strength, Hb and PSS form thicker layers. At pH 5.0, Hb is positive (isoelectric point (pI) of the protein is 6.8 and PSS is negative ($pK_a = 1$)).[7,13] The first layer was always Hb, since it can easily adsorb on a glass surface.[11a] Deposition of PSS on the top of an hemoglobin layer significantly suppresses the thickness of a previously deposited Hb layer, while the next protein layer increases it again (Figure 2). PSS probably peels off some of the outermost proteins and recharges the surface.

The thickness of $(Hb/PSS)_n$ films was estimated by QCM in three different ways: with drying after each layer, after each bilayer, or single drying after 10 bilayers. For the films with intermediate dryings, a linear growth was observed (Figure 2) with equal distribution of Hb along the vertical cross-section of the films; the same was assumed for the films prepared without drying (Table 1). The thicknesses of Hb/PSS bilayers were estimated as 6.1 ± 0.8, 3.9 ± 0.2, and 2.5 ± 0.2 nm for the films as above. The results are in good agreement with previously obtained data on Hb/PSS film deposition at different conditions[7] and sizes of Hemoglobin tetramer which is usually approximated as a sphere of 5.2−5.5 nm diameter,[7a] although some theoretical estimations on the basis of different ideal shapes give the range from 2.9 to 6.4 nm.[14] The thickness value presumably varies due to the adsorption of different amounts of hemoglobin resulting in incomplete defect layers as it was confirmed by AFM.

The AFM image of a dried untreated $(Hb/PSS)_{10}$ film obtained with intermediate drying after each layer is presented in Figure 3a. Relatively uniform coverage of the slide with the enzyme/polyelectrolyte layers was observed with a rough surface typical for LbL multilayers.[15] The depth of the majority of defects (holes) observed on the film surface evaluated from the

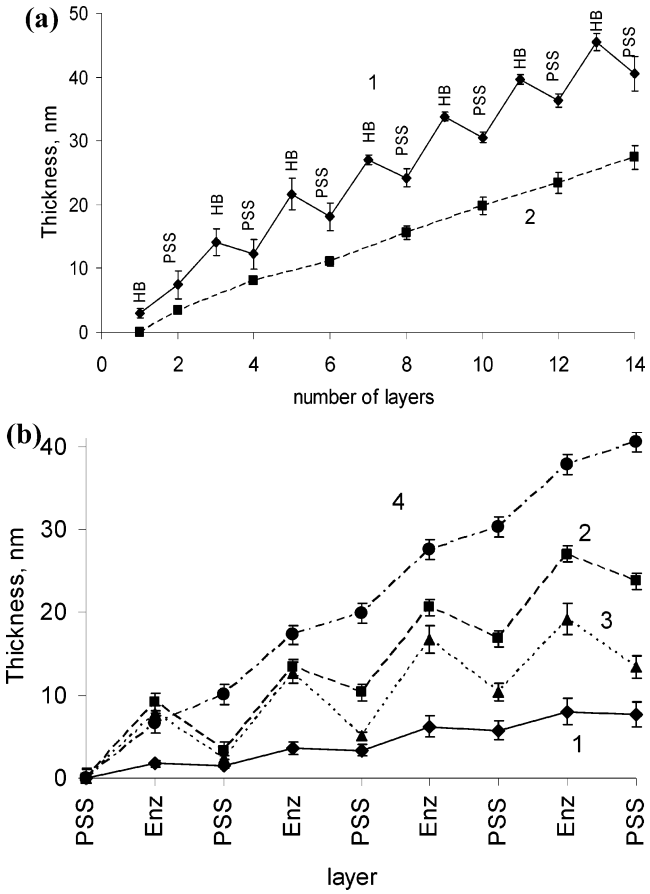

Figure 2. Film thickness in the process of LbL assembly: (a) Hb/PSS films, 1 - drying after each layer, 2 - drying after each (Hb/PSS) bilayer; (b) 1 - PSS/Cat, 2 - PSS/Cat-inact, 3 - PSS/HRP, 4 - PSS/Prot, drying after each layer. The average values with standard deviations (bars) obtained from three parallel experiments are shown.

Table 1. Thickness of Protein/PSS Films

		thickness, nm	
protein	pI[7,13]	protein/PSS bilayer	protein layer on PSS
Hb	6.8	6.1 ± 0.8	9.3 ± 0.9
		3.9 ± 0.2[a]	
		2.5 ± 0.2[b]	
Cat	5.5	1.9 ± 0.2	2.3 ± 0.5
Cat-inac		5.9 ± 0.4	9.9 ± 0.5
HRP	7.0	3.4 ± 0.3	9.7 ± 0.8
Prot	11.0	10.1 ± 0.3	7.2 ± 0.4

[a] Drying after each PSS layer. [b] Drying after 10 bilayers.

