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Notes

Multi-Biocatalytic Properties of Layer-by-Layer Assembled Lysozyme/Catalase Multilayers

Bokyoung Lee[1], Sungwoo Kim[1], and Jinhan Cho*[,2]

[1]*School of Advanced Materials Engineering, Kookmin University, Seoul 136-713, Korea*
[2]*Department of Chemical & Biological Engineering, Korea University, Seoul 136-713, Korea*

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Introduction

The area of electrochemical biosensors has been significantly advanced over the past few years with the development of enzyme biosensors, immunosensors, and DNA sensors.[1-6] Although these sensors are being successfully used in clinical chemistry, the food industry and various environmental fields, much research remains for the realization of sensing devices with high specificity, high sensitivity, multi-sensing capabilities and flexibility of use. Recently, the use of layer-by-layer (LbL) assembly for the design of electrochemical biosensors has attracted extensive attention.[7-16] An important advantage of this method is that it allows for the preparation of films with controlled thickness, composition and functionality on substrates of different shapes and sizes. The basic principle of LbL assembly is the alternate adsorption of oppositely charged polyelectrolytes (PEs) on solid surfaces using complementary interactions (i.e., electrostatic, hydrogen-bonding or covalent interactions); this technique has recently been extended to fabricate multilayered protein films. For example, electrochemical sensors based on enzyme/PE[9,10] or enzyme/inorganic nanoparticle[11,12] multilayers have notably improved sensitivities compared to devices with single enzyme layers as a result of increased enzyme absorption within the 3D nanoarchitecture. However, it should be noted that such approaches have mainly focused on a single enzymatic function. That is, oppositely charged components, such as conventional PEs and inorganic components, have been used as building blocks for enzyme multilayers. Generally, enzymatic activity is strongly dependent on the substrate diffusion limitations caused by the amount of enzyme adsorbed and the diffusion limitation of the encapsulated films.[13] Therefore, considering that next generation sensing devices will require more and more multi-detection properties with higher sensitivities and simpler operation, it would be advantageous for various enzyme layers with different enzymatic functions to be electrostatically assembled for enzyme/enzyme multilayer structures, excluding unnecessary building blocks such as conventional PEs. Additionally, both the electrostatic bonding between two different enzymes and the amount of enzyme adsorbed should be optimized for a multilayer structure and a high degree of sensitivity. In light of these requirements, the pH-dependent electrostatic properties of amino acid residues in enzymes that allow for electrostatic bonding are important in designing enzyme/enzyme multilayers. However, to our knowledge, few studies have investigated the preparation and optimized multi-sensing functions of enzyme/enzyme multilayers using pH control of two different enzyme solutions. Therefore, our motivation is to employ two different kinds of enzymes with cationic and anionic charges as building blocks in the given pH range to realize the muti-biocatalytic properties of LbL-assembled enzyme/enzyme multilayers.

Here, we investigate the pH-dependent electrostatic and multi-sensing characteristics of LbL-assembled multilayers prepared from two different enzymes with different isoelectric points (*pI*). Catalase (CAT), with *pI*~5.6 and lysozyme (LYS), with *pI*~10.1, were employed as anionic and cationic species in the solution pH range of 6 to 9, respectively. The adsorbed amounts of the CAT/LYS multilayers were found to be significantly increased at pH 6 and 9 with low charge density (i.e., pH 6 for CAT and pH 9 for LYS).

CAT exhibits electrocatalytic behavior toward H_2O_2, while LYS breaks down bacterial cell walls by facilitating the hydrolysis of glycosidic bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid. Therefore, the films composed of these enzyme multilayers would have integrated biocatalytic properties that could decompose both reactive oxygen species and the cell walls of gram-positive bacteria such as *Micrococcus lysodeikticus*, simultaneously. This work is very significant in that the enzyme multilayers formed through solution pH control can provide a basis for multi-sensing devices with high sensitivities. Therefore, we believe that our approach can be used to exert a wide range of control over the preparation of various enzyme-based multilayers displaying high biocatalytic and integrated activities.

Experimental

Materials. Catalase (CAT) (from bovine liver) and cationic lysozyme (LYS) (from chicken egg white) were purchased from Aldrich. In this case, the solution pH of CAT

*Corresponding Author. E-mail: jinhan71@korea.ac.kr

(*pI*~5.6) and LYS (*pI*~10.1) was controlled in the range of 6~9 and resultantly CAT and LYS were used as anionic and cationic components for the build-up of enzyme/enzyme multilayers in the employed pH range, respectively. The solution concentration of CAT and LYS was fixed at 1 mg·mL^{-1}. More detailed experimental methods were shown in Supporting Information.

