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A Polymer Platform Composed of Structurally Well-defined Stereocomplex Structures for Efficient Enzymatic Reactions

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Previously we reported that β-galactosidase immobilized on the structurally well-defined poly(methyl methacrylate) (PMMA) stereocomplex films fabricated by layer-by-layer (LbL) assembly of isotactic (it) and syndiotactic (st) PMMAs on solid substrates were highly active when compared to the enzyme immobilized on the single-component films, and that a slight difference of polymer surface structures strongly affects activities of immobilized enzymes, even though polymers have the same chemical component (*Chem. Mater.*, **19**, 2174-2179 (2007)). In this paper, we demonstrated activities of other enzymes immobilized on the PMMA films. Activities of immobilized alkaline phosphatase (ALP) were measured by following hydrolysis rate of fluorescence substrate 4-methylumbelliferyl phosphate (4-MUP). Initial velocity of ALP hydrolysis reaction immobilized on the complex film was approximately 2-fold faster than those on the single-component it-PMMA and atactic (at) PMMA films. Michaelis-Menten analyses revealed that catalytic efficiency (k_{cat}/K_m) of ALP was strongly dependent on conformational regulation of PMMA chains at film surfaces. Quartz crystal microbalance (QCM) analyses revealed that immobilization amount of ALP on the complex was greater than those on the single-component films. Similar dependencies were also observed in the case of cellulases that degrade polymeric cellulose under acidic condition. Secondary structural analyses using an attenuated total reflection infrared (ATR-IR) spectroscopy revealed that structural denaturation of the enzyme after immobilization processes was well-suppressed on the complex film, although the enzymes on the single-component PMMA films were relatively denatured. We propose here that PMMA stereocomplex films are a generally convenient platform for immobilization of functional proteins and could be used under various conditions without loss of their activities.

Key words: poly(methyl methacrylate), stereocomplex, layer-by-layer assembly method, enzyme reaction

1. INTRODUCTION

The surface modification of solid supports has attracted much attention in biomedical and bioengineering fields such as immunoassays [1-3], diagnostics [4], and DNA/protein microchips [5-7]. Unlike DNA, the chemical and physical properties of protein are often changed upon immobilization on solid. It is important to choose a suitable surface as a support matrix as well as an appropriate immobilization method to minimize losses in activities of immobilized proteins.

Recently, we reported that stereocomplex films composed of structurally well-defined PMMA with double-stranded helical structures [8-9] were suitable platforms for immobilization of functional proteins such as antibodies [10] and enzyme (β-galacotisdase) [11], and proposed that a more ordered conformation of PMMA on the coating surface is more effective for maintaining the native activity of the immobilized proteins. The complex films composed of stereoregular it- and st-PMMA chains could be easily fabricated by the simple LbL assembly method [12-13] on any solid supports [14-18]. And physical approaches for protein immobilization are simple methods that allow effective loading without chemically modifying of protein molecules and could be applied for almost of proteins.

To further demonstrate generality of our proposal, in this study, we investigated activities of other enzymes immobilized on PMMA-coated surfaces. ALP [19] and cellulase [20], of which the three dimensional structures and the optimum reaction conditions are different from those of β-galactosidase, were chosen as model proteins, and the effect of the surface PMMA conformation on activities of enzymes has been characterized.

2. EXPERIMENTAL

2.1 PMMA films fabrication

PMMA stereocomplex ultrathin films were prepared on 96-well multiplates (Immuno 96 Microwell Plates, Nunc) by the LbL method as previously described [11]. Each well was filled with 200 μL of it-PMMA solution in acetonitrile (the starting polymer, M_n = 19 000, M_w/M_n = 1.10, *mm* > 98%, Polymer Source, Inc., 1.7 mg