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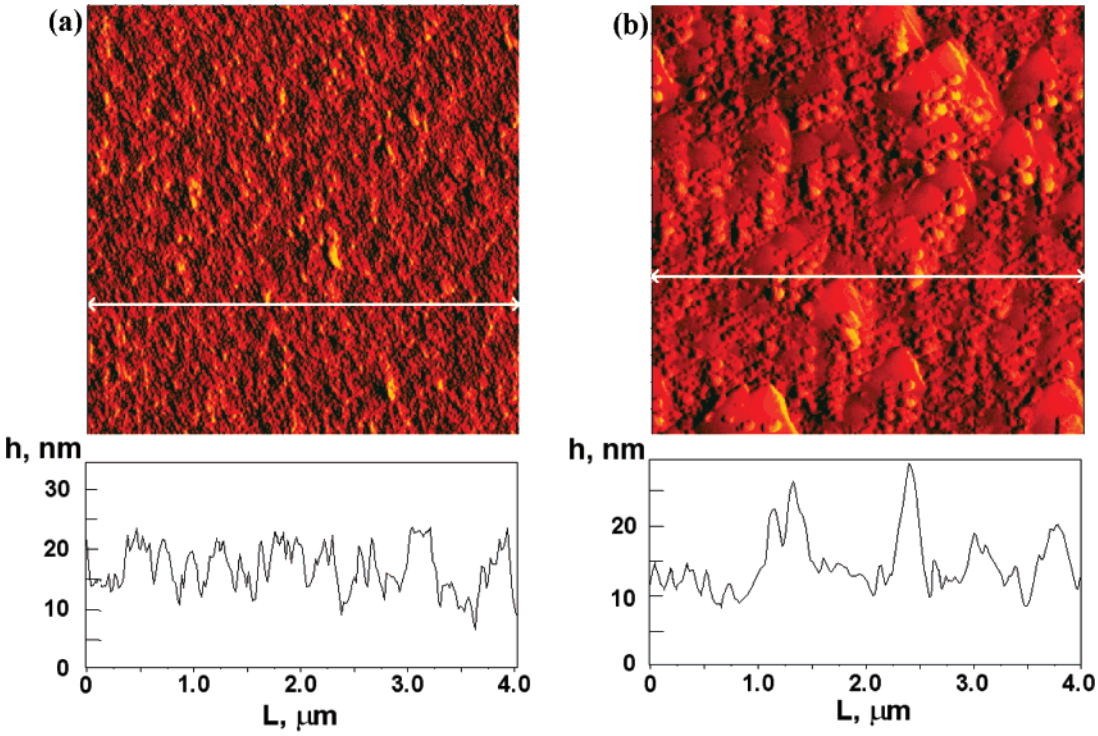

Figure 3. AFM images of a dried $(Hb/PSS)_{10}$ film before (a) and after 20 min in a 0.02 M H_2O_2 solution, pH 5.0 (b).

film cross-section profile is around 7 nm. The value is close to the thickness of the Hb/PSS bilayer obtained for the films by QCM (Table 1). At the same time, some of the defects penetrate deeper into the film reaching the second and probably the third Hb/PSS bilayer at some points.

The UV−vis spectra of an $(Hb/PSS)_{10}$ film in the process of assembly are shown in Figure 4a. A maximum at 409 nm attributed to the Soret band of hemoglobin and another one, a wide low-intensity maximum in the range 500−600 nm, which corresponds to the visible band of hemoglobin,[11,12] are observed. Although the visible range band of Hb is more sensitive to oxidation state of the enzyme,[11] its intensity in the film spectra is not sufficient to follow the reaction kinetics. Hence, the Soret band was chosen for the hemoglobin nativity control. Acids can drastically affect the hemoglobin absorbance causing intensity decrease and shift of the bond position.[11b−e] Moreover, the Soret band of heme-containing proteins is sensitive to matrix drying[11d−e] and protein binding.[11f−g] At the pH under investigation no noticeable shift of the Soret band in the film spectra was found (as compared with that of solutions). We assume that no sufficient change of hemoglobin structure or its denaturation takes place upon complexation with polyelectrolytes.[11h] The growth of multilayer absorbance at 409 nm accelerates with an increasing number of hemoglobin/PSS bilayers (Figure 4b). This is somewhat different from the results of QCM measurements for (Hb/PSS) films, where a linear film growth was observed. At the same time, with deposition of each hemoglobin layer, visible haziness increases and the background level rises over all the UV−vis range, being more pronounced at lower wavelengths. This effect may be caused by formed barely soluble Hb/PSS complexes scattering light.

Reaction of Hb/PSS Multilayers with H_2O_2. No noticeable change of $(Hb/PSS)_n$ film spectra was found in solutions without hydrogen peroxide under experimental conditions (within 8 h). In the presence of H_2O_2 the intensity of the Soret band decreases essentially in 20 min (Figure 5). The kinetics of hemoglobin decay in the LbL films show a fast 1−2 min initial drop followed by a slow decrease with about a 50 times smaller rate (Figure 6a). Similar biphasic kinetic curves were observed for hemoglobin autoxidation and its oxidation with potassium ferricyanide and explained by existing two different chains of hemoglobin.[12,16] From our point of view, the observed kinetics for LbL films can be also related to some degree to the limitation of H_2O_2 flux into the film interior, since a dependence of hemoglobin decay on film thickness was found.

According to AFM, after 20 min in a 0.02 M H_2O_2 solution the film surface structure is changed (Figure 3b). It is not a uniform film but agglomerates of different sizes probably consisting of hemoglobin, its simple complexes with PSS, and aggregates of several hemoglobin and polyelectrolyte macromolecules are observed on the slide surface. We assume that a complex process including enzyme denaturation, film restructurization, and decomposition takes place in a media containing hydrogen peroxide.

Influence of Hb/PSS Film Thickness on Hemoglobin Decay. The effective rate of hemoglobin decay (W^{Hb}) depends on the number of deposited Hb/PSS bilayers (Figure 6b). The decomposition of one bilayer film is very fast; hemoglobin is completely denaturated after 2−3 min in 0.02 M H_2O_2. The Hb decay slows down with an increasing number of Hb/PSS bilayers, and for 10−20 bilayer films the initial rate of Hb decay practically does not depend on film thickness (Figure 6b).