Results and Discussion

The *pI* measured from the zeta (ζ) potential of CAT was 5.6, which implies that CAT has an overall positive charge at pH < 5.6 and an overall negative charge at pH > 5.6. On the other hand, the *pI* of lysozyme was measured to be about 10.1. This phenomenon suggests that CAT can be electrostatically assembled with LYS in the pH range of 6 to 9, and, furthermore, the adsorbed amount of these enzymes could be significantly influenced by the solution pH owing to the presence of amine (-NH_2) and carboxylic acid groups (-COOH) on the amino acid residues of the enzymes. To confirm these pH-dependent properties, we measured the amount of CAT and LYS adsorbed as the bilayer number (n) of $(LYS/CAT)_n$ multilayers increased as a function of enzyme solution pH. The LbL assembly of CAT and LYS was performed on quartz substrates, and the growth of the LYS/CAT multilayers was monitored by UV-vis spectroscopy and quartz crystal microbalance (QCM) measurements (Figure 1(a) and (b)). In this case, it was observed that the average incremental absorbance per bilayer significantly increased with the pH value for enzymes with low zeta potentials. More specifically, for the LYS/CAT multilayer assembly at pH 6, the zeta potentials of LYS and CAT displayed the highest positive value (about +15 mV) and the lowest negative value (about -5 mV), respectively, in the given pH range (pH 6-9) (Supporting Information, Figure S2). Therefore, for charge overcompensation, a relatively large amount of CAT was deposited on the LYS layer with highly positive charges.[17,18] Conversely, at pH 9, the large increase in the adsorbed amount of LYS (ΔF≈68 Hz and Δm≈1,180 ng·cm^{-2} per layer) and CAT (ΔF≈43 Hz and Δm≈757.8 ng·cm^{-2} per layer) was caused by LYS containing

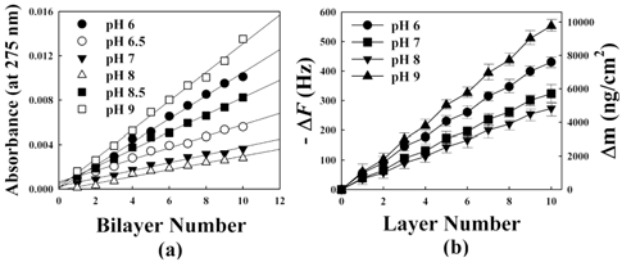

Figure 1. UV-vis absorbance of $(LYS/CAT)_n$ multilayers measured with increasing bilayer number (n) as a function of solution pH. (b) Frequency and mass changes in the LYS/CAT multilayers with increasing the layer number.

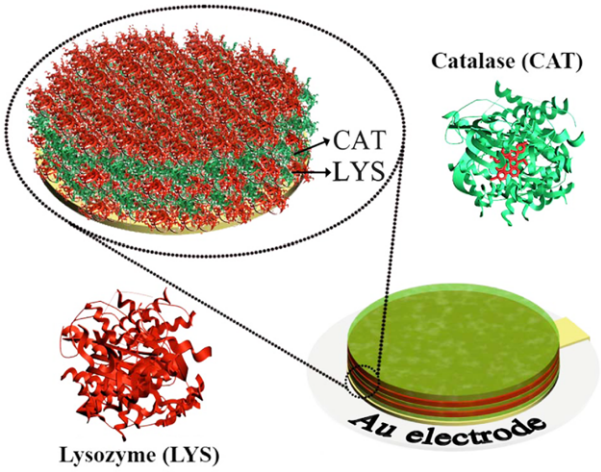

Figure 2. Schematic for LYS/CAT multilayer-based electrochemical sensors prepared onto gold electrode.

a low amount of positive charges. Additionally, the adsorbed amount of enzymes deposited at pH 7 and 8 was relatively small in comparison with that at pH 6 and 9. For more clarifying our results, we investigated the film thicknesses using ellipsometry. In this case, LYS/CAT multilayers were deposited onto Si wafer. Although two different kinds of substrates (i.e., gold substrate for QCM and Si wafer for ellipsometry) were used for QCM and ellipsometric tests, the total adsorbed amount of LbL-assembled layers were not significantly influenced by a kind of used substrates. As a result, the total film thicknesses of $(LYS/CAT)_5$ films were measured to be 58.5, 44.0, 37.2, and 75.3 Å at pH 6, 7, 8 and 9, respectively.