mL^{-1}) and left for 5 min at room temperature. After removing the solution, each well was rinsed with 200 μL of acetonitrile three times. Subsequently, it-PMMA-physisorbed wells were filled with 200 μL of st-PMMA solution in acetonitrile (M_n = 18 600, M_w/M_n = 1.23, *rr* > 85%, Polymer Source, Inc., 1.7 mg mL^{-1}) and left for 5 min at room temperature (at this process, the stereocomplexation was completed), followed by removing the solution and rinsing in the same manner. After fourteen steps of LbL assembly, the stereocomplex films (it-PMMA/st-PMMA)$_7$ with approximately 4.8 nm thickness were obtained. Single-component films were fabricated on wells by filling with 200 μL of it- or at-PMMA (M_n = 22 500, M_w/M_n = 1.03, Polysciences, Inc.) solutions at a 0.17 mg mL^{-1} and left at room temperature until acetonitrile had completely evaporated, followed by rinsing with Milli-Q water.

2.2 Immobilization and activity analysis of enzymes on PMMA films

Enzyme immobilization on the fabricated PMMA films were performed as previously described [11]. A solution of ALP from *Escherichia coli* (96 kDa, lot CEH0261, Wako) at 1 μM was prepared in 100 mM of Tris-HCl buffer (pH 8.0). A solution of cellulase from *Tricoderma viride* (lot 114K1463, Sigma) at 1 mg mL^{-1} was prepared in acetate buffer (pH 5.0). Cellulase used here was an enzyme complex which breaks down cellulose to β-glucose and contained various types of enzyme such as endogulcanase, exglucanase, and β-glucosidase. The PMMA-coated wells were filled with enzyme solution and incubated for 1 h at 37 °C, followed by rinsing with the buffer solution. Amounts of enzymes physically adsorbed on the film surfaces were estimated by QCM. PMMA films fabricated on a 9-MHz QCM substrate with gold electrodes (4.5 mm diameter, USI) were immersed into enzyme solutions under the aforementioned conditions and mass increases of adsorbent (Δm) were estimated from fundamental frequency decreases ($-\Delta F$) as follows: $-\Delta m$ (ng) = 0.87 × ΔF (Hz) [21]. The enzymatic activity of physisorbed ALP was determined by following ester bond cleavage of 4-MUP. The solution of 4-MUP in CAPS buffer (pH 10.0) at various concentrations was put into the well, and the changes of fluorescence emission intensity derived from hydrolytic products 4-methylumbelliferone (4-MU) were followed as a function of time using a fluorescence microplate reader (Infinete F200, Tecan, Switzerland). Reaction mixture was excited at 360 nm, and the emission intensity at 465 nm was recorded. Experiments were performed triplicate. Cellulase activity was also investigated with similar manner by using Enzchek® Cellulase Substrate blue fluorescent, 339/452 (ECS, Invitrogen).

2.3 ATR-IR analysis of ALP on PMMA films

ATR spectra of ALP physisorbed on PMMA films were obtained in air using the refractive surface of 100 nm thick gold-coated poly(ethylene terephthalate) substrates (Tanaka Precious Metals, Japan) with a Perkin-Elmer Spectrum One (USA) in air at ambient temperature. Interferograms were co-added 50 times, and Fourier transformed at a resolution of 4 cm^{-1}. Experiments were repeated three times.

3. RESULTS AND DISCUSSION

3.1 Hydrolysis reaction of 4-MUP by immobilized ALP

Stereocomplex and single-component PMMA films were prepared on solid substrates, and activities of immobilized enzymes on PMMA films were investigated quantitatively. We selected two kinds of enzymes that were active under different conditions for demonstrating the wide potential of the complex surface compared to those of single-component it- and at-PMMA surfaces and the generality of effects of surface polymer chains with regular conformation on activities of immobilized proteins.