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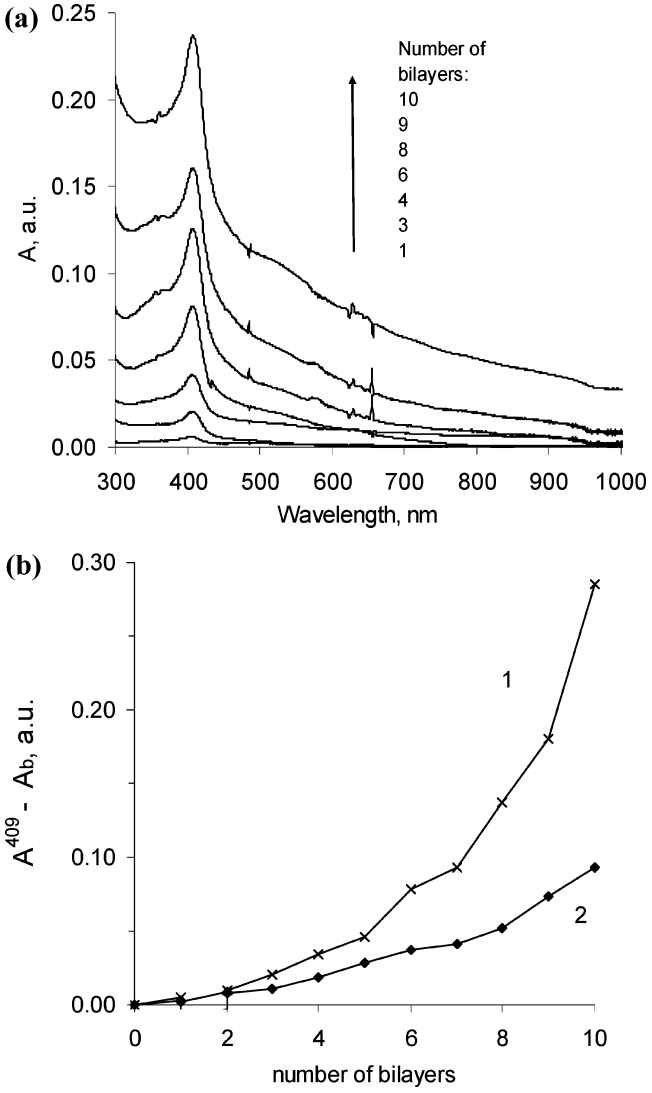

Figure 4. (a) UV−vis spectra of an Hb/PSS film in the process of LbL assembly. (b) $A^{409} - A_b$ as a function of the number of bilayers for HB/PSS films; background: 1 - $A_b = A^{750}$; 2 - $A_b = {}^1/_2(A^{369} - A^{449})$. Upper layer is always PSS.

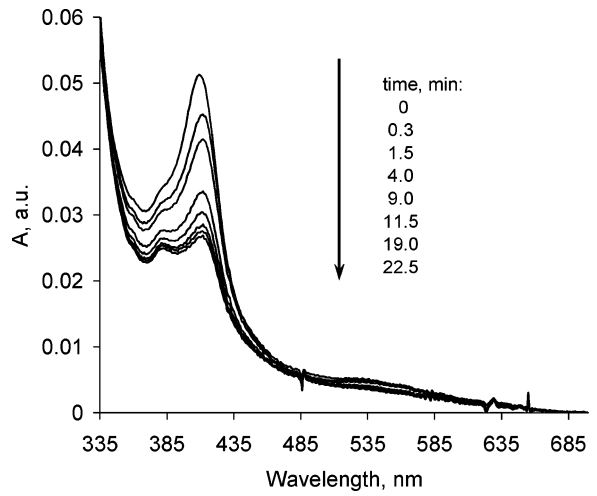

Figure 5. Spectra of an $(Hb/PSS)_{10}$ film in the process of treatment with 0.02 M H_2O_2 at pH 5.0.

Taking into account that reaction rate is proportional to reagent concentrations and initially the films do not contain any H_2O_2,

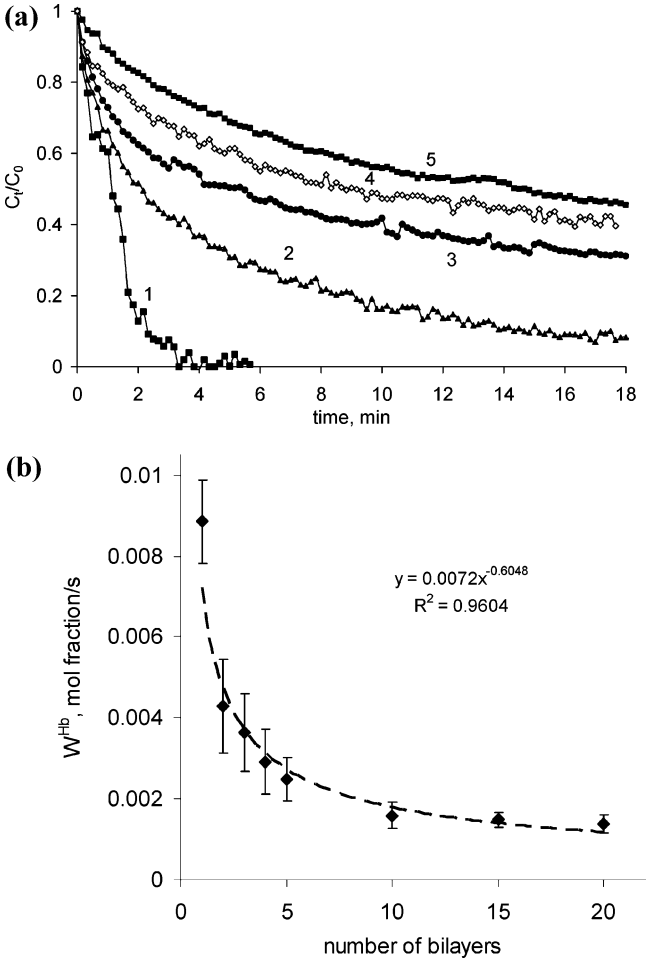

Figure 6. (a) Hb decay in 1 (*1*), 3 (*2*), 5 (*3*), 10 (*4*), and 20 (*5*) Hb/PSS bilayer films ($[H_2O_2] = 0.02$ M). (b) Dependence of initial Hb decay rate on number of Hb/PSS bilayers. The data points are approximated with a power equation (dashed line).

we believe that there is a significant H_2O_2 concentration gradient within the first five Hb/PSS bilayers, while in the rest of the film (in depth) its concentration changes not as rapidly (Figure 1).