Based on these thicknesses, the hybrid electrochemical sensors composed of LYS and CAT were designed as shown in Figure 2. The notable characteristic of the $(LYS/CAT)_n$ multilayers is that these films exhibited excellent electrocatalytic sensing toward H_2O_2, as CAT is an efficient catalyst for H_2O_2,[6,12] as found by observing the oxidation of tryptophan (Trp) residues in hen egg white lysozymes by H_2O_2.[19-21]

In order to test our hypotheses, three different kinds of multilayer-modified electrodes were prepared at pH 9: (LYS/poly(styrene sulfonate), (PSS))$_5$, (poly(allylamine hydrochloride), (PAH/CAT))$_5$, and $(LYS/CAT)_5$ multilayer-coated gold electrodes. In this case, PSS and PAH were used as negatively and positively charged PEs without any electrochemical properties, respectively. As shown in Figure 3(a), the electrode modified with the $(LYS/CAT)_5$ multilayers showed the strongest redox current, suggesting that the LYS/CAT multilayers had the highest sensitivity to H_2O_2. It should be noted here that the adsorbed amounts of LYS (Δm≈1,180 ng·cm^{-2} per layer) and CAT (Δm≈757.8 ng·cm^{-2} per layer) in the LYS/CAT multilayers were lower than those of LYS (Δm≈1,205 ng·cm^{-2} per layer) in the LYS/PSS multilayers and CAT (Δm≈1,278 ng·cm^{-2} per layer) in the

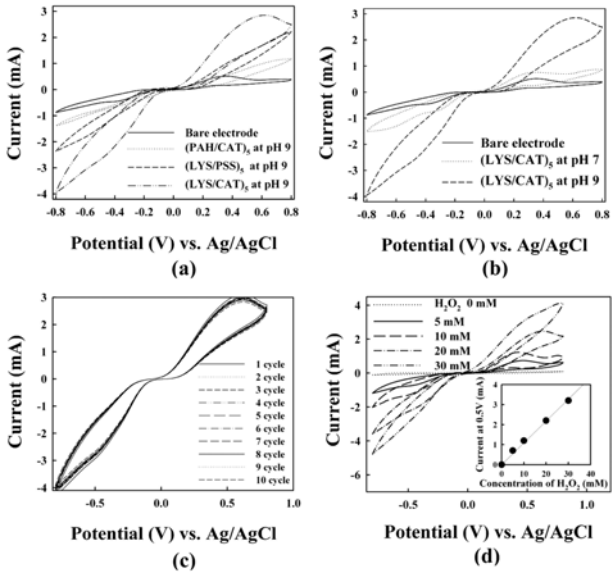

Figure 3. (a) Cyclic voltamograms of bare electrode and $(LYS/CAT)_5$, $(LYS/PSS)_5$, and $(PAH/CAT)_5$ multilayer-modified electrodes. In this case, the multilayers were alternately deposited at solution pH 9. (b) Cyclic voltamograms of bare electrode and $(LYS/CAT)_5$ multilayer-modified electrodes prepared at solution pH 7 and 9, respectively. (c) Cycling test in cyclic voltamograms of $(LYS/CAT)_5$ multilayer-coated electrode. Here, the cyclic voltamogram measurements shown in Figure 3(a)-(c) were performed in PBS solution of pH 7.0 containing 21 mM of H_2O_2 and the scan rate was 50 $mV \cdot s^{-1}$. (d) Cyclic voltammograms of $(LYS/CAT)_5$ modified electrode in pH 7 containing 5, 10, 20, and 30 mM H_2O_2. The inset indicates the calibration curve of the amperometric responses of electrode modified with multilayes.

PAH/CAT multilayers. These results imply that the multilayers composed of both enzymes were the most effective in sensing reactive oxygen species, such as H_2O_2, due to synergetic effects between LYS and CAT. In addition, the electrochemical sensitivity of the enzyme multilayers containing the largest adsorbed amounts of LYS and CAT at pH 9 was superior to that of the multilayers assembled at pH 6, suggesting that the sensitivity was proportional to the amount of CAT and LYS adsorbed (Figure 3(b)). Furthermore, continuous cycling tests confirmed that the enzyme multilayers were quite stable. That is, the negligible change observed in the redox peaks during cycling tests indicated that these films were electrochemically and structurally stable (Figure 3(c)). Additionally, the peak current at $(LYS/CAT)_5$ multilayer-coated electrode was linearly enhanced with increasing the concentration of H_2O_2 from 5 to 30 mM (Figure 3(d)).

The additional advantage of the $(LYS/CAT)_n$ multilayers is that the inserted LYS layers had anti-bacterial properties achieved by breaking down the polysaccharide walls of many kinds of bacteria. For this study, the catalytic property of LYS in multilayers was investigated with an Enzchek lysozyme assay kit (Invitrogen) using fluorescence-labeled

