High Activity Enzyme Microcrystal Multilayer Films

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Received March 13, 2001
Revised Manuscript Received June 13, 2001

Research focused on the preparation of protein-containing thin films is largely driven by their wide applicability in biotechnology.[1−3] In addition to the more traditional methods (e.g., physical adsorption, covalent attachment, biospecific recognition, sol−gel/polymer entrapment etc.), the layer-by-layer (LbL) technique[4] has proven to be an alternative and versatile approach for the construction of protein films. Biomolecular assemblies with specifically designed architectures and thickness have been fabricated by the alternate (i.e., LbL) adsorption of various (solubilized) proteins and oppositely charged polyelectrolyte both on planar substrates[5] and colloid particles.[6] Although there are virtually no practical limitations with respect to the number of protein layers that can be assembled using the LbL strategy, the biocatalytic performance of these films is highly dependent on the film structure and protein loading.[5a,c,e,f,6] Thicker films suffer from limited substrate accessibility and diffusion effects, thus limiting their enzymatic reaction efficiencies.[5a,6b] Here, we report the facile and general preparation of novel enzyme multilayer films based on encapsulated enzyme (catalase) microcrystals. These stable, relatively thin (few micrometers), high enzyme content films display biocatalytic activities of up to 50 times higher than those prepared by conventional LbL deposition of solubilized enzyme, thus making them attractive candidates for use in various biotechnological applications.

The catalase microcrystals were first encapsulated by the alternate adsorption of poly(styrenesulfonate) (PSS) and poly(allylamine hydrochloride) (PAH) on their surface, yielding an extremely high enzyme loading (with retained activity) in the polyelectrolyte multilayer capsule.[7] Multilayer films were then constructed on quartz crystal microbalance (QCM) electrodes or quartz slides by the LbL deposition of the polyelectrolyte-coated catalase crystals and oppositely charged polyelectrolyte.[8] Figure 1 shows the QCM frequency changes (ΔF) for the alternate assembly of PSS-coated catalase crystals and PAH.[9] An average ΔF value of −4462 (±1171) Hz was observed for each catalase crystal adsorption step, corresponding to an enzyme loading of about 3870 ng per layer,[10] or 120 mg m^{-2} per layer. The number of PSS/PAH layers (from one to nine) coating the catalase crystals did not influence the amount that was deposited. LbL-assembled films of *uncoated* catalase crystals deposited alternately with PSS[8] yielded ΔF values of −435 (±158) Hz (or 378 ng) per enzyme layer. The difference in the above values can largely be attributed to the lower charge density on the enzyme crystal surface compared to that of the polyelectrolyte-coated crystals.[7] The deposition of solubilized catalase from solution, assembled in alternation with PAH, gave a ΔF per enzyme layer of −118 (±27) Hz, corresponding to a mass coverage of around 3 mg m^{-2}.[11] Protein films prepared by the LbL assembly (as well as other methods) typically yield surface coverage values of about 3−10 mg m^{-2} per protein layer,[1−3] which are at least 1 order of magnitude lower than those obtained by using polyelectrolyte-encapsulated enzyme crystals (∼120 mg m^{-2}).

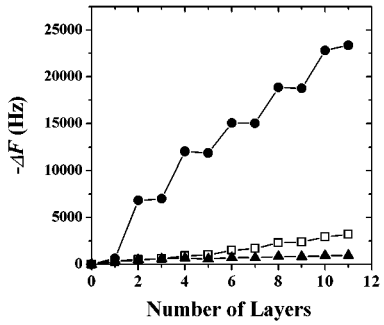

Figure 1. QCM frequency change as a function of number of layers for the assembly of PSS-coated catalase crystals (circles), uncoated catalase crystals (squares), and solubilized catalase (triangles), each deposited in alternation with polyelectrolyte (PAH or PSS).