Influence of H_2O_2 Concentration on Hemoglobin Decay Rate. Figure 7a shows kinetic curves of hemoglobin decay in $(Hb/PSS)_{10}$ films at different H_2O_2 concentrations in surrounding solutions. In diluted solutions, the initial rate of hemoglobin discoloration linearly depends on the concentration of hydrogen peroxide (Figure 8). However, the reaction slows down at high concentrations. The dependence seems to be well described by a Michaelis−Menton kinetic curve (Figure 8), which is an indication of a complex multiple-stage reaction scheme of the $Hb-H_2O_2$ interaction in the LbL films. It has to be mentioned that only freshly prepared Hb/PSS films were used for the kinetic measurements. If the films were preliminarily kept several days in water (pH 7.0), the intensity of the 409 nm band sufficiently decreased. No fast initial step was observed on the kinetic curves. The initial rates of intensity decrease in this case correspond to that on later steps of oxidation for freshly prepared films (data not shown). A slow oxidation of hemoglobin seems to take place during film storage.

Influence of Catalase Layers on Hemoglobin Decay in LbL Multilayers. The isoelectric point of catalase is equal to 5.5,[7] and therefore, at pH 5.0 it is positive. Deposition of the

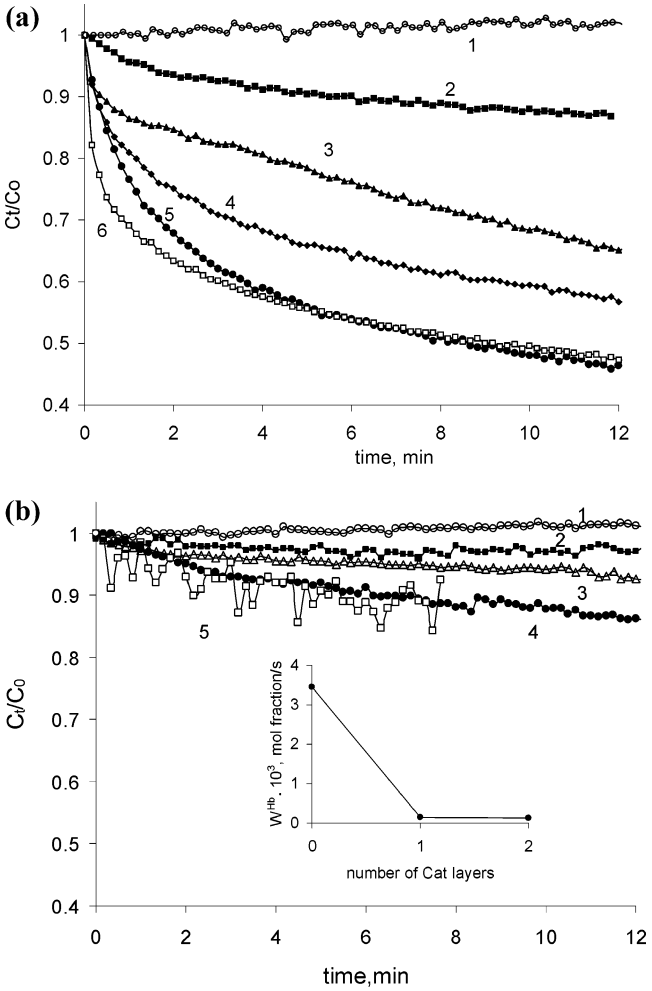

Figure 7. Hb decay in $(Hb/PSS)_{10}$ (a) and $(Hb/PSS)_{10}$/Cat (b) films at different H_2O_2 concentrations. On (a) 1 - 0 M; 2 - 0.001 M; 3 - 0.004 M; 4 - 0.007 M; 5 - 0.02 M; 6 - 0.04 M. On (b) 1 - 0 M; 2 - 0.004 M; 3 - 0.02 M; 4 - 0.04 M; 5 - 0.2 M; the insert shows dependence of initial decay rate on the number of catalase layers for 0.02 M H_2O_2.

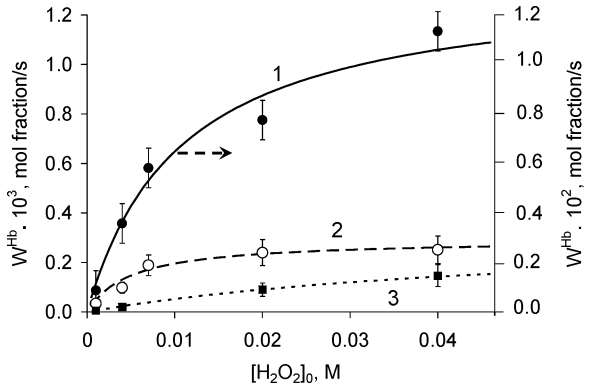

Figure 8. Initial rates of Hb decay within $(Hb/PSS)_{10}$ (1 and 2) and $(Hb/PSS)_{10}$ /Cat (3) at different H_2O_2 concentrations: 1,3 - initial rates; 2 - the same as 1 but over the 5−10 min range. The lines show approximations of the obtained data by Michaelis−Menton equations.

enzyme in alternation with PSS was proven by QCM measurements of the film thickness increase (Figure 2b). The estimated thickness of the layer deposited on the top of a PSS layer is 2.3 ± 0.5 nm for an active enzyme and 9.9 ± 0.5 nm for an inactivated specimen (Table 1).

