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A control over accessibility of immobilized enzymes through porous coating layer

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Abstract

We report immobilization of an enzyme by layer-by-layer (LbL) film deposition technique. All the enzyme layers, including the inner ones, contributed to the activity. We put-forwarded additional coating layers to protect the enzymes. To control the accessibility of the enzymes beneath the coating layer, pores have been introduced. Our results show controlled accessibility of immobilized enzymes in solid-state matrices.

Keywords: Layer-by-layer assembly; Immobilization; Enzymes; Horseradish peroxidase

1. Introduction

One of the key challenges in the forefront of rapidly emerging biomaterial science and biotechnology is to tailor the spatial organization of protein molecules within artificial molecular systems. The task found an immense application in the development of protein-based electronic devices, such as biosensors [1–5]. Fast detection speed, high sensitivity and inherent selectivity have made the enzymatic biosensors very attractive and useful in analytical work. The immobilization of the enzyme on a solid support is a fundamental step in the design of stable and sensitive biosensors. Traditional immobilization techniques include Langmuir–Blodgett deposition method or certain biospecific interactions like antigen–antibody interactions or avidin–biotin interactions [6–8]. However, denaturation of the protein molecules as well as the restricted permeability of the substrate inside those layered architecture limit the extensive applicability of these methods.

Layer-by-layer (LbL) film deposition technique, which relies on alternate adsorption of cationic and anionic polyelectrolytes, has recently emerged as a promising approach for the preparation of ultrathin films with specifically designed architecture [9–11]. The simple and elegant deposition technique was first introduced by Decher et al. [12] for linear polyelectrolytes and later extended to proteins [13–16], enzymes and nanoparticles [17–20]. In general, film growth proceeds through electrostatic interaction by alternatively exposing a solid substrate to polycationic and polyanionic solutions. Each exposure results in an overcompensation of charges and could be continued over many layers, allowing an unprecedented level of control over the composition of multilayer assemblies. Several reports on the use of such LbL assembly of enzymes showed that proteins or enzymes embedded within polyelectrolyte multilayer (PEM) films retain their biological activities [21–23] indicating that the secondary conformation of the enzymes are restored to a major extent within those multilayered assemblies [10,24–26]. In addition, these polyelectrolytes also increase the electron current within the layered structure by overcoming the intrinsic barrier of direct electron transfer at the active site of enzymes which is mostly shielded inside the hydrophobic domain of proteins [27]. Thus, LbL self-assembling has been evolved as a potential method for investigating the structure–function relationship between biomolecules and biomembranes [28].

Horseradish peroxidase (HRP) is an oxidoreductase, which catalyses the reaction between a hydrogen donor and an ac-

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ceptor, mostly H_2O_2. Incorporation of HRP within PEM films hence offers an excellent opportunity for tailoring the behavior of corresponding enzyme multilayer electrodes as H_2O_2 sensors [29].

Previously it has reported that nanopores can be incorporated in some specific polymer films by changing its acid environment. The pore volume can be tuned simply by controlling pH of medium. Poly(allylamine hydrochloride)/poly(acrylic acid) (PAH/PAA) films, adsorbed at certain condition, show this type of behavior [30].

In the present paper, we have used PAH/PAA films as a coating layer on the enzyme layer, so that enzymes can be accessed through these nanopores. Since size of pores can be tuned, the accessibility of the enzymes beneath the PAH/PAA coating layer can also be controlled by changing the pH environment of these coated films. We have shown here that the enzyme activity accessed through the pores may reach a maximum when plotted as a function of pH of the environment.

The controlled accessibility of enzyme can restrict catalyzing process in various reactions. The activity of enzyme can be controlled through this process according to our demand. Furthermore this method can be extended to drugs, pesticides, cosmetics and many other materials with polyelectrolytes on their surface for adsorption on solid substrates. The theme will open up a new route in achieving controllability of such materials.