Scanning electron[12] and optical[13] microscopy were used to examine the morphology of the enzyme multilayer films. Figure 2 shows the SEM images of a multilayer film that comprises four layers of PSS-coated catalase crystals, assembled in alternation with PAH, on QCM electrodes. The catalase crystals are quite evenly distributed on the surface, but they are not densely packed. The surface image also shows the polydispersity of the micrometer-sized catalase crystals, while the cross-sectional image (inset) shows the film roughness and thickness (ca. 2−5 μm). The optical micrograph of a one-layer enzyme film formed by the deposition of catalase crystals coated with five PAH/PSS layers on a polyelectrolyte-modified quartz slide shows sparser enzyme coverage of the surface.[14]

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(8) Multilayer films of polyelectrolyte-encapsulated catalase (Sigma, C-100) microcrystals were prepared on substrates modified with a precursor film of poly(ethyleneimine)(PEI)/(PSS/PAH)$_2$, which was deposited from a 3 mg mL^{-1} aqueous solution of the corresponding polyelectrolyte containing 0.5 M NaCl. The substrates were then immersed alternately in a suspension of the polyelectrolyte-coated catalase crystals (ca. 5 mg mL^{-1} in 1 M potassium acetate buffer pH 5) for 60 min and PAH (5 mg mL^{-1} in 1 M potassium acetate buffer pH 5) for 15 min, with intermediate water (at 4 °C) washing. Finally, three additional polyelectrolyte layers were deposited on top to prevent desorption of the crystals with time. The films were rinsed with water (4 °C) and dried with a gentle stream of nitrogen after deposition of each layer. The LbL assembly of uncoated catalase crystals was performed in the same way, with the exceptions that the precursor film was PEI/PSS/(PAH/PSS)$_2$ and the alternating polyelectrolyte was PSS. Polyelectrolytes used: PSS (M_w 70000, Aldrich), PAH (M_w 15000, Aldrich), PEI (M_w 25000, Aldrich).

(9) The piranha-cleaned, polyelectrolyte-coated QCM electrodes (9 MHz, Kyushu Dentsu, Japan) were incubated in the crystal suspension for 60 min. No difference in ΔF was observed if the electrodes were incubated in the crystal suspension for longer times, indicating that saturation coverage was reached after 60 min. All frequencies were measured in air.

(10) The amount of enzyme deposited was calculated from the decrease in ΔF using the Sauerbrey equation [Sauerbrey, G. *Z. Phys.* **1959**, *155*, 206]. A 1 Hz decrease in frequency corresponds to a 0.868 ng increase in mass (electrode area = 0.16 cm^2 per side; two sides used). The mass of encapsulating polyelectrolyte was assumed to be negligible.

(11) Solubilized catalase was assembled alternately with PAH at pH 7.0 on a precursor film of PEI/(PSS/PAH)$_2$.

(12) SEM measurements were performed on Au sputter-coated samples prepared on QCM electrodes with a Zeiss DSM 940 instrument.

(13) The optical micrographs were obtained with an Olympus *Provis* (Japan) microscope.

10.1021/ja015807l CCC: $20.00
Published on Web 07/27/2001

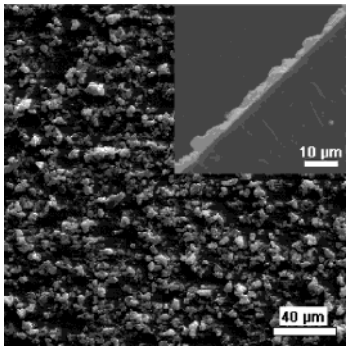

Figure 2. SEM micrographs of the surface and a cross-section (inset) of multilayer films composed of four alternating layers of PSS-coated catalase crystals and PAH, deposited on polyelectrolyte-coated Au QCM electrodes.

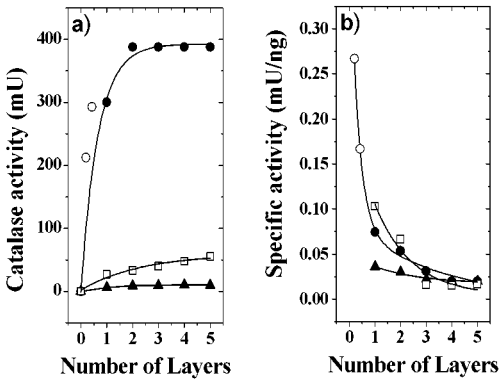

Figure 3. (a) Total film and (b) specific enzymatic activity as a function of number of layers for $(PAH/PSS)_2$/PSS-coated catalase microcrystals (circles), uncoated catalase microcrystals (squares), and solubilized catalase (triangles). Curves are drawn to guide the eye.