The deposition of a catalase layer on the top of an $(Hb/PSS)_{10}$ film does not change its spectrum significantly in the range 350−500 nm. The extinction coefficient of catalase at 409 nm is 3 times smaller than that of hemoglobin (1.7 and 5.1 L g^{-1} cm^{-1}, respectively), and only one layer of catalase was deposited.

If a layer of catalase is adsorbed on the top of an Hb/PSS film, the intensity of the 409 nm band in H_2O_2 solutions decreases very slowly (Figure 7b) indicating its protective antioxidant properties. The rate is about 100 times smaller than that in the initial decay stages of the films without catalase (Figure 8). In this case the hemoglobin decay also seems to follow Michaelis−Menton kinetics in 0.004−0.2 M H_2O_2 solutions. Initially, it was assumed that a catalase layer has a limited capacity to consume H_2O_2 and, at high concentration of hydrogen peroxide, the value of initial reaction rate of Hb decay in unprotected films can be reached. Some increase of the hemoglobin decay rate was observed at high H_2O_2 concentrations; however, up to 0.2 M H_2O_2, the decay remains much lower than that for unprotected films. Unfortunately, intensive formation of oxygen bubbles at high concentrations of hydrogen peroxide resulted in high noise-to-signal ratio, and it made conducting the experiments in these extreme conditions impossible. The second layer of catalase alternated with PSS does not noticeably influence the rate of the decay (Figure 7b, insert). Therefore, a single catalase layer significantly lowers the the amount of hydrogen peroxide reaching the hemoglobin layers in the film depth.

When a layer of inactivated catalase was deposited on the top of the films, the hemoglobin decay in the presence of H_2O_2 still takes place and its kinetics is almost identical to the one measured for the films without the catalase layer (Figure 9). One can conclude that the protective action of catalase is related to its activity in H_2O_2 decomposition but not to additional diffusion limitation through the enzyme layer.

Effect of Multilayer Architecture on Hemoglobin Protection in $(Hb/PSS)_{10-x}/(Cat/PSS)/(Hb/PSS)_x$ Films. Figure 10a shows time profiles of hemoglobin decay in $(Hb/PSS)_{10-x}/(Cat/PSS)/(Hb/PSS)_x$ films with different positions of the Cat/PSS bilayer in the assemblies. If the Cat/PSS bilayer is located on the top of $(Hb/PSS)_{10}$ films as an outer layer ($x = 0$), the film discoloration in a 0.004 M H_2O_2 solution is much slower than that for the films without a catalase layer or with a catalase layer located within Hb/PSS layers. The Hb decay varies with the position of the catalase layer in the film (Figure 10b). The more Hb/PSS bilayers are placed on the top of a catalase layer, the faster decay is in all other similar conditions. Finally, for the films with nine Hb/PSS bilayers on the top of a Cat/PSS layer, the decay kinetics is close to that given earlier for the films with no catalase at all. We believe that this result can be explained by a declined flux of H_2O_2 into inner film layers due to its catalytic decomposition by the catalase layer. One can notice that no such effect can be seen for films with similar structures but prepared using inactivated catalase (Figure 10b).

Effect of Other Enzymes on Decay of Hb/PSS Films. For further experiments, two other proteins were chosen: peroxidase (HRP), an enzyme with well-known activity toward H_2O_2 and protamine sulfate (Prot), a protein which does not possess such activity. According to their pI, HRP (pI = 7.0)[7] and protamine sulfate (pI = 11)[13] are positively charged at pH 5.0 and can be alternated with negatively charged PSS. Deposition of the proteins was confirmed by QCM; Table 1 contains data on bi-

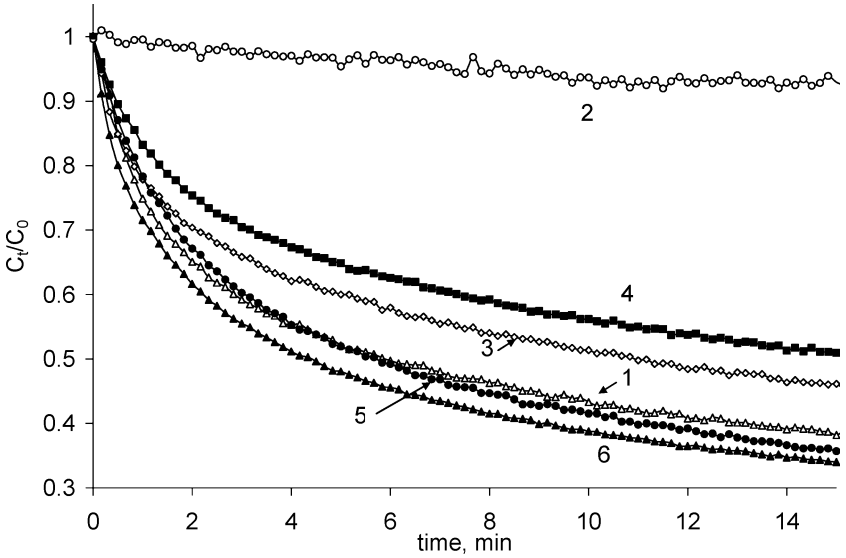

Figure 9. Influence of different enzyme and protein layers on Hb decay within $(Hb/PSS)_{10}$/Enz films in 0.02 M H_2O_2. 1 - no layer; 2 - Cat; 3 - Cat-inact; 4 - HRP; 5 - Prot; 6 - Hb.