2. Experimental

2.1. Materials and equipments

Poly(allylamine hydrochloride) (PAH, $M_W = 15{,}000$, cationic polyelectrolyte); Poly(sodium 4-styrene sulfonate) (PSS, $M_W = 70{,}000$, anionic polyelectrolyte); Poly(acrylic acid) (PAA, $M_W = 240{,}000$, anionic polyelectrolyte) and Guaiacol were purchased from Aldrich Chemical Company. Horseradish peroxidase (HRP, RZ > 2.0, 205 U/mg) was obtained from SRL, India and was used as received. Hydrogen peroxide (30% w/v solution) was purchased from Ranbaxy, India. All other reagents were of high analytical grade and used without any further purification. The polyelectrolyte solutions as well as buffer solutions were prepared using Millipore MilliQ deionized water ($\rho = 18\ \mathrm{M\Omega\,cm}$) and pH of the solutions was adjusted with either HCl or NaOH. UV–visible absorption spectra as well as enzymatic activities were measured using a Shimadzu 1700 spectrophotometer. pH balance and measuring was carried out with a Kent EIL-7045/46 pH meter. Atomic force microscopic (AFM) images were acquired by Molecular imaging, USA, model Picoplus. Scanning electron microscope (SEM) images were taken by a Hitachi Scanning Electron Microscope.

2.2. Methods

2.2.1. Fabrication of multilayer films containing HRP on glass slides

LbL film deposition technique used for deposition of multilayer films of enzyme with polyelectrolytes is as follows. First, aqueous solutions of the polyelectrolytes (PAH, PSS, PAA) were prepared at a concentration of 10^{-2} M. The pH levels of PAH and PSS solutions were adjusted at 5.5 and that of PAA solution at 3.5 with HCl and NaOH solution, respectively [9,24, 31,32]. The strength of HRP solution was kept at 0.5 mg ml^{-1} and its pH was maintained at 5.5. At this pH, the enzyme was positively charged [1,10,22]. Glass slides were cleaned through sonication with soap solution, deionized water, followed by methanol and again deionized water for 30, 20, 20, and 30 min, respectively. The clean slides were then kept in a solution containing NH_4OH, H_2O_2, and H_2O in a 1:1:5 (v/v) proportion for 30 min in order to make the surfaces of the slides completely hydrophilic. Finally, the slides were dried through hot air gun.

Film deposition process initiated by dipping the slides in a PAH solution for 10 min followed by rinsing in three water baths with light agitation for 2, 2, and 1 min, respectively. The slides were then immersed in an oppositely charged polyelectrolyte (PSS) solution for another 10 min followed by same rinsing protocol to complete one bilayer. The dipping processes were repeated until a desired number of bilayers were formed. The PAH/PSS precursor layers gave a surface, sufficiently charged to allow a better adsorption of the proteins. HRP was deposited in alternation with PSS or PAA. As compared to the polyelectrolyte adsorption, the dipping time for the enzyme was a little longer (15–20 min) due to its high molecular weight and less charge content. Moreover, the dipping process for HRP/PSS or HRP/PAA was carried out 4 °C. One to three bilayers of HRP/PSS or HRP/PAA were deposited. After HRP adsorption as the outermost layer, the film was again dipped in the PSS solution in certain cases. Finally, the substrates were dried by blowing cold air.

2.2.2. Formation of micropores in enzyme multilayers

Ultrathin protein films thus formed as outlined above were further coated with 1–3 bilayers of PAH/PAA. PAH and PAA solution used for this step had a pH of 7.5 and 3.5, respectively. To introduce pores in the PAH/PAA layers, the films were dipped in a pH-controlled water bath for 15 s and rinsed in the deionized water for 60 s [30]. A series of solutions with pHs ranging from 1.8 to 3.2 in step of 0.2 was used to control pore formation in the PAH/PAA films.

2.2.3. Enzyme activity assays

HRP is a redox enzyme, which catalyzes the reaction between a hydrogen donor and an acceptor. HRP films therefore were assayed with guaiacol and H_2O_2 as substrates. The kinetics of enzymatic oxidation of guaiacol to guaiacol dehydrogenation product (GDHP) was monitored spectrophotometrically. To measure the activity of HRP multilayers, the films were immersed in a 3 mL 0.1 M sodium phosphate buffer (pH = 7.0, optimum value for highest activity of HRP) solution at 25 °C in a cuvette, to which 83.3 μL guaiacol stock solution (18 mM) was added followed by 40 μL H_2O_2 stock solution (8 mM) to attain final concentration of 0.5 and 0.1 mM, respectively. The progress of the reaction was monitored for 10 min by the change in absorbance at 436 nm, which was attributed to the λ_{Max} of the oxidized product of guaiacol, GDHP