Prior to the measurement of the biocatalytic activity, the films were immersed in 50 mM potassium phosphate buffer at pH 7.0 for 2 h to solubilize the enzyme (catalase can be solubilized at pH < 4 and pH > 6.[7]) The polyelectrolyte capsules containing the catalase were enlarged and became more spherical.[14] This morphology change is due to the osmotic pressure buildup in the capsules, caused by the high concentration of dissolved enzyme. Although solubilization of the enzyme crystals occurs because the polyelectrolyte multilayers are permeable to small molecules (e.g., ions and water),[15] the catalase is retained within the polyelectrolyte capsules.[7] Proof that the solubilized enzyme was retained in the film was verified by QCM measurements on air-dried electrodes, which showed no change in ΔF before and after enzyme crystal solubilization. The films were also found to be stable for periods greater than several weeks.

The catalase activity[16] of multilayer films prepared from $(PAH/PSS)_2$/PSS-coated catalase crystals, uncoated catalase crystals, and solubilized catalase are shown in Figure 3. The first layer of encapsulated crystals shows an activity of 300 mU, which is approximately 10 and 50 times higher than those of the corresponding films formed from uncoated crystals and solubilized catalase, respectively. Deposition of a second layer of polyelectrolyte-encapsulated catalase crystals increased the total film activity by a further 30%, after which a plateau was reached. It is highlighted that even films formed by the deposition of polyelectrolyte-coated catalase crystals for only 10 or 30 s (open circles)[17] show enzymatic activities about 8 times higher than those prepared by the LbL assembly of uncoated crystals, and 20−30-fold larger than films of multiple (e.g., five) layers of solubilized catalase. For LbL-assembled films of solubilized enzyme and polyelectrolyte, substrate accessibility becomes more restricted as the number of enzyme layers is increased due to permeation and film structure influences.[5a,6b] For the films of polyelectrolyte-encapsulated enzyme microcrystals, where there is a large (saturation) amount of enzyme on the surface, the measured film activity plateaus because of the limited diffusion of substrate into the enzyme film (diffusion-limitation).[18] The multilayer films of encapsulated crystals, however, clearly display the highest total film enzymatic activities because they contain more catalase. The specific enzymatic activity decreases with increasing enzyme layer number for the films prepared (Figure 3b). The specific activity is similar for both types of catalase crystal films, showing that encapsulating the enzyme crystals does not cause significant denaturation of the enzyme.[7] The lower specific activity for the solubilized catalase is likely due to its direct interaction with polyelectrolyte. In contrast to enzyme entrapment methods (e.g., immobilization within polymer matrixes), where high enzyme loadings can also be obtained, the films presented here are relatively thin (few μm vs hundreds of μm), thereby providing faster response times when used as biosensors.[19]

In summary, the LbL preparation of multilayer films from polyelectrolyte-encapsulated enzyme crystals provides a promising and facile route to high enzyme content films with high enzymatic activities. The generality and simplicity of the approach makes it attractive for the preparation of related films from crystallized (and even amorphous) compounds. In addition, multicomponent films with complex architectures can be produced by judicious selection of enzyme microcrystals and film design, hence representing a major improvement over other techniques currently employed.

Acknowledgment. Financial support from the BMBF (Biofuture initiative), the DAAD, and the DFG (INK 16B1-1) is acknowledged. D. Wang is thanked for help with the SEM measurements, and U. Wollenberger and Y. Lvov for valuable discussions.

Supporting Information Available: Optical micrographs showing the polyelectrolyte-encapsulated catalase crystals on substrates in crystalline and solubilized form (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) See Supporting Information.

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(16) The activity of catalase in the film was determined by measuring the kinetics of the substrate H_2O_2 decomposition on an UV−vis (HP 8453) spectrophotometer. After solubilization of the enzyme in the film, the QCM electrode was mounted vertically on the top of a quartz cuvette filled with 2 mL of 21 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0), with the top edge of the QCM electrode just under the solution. The absorbance change at 240 nm was recorded under conditions of vigorous stirring of the solution. The total enzymatic film activity was corrected to correlate to the enzyme deposited on the gold-coated section of the quartz electrode, as only this part of the QCM gives rise to the measured frequency change. One unit of catalase will decompose 1 μmol of H_2O_2 per minute at pH 7 and 25 °C. The activity of the immobilized catalase was calculated from the decrease in absorbance by using the extinction coefficient of 0.041 $mmol^{-1}$ cm^{-1} at 240 nm. [Boehringer Mannheim, Biochemica Information, first edition, p 15, 1987].

(17) "Layer numbers" for the 10 and 30 s data were calculated as the ratio of catalase amount deposited (for these times) to those for a single deposition step at equilibrium adsorption (layer number = 1) from QCM.

(18) Substrate (H_2O_2) diffusion to the catalase appears to be also limited by the O_2 (bubbles) produced within the film.

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