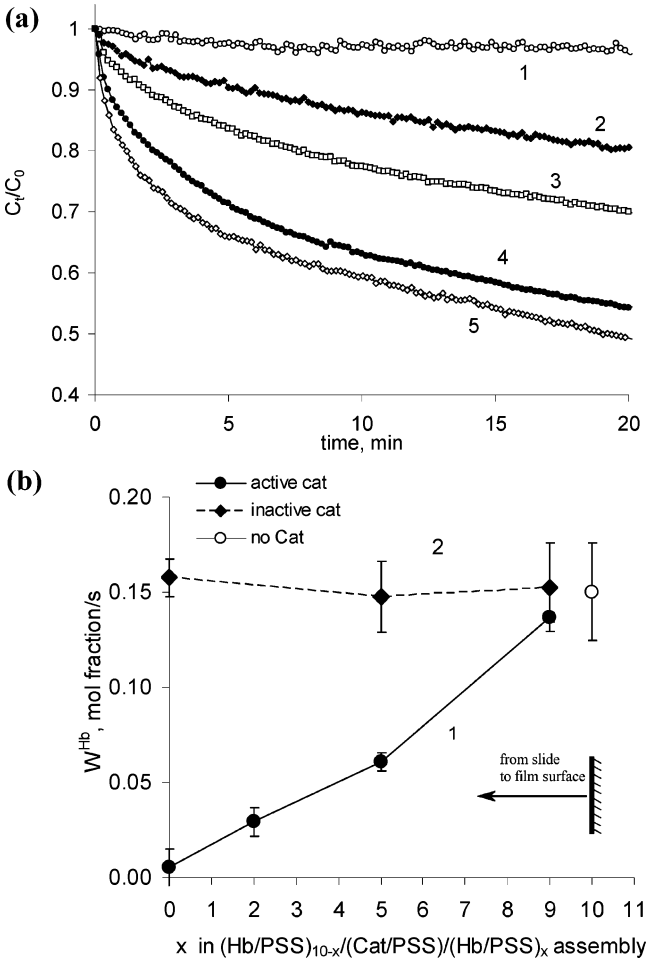

Figure 10. (a) Hb decay in $(Hb/PSS)_{10-x}/(Cat/PSS)/(Hb/PSS)_x$ films, $x =$ 0 (1), 2 (2), 5 (3), 9 (4), and $(Hb/PSS)_{10}$ films (5) in 0.004 M H_2O_2. (b) Dependence of the initial rate of Hb decay in $(Hb/PSS)_{10-x}/(Cat/PSS)/(Hb/PSS)_x$ films on the position of a Cat layer in the assembly: 1 - active Cat, 2 - inactivated Cat. The open circle corresponds to $(Hb/PSS)_{10}$ films. The results shown represent data of three independent experiments at each x value.

layer thicknesses of the protein/PSS films. It has to be mentioned that, for all the proteins under investigation, overequilibrium enzyme adsorption on a PSS layer was observed in the experimental conditions, which can be a result of increasing hydrophobic interaction between proteins and polyelectrolytes.

Table 2. Effective Constants of H_2O_2 Decomposition on Polyelectrolyte/Enzyme Films

film	$k_{eff} \times 10^5$, s^{-1} cm^{-2}
$(Hb/PSS)_{10}$	1.1 ± 0.6
$(Hb/PSS)_{10}$/hemoglobin	1.6 ± 0.3
$(Hb/PSS)_{10}$/Cat-inact	0.9 ± 0.4
$(Hb/PSS)_{10}$/HRP	4.9 ± 0.3
$(Hb/PSS)_{10}$/Prot	1.4 ± 0.2
$(Hb/PSS)_{10}$/Cat	24.4 ± 1.8

We tried another enzyme active toward H_2O_2 as a possible protective layer. A horseradish peroxidase layer slightly slows the decay of hemoglobin in the $(Hb/PSS)_{10}$ films (Figure 9); however, the decay is still sufficient, and no protection was registered. Peroxidase requires a separate acceptor to decompose hydrogen peroxide, while catalase uses H_2O_2 as a substrate as well as a hydrogen acceptor.[10] In addition, the reaction of peroxidase with peroxide produces highly active oxygen-centered free radicals, which can cause secondary damage of the films. The reaction of HRP with H_2O_2 is much slower than that of catalase, and sufficient amounts of H_2O_2 still penetrate inside, causing the fast drop of film absorbance (Figure 9). At the same time, a layer of protamine sulfate, which does not possess any enzymatic activity toward hydrogen peroxide, does not change the decay of hemoglobin in the film as compared with those without this layer.

Decomposition of H_2O_2 in Polyelectrolyte/Enzyme Multilayers. Since it was assumed that peroxide decomposition by enzymes plays an important role in the protection of hemoglobin in LbL films, the decomposition of H_2O_2 on the enzyme/polyelectrolyte films was investigated. The H_2O_2 consumption in the presence of $(Hb/PSS)_{10}$ films was studied at the same conditions as those for where the film spectral changes were registered. It was found that very slow decay of hydrogen peroxide takes place in the presence of Hb-containing films (Table 2). The peroxidase-like behavior of Hb is well-known and usually attributed to the reaction of iron-containing heme