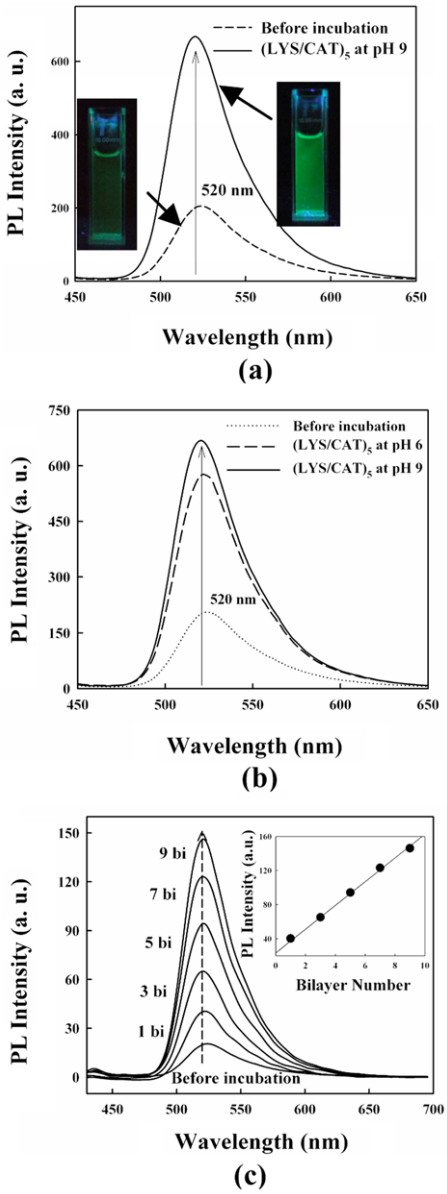

Figure 4. (a) PL spectra of $(LYS/CAT)_5$ multilayers deposited at pH 9 before and after incubation. The inset indicates fluorescence images of buffer solution before and after incubation. (b) PL spectra of $(LYS/CAT)_5$ multilayers assembled at pH 9 and 6 after incubation. (c) PL spectra of $(LYS/CAT)_n$ multilayers at pH 9 as a function of bilayer number after incubation. Here, all the PL spectra shown in Figure 4(a)-(c) was measured in pH 7.5 reaction buffer solution containing 0.02 mg/mL fluorescein binding micrococcus lysodeikticus. Excitation wavelength was 380 nm.

Micrococcus lysodeikticus cells (Figure 4(a)). Briefly, the assay measures lysozyme activity (i.e., hydrolysis) on *Micrococcus lysodeikticus* cell walls through fluorescence quenching.[22-24] The biocatalytic property of lysozymes relieves this quenching, yielding an increase in fluorescence intensity that is proportional to lysozyme activity. When the (LYS/

$CAT)_5$ multilayer film at pH 9 was dipped into a buffer solution containing fluorescence-labeled *Micrococcus lysodeikticus* cells and was incubated at 37 °C for 30 min, the fluorescence intensity of the buffer solution measured at 520 nm was 3.5 times higher than that of the buffer solution measured before incubation. Considering that the lysozyme activity is closely related to the amount of LYS adsorbed in the multilayers, it is reasonable then to expect the fluorescence intensity from the $(LYS/CAT)_5$ multilayers at pH 6 to be lower than that from the (LYS pH9/CAT pH9)$_5$ multilayers mentioned above (Figure 4(b)).

We therefore tried to control the lysozyme activity as a function of the bilayer number (n) of $(LYS/CAT)_n$ multilayers at pH 9. As shown in Figure 4(c), the fluorescence intensity from the lysozyme activity increased linearly with increasing bilayer number of the multilayers. We also did not observe any notable lysozyme denaturation for incubation time up to 60 min in view of surface structure and kinetic study (Supporting Information, Figure S2). These observations suggest that this approach based on LYS/CAT multilayers could easily be optimized for biocatalytic sensitivity of enzyme sensors by controlling the solution pH and bilayer number and could additionally be used to design integrated biocatalytic properties. Furthermore, we would like to highlight the fact that enzyme/enzyme multilayers can simplify multilayer structures and maximize integrated biocatalytic properties with a defined deposition cycle as compared to conventional enzyme/PE (e.g., cationic PAH or anionic PSS without any biocatalytic properties) multilayers.

Conclusions

We have demonstrated that LYS/CAT multilayers with highly integrated biocatalytic properties can be prepared through solution pH control by considering the *pI* of the two enzymes utilized. The adsorbed amount of the respective enzymes was significantly increased at the solution pH condition corresponding to relatively low zeta potentials for CAT (i.e., pH 6) or LYS (i.e., pH 9). Particularly, the (LYS pH 9/CAT pH 9)$_n$ multilayers with maximized loading amounts of the two different enzymes displayed a high degree of electrocatalytic sensing toward H_2O_2 and of lysozyme activity toward *Micrococcus lysodeikticus* cells. Our approach based on pH-controlled enzyme/enzyme multilayers is expected to be beneficial in the preparation of various biocatalytic instruments for applications requiring more integrated biocatalytic properties and simplified multilayer structures with a high degree of sensitivity.

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Supporting Information: Information is available regarding the experimental details, zeta potentionals, and surface morphology. The materials are available via the Internet at http://www.springer.com/13233.

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