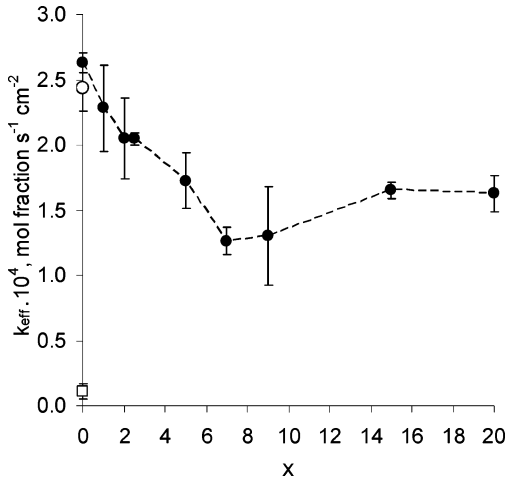

Figure 11. Dependence of rate constant of H_2O_2 decay on number of (Hb/PSS) bilayers (x) assembled on the top of a (Cat/PSS) layer. The open symbols show the values obtained for $(Hb/PSS)_{10}$ (□) and $(Hb/PSS)_{10}$/Cat films (○).

with peroxide.[12] In the range of concentrations used in the study (0.001−0.04 M), the decay of H_2O_2 follows first-order reaction kinetics, and no deceleration of peroxide decomposition on $(Hb/PSS)_{10}$ films were observed at high peroxide concentrations (although Michaelis−Menton-like kinetics of Hb decay in the films were found).

Hydrogen peroxide decomposition significantly increases in films with a protective catalase layer (Table 2). In this case the decomposition also follows a first-order reaction equation. The multilayers covered with a catalase layer show the highest activity in peroxide decomposition among the investigated samples (Table 2). At the same time, decomposition of peroxide in films with other investigated layers (including inactivated catalase) is comparable with that on $(Hb/PSS)_{10}$ films. Peroxidase, which shows some protective properties on hemoglobin decay, decomposes peroxide faster than other layers, but several times slower than the catalase layer does.

The decay of H_2O_2 depends on film architecture. Figure 11 shows the dependence of the effective constant of hydrogen peroxide decay k_{eff} on a number of Hb/PSS layers on the top of a catalse layer. If a catalase layer is located above the Hb/PSS film and in direct contact with the surrounding solution, the decomposition of hydrogen peroxide is fast. At the same time, if the catalase layer is covered with several Hb/PSS bilayers, H_2O_2 still decomposes with a rate constant at least 10 times higher than that for unmodified Hb/PSS films. Within the first 5−7 bilayers, the deeper the catalase layer is located in a film, the lower the H_2O_2 decomposition. This fact indicates that LbL-assembled Hb/PSS films are permeable for low molecular weight hydrogen peroxide. On the other hand, it shows that effective concentration of hydrogen peroxide, which can reach the catalase layer in the depth of the films, decreases. H_2O_2 decomposes on catalase layers as well as on Hb/PSS multilayers. It makes the observed bulk decomposition of H_2O_2 on thick mixed films more complicated. The decay of H_2O_2 increases again for the film containing 10−20 layers of Hb/PSS on the top of a Cat/PSS bilayer (Figure 11). In this case the decomposition on significantly increased amounts of hemoglobin compensates the fall of H_2O_2 concentration reaching the catalase layer located deep in the films. Considering all of the above architectural variations in the multilayer composition, it can be concluded that positioning the catalase layer on the top of the film is the best protection strategy.

Discussion

In this study, we explored the possibility of using surface molecular architecture with a LbL nanoassembly method for positioning catalytic enzyme layers at different depths of polyelectrolyte films and analyzed its efficiency in hydrogen peroxide decomposition. Therefore, we optimized the multilayer composition for the best protection of the film inner section, which is the basic research step for designing polyelectrolyte nanocapsules with protected interiors.[8a]

Two assumptions concerning LbL multilayer properties were made: first, we believe that the presence of a negatively charged polyelectrolyte (PSS) does not significantly change the mechanism of an Hb reaction with H_2O_2 compared with that in solution.[11,12] Retaining reaction mechanisms have been confirmed for several other systems based on LbL assemblies.[2,7] The second assumption made is that the structure and properties of Hb/PSS layers do not depend on film thickness, and local kinetic rate constants within the films (k_d for Hb discoloration and k'_{Cat} and k'_{Hb} for H_2O_2 decomposition on catalase and hemoglobin accordingly) are independent of the number of layers and remoteness of a layer from the film surface. The observed kinetics of film discoloration is a sum of local rates of Hb decay through the film; $W^{Hb} = \int W_v^{Hb}\, d\upsilon$. The reaction rate in each small volume is proportional to the concentrations of Hb and H_2O_2 in the volume: $W_v^{Hb} = k_d C_{Hb} C_{H_2O_2}$. In a similar way, for H_2O_2, the experimentally observed effective rate of H_2O_2 decay is a summarization of the reactions on all catalase and hemoglobin layers (Figure 1). On each of them the local decomposition rate $W_v^{H_2O_2}$ depends on hydrogen peroxide concentration and the catalyst amount. Unfortunately, the local values of C_{Hb} and $C_{H_2O_2}$ are not possible to measure, and corresponding bulk values, such as the fraction of hemoglobin decomposed through the film C_t/C_o and H_2O_2 concentration in the surrounding solution C_t^{sol}, can be used.

The decay of hemoglobin in Hb/PSS films under H_2O_2 treatment can be considered a two-stage process. The first stage is the diffusion of H_2O_2 into an Hb/PSS film, followed by reaction of H_2O_2 with available hemoglobin in the second stage (Figure 1). One can see that a relative rate of hemoglobin decay is maximal for one bilayer Hb/PSS film (Figure 6). In this case, the oxidation of hemoglobin seems to be the limiting stage of the reaction. The process occurs in the kinetic region without any diffusion limitations for H_2O_2 to reach the first hemoglobin layer. However, with increasing numbers of Hb/PSS bilayers, the rate of hemoglobin decay decreases. The dependence is more prominent for 2−5 bilayer films, while for 10−20 bilayers there is no significant difference in the relative rate of oxidation. Taking into account that initially the films do not contain any H_2O_2, we believe that there is a significant H_2O_2 concentration gradient within the first five Hb/PSS bilayers, while, in the layers located deeper in the films, H_2O_2 concentration changes less rapidly (Figure 1a).

An additional support for a H_2O_2 concentration gradient is the kinetics of its decomposition on films with different architectures (Figure 11). If a catalase layer is assembled on the top of Hb/PSS layers, the decomposition of H_2O_2 on such films is at least 2 times higher than that for the films with a

catalase layer separated from the solution by several Hb/PSS bilayers. It can be explained by contact of the outermost catalase layer with a higher concentration of hydrogen peroxide in the surrounding solution, while an effective concentration of H_2O_2 in the films is smaller. The data also seem to fit into a model which includes a drop of H_2O_2 concentration in few outermost layers followed by its relatively slow decrease through the deeper layers. One can see that the results somehow disagree with the common point of view on properties of LbL structures assembled as microcapsule walls. The polyelectrolyte microcapsules are usually considered completely permeable for substances with a molecular weight less than 3000.[3,8] Those observations were made for free-standing LbL-film walls of a 4−5 bilayer thickness, probably containing defects in the structure originated from template microcore dissolution. However, for several other systems assembled on flat templates, dependences of fluxes of uncharged[17] and charged[18] solutes through LbL films on the numbers of polyelectrolyte layers were shown. Recent studies on LbL multilayers show that upper layers in the films have a less dense structure as compared with inner parts of the films;[19] it can favor diffusion through a few outer layers. From an AFM image (Figure 3a), one can see that upper layers of an Hb/PSS film have numerous defects in the structure which support diffusion of hydrogen peroxide into the film. The existence of a H_2O_2 gradient in Hb/PSS films seems to be a co-influence of the physical diffusion limitation and its slow decay on consequently assembled hemoglobin layers.

The strong catalase effect is related to its activity in hydrogen peroxide decomposition but not to physical diffusion into the film decreased by additional bilayers because neither inactivated catalase nor other enzymes or proteins affect the hemoglobin decay in a similar way (Figure 9). Experimental data show that for such an active enzyme as catalase a single layer is already enough to lower substantially the decay of hemoglobin in the LbL films.

The influence of catalase on hemoglobin decay depends on its location within Hb/PSS layers (Figure 10). The catalase layer assembled on a top of the films shows the highest protective properties because it prevents contact of the outermost layers of hemoglobin with hydrogen peroxide (Figure 1b). If a catalase layer is located within Hb/PSS layers, the layers above the catalase still are accessible for H_2O_2 and are easily destroyed. At the same time, the layers shielded by catalase retain their color. In the last case, catalase decomposes H_2O_2 decreasing its concentration in the depth of the film and preventing hemoglobin damage (Figure 1c). The observed kinetics of hemoglobin decomposition in the presence of catalase layers is a superposition of decays of inner and outer layers of the film.

Finally, some general comments on reactivity of substances assembled into LbL films with respect to low molecular weight substances in a solution. If the reaction between the reagents is relatively fast with a high reaction rate constant, for example, as that for the reactions of catalase or Fe_3O_4 nanoparticles with hydrogen peroxide,[8a] free radicals with inhibitors, etc., the upper layers of the films should be affected more than the layers located deeper in the film. An active ingredient assembled on top of the film can significantly decrease the flux of a low molecular weight substance into the deeper film layers. But if the activity of the layer is lower (or the layer does not possess such activity), the reaction does not compete with substance diffusion into the films, and inner layers are affected (or participate in the reaction).

It may yield some surprising but predictable practical results, such as a drastic loss of apparent activity of some highly active catalysts assembled into LbL films with increasing thickness (which was found experimentally for glucose oxidase LbL multilayers),[8d] a competition of different reaction pathways in mixed films, and complete inhibition of certain reactions in such films, as well as asymmetric product formation through the films. Specificities of reaction kinetics and diffusion have to be taken into account while building active devices and microreactors based on LbL polyelectrolyte shells.

Interior protection with LbL multilayers needs a very low amount of a protective ingredient (polyelectrolyte, enzyme, catalytic nanoparticles, etc.) into the system, and since the deposited layer is molecularly thin, it does not influence the bulk system properties. One can call such protection as an active catalytic defense as an opposite to the recently demonstrated passive protection (such as TiO_2 monolayer nanocoating for light protection of encapsulated drug).[20] At the same time, high local concentration of the active ingredient on the surface creates unique barrier conditions allowing us to cut off interfering substances, pollutants or oxidants. LbL-assembled films with protective layers in their structure can find applications in new drug delivery systems to increase the stability and storage time of the formulations, in biosensors to minimize the action of pollutants and interfering substances, and in enzymatic microreactors and membrane reactors to remove impurities, which can result in formation of byproducts. However, most prospective area of application of LbL-assembled protective layers seems to be preventing oxidation and biodegradation of devices based on biomaterials. Antioxidant enzymes, natural polyphenols, and polymers with antimicrobial activity are promising materials for such thin coatings which may be applied, for example, at the surface of drug microcrystals.

In conclusion, the influence of a catalase layer located at different depths in hemoglobin/polystyrene sulfonate films assembled via the layer-by-layer technique on the kinetics of hemoglobin degradation under hydrogen peroxide treatment and features of H_2O_2 decay has been studied. The catalase layer on the top of the multilayers shows the highest activity in hydrogen peroxide decomposition. Hemoglobin in such films retains its nativity for a longer period of time. It makes positioning a layer with catalytic properties as an outer layer the best protection strategy for layer-by-layer assembled films.

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