ALP activities on PMMA films were estimated by

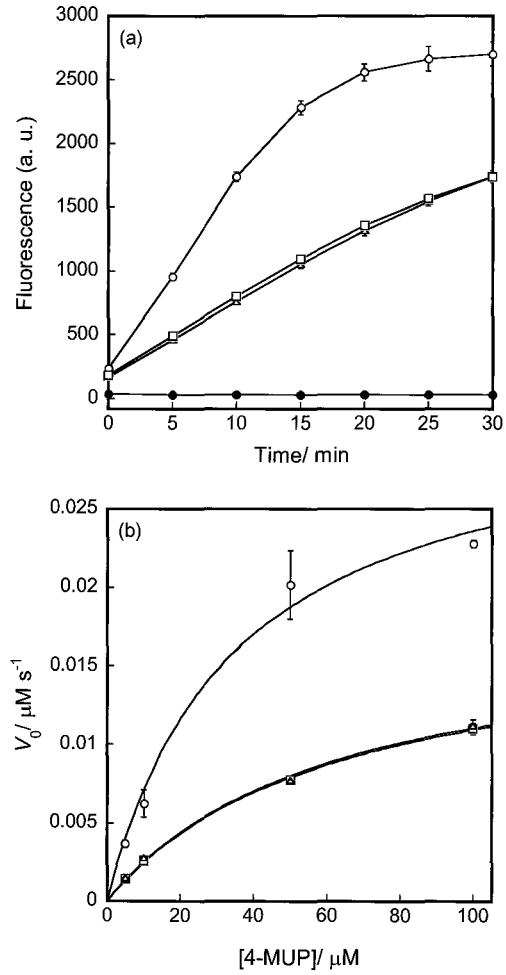

Fig. 1 (a) Enzymatic activity of ALP immobilized on PMMA-coated surfaces as a function of time; stereocomplex (open circle), it-PMMA (open triangle), and at-PMMA (open square), spontaneous on stereocomplex (closed circle). Before hydrolysis, enzyme was immobilized onto PMMA-coated surfaces at 1.0 μM of ALP. Hydrolysis conditions: 100 mM CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid, pH 10.0), 400 mM NaCl, 10 mM $MgCl_2$, [4-MUP] = 5 μM. Samples were excited at 360 ± 17.5 nm, and the fluorescence emission intensity was measured at 465 nm ± 17.5 nm. (b) Effect of substrate concentration on the initial rate of the enzyme-catalyzed reaction; stereocomplex (circle), it-PMMA (triangle), and at-PMMA (square). Lined curves are the fitting to the Michaelis-Menten equation. Plots show the mean values (n = 3) ± standard deviation.

Table I Reaction kinetics of the immobilized ALP

coating	K_m [a] (μM)	V_{0max} (μM s^{-1})	k_{cat} [b] (s^{-1})	k_{cat}/K_m [c] ($\mu M^{-1} s^{-1}$)	R^2 [d]
complex[e]	35	0.031	1.5[f]	0.043	0.99
it-PMMA[e]	62	0.018	1.2[f]	0.019	0.99
at-PMMA[e]	61	0.018	1.0[f]	0.017	0.99

[a] K_m, Michaelis constant, the substrate concentration at which the reaction rate is half-maximal. [b] k_{cat}, turnover number, the number of reaction events per enzyme molecule and seconds. [c] k_{cat}/K_m, catalytic efficiency of enzyme. [d] R^2, coefficient of determination. [e] Enzyme was immobilized onto PMMA-coated surfaces at 1.0 μM. [f] The amounts of enzyme estimated by QCM were used.

following initial rates (V_0) of the hydrolytic reaction of 4-MUP. Fig. 1a shows hydrolysis rates of 4-MUP to 4-MU by physically immobilized ALP. Reaction rate on the complex surface was approximately 2-fold faster than those on it- and at-PMMA surfaces and apparently depended on species of PMMA surfaces. Autohydrolysis of substrate on PMMA stereocomplex surfaces lacking ALP was not observed, also suggesting that products were produced by reactions of immobilized enzymes. Since reaction rate depends on total amounts of ALP immobilized (note that the practical area for enzyme immobilization was 0.94 cm^2), ALP amounts were estimated by using the QCM substrate to be 216 ± 19, 153 ± 11, and 173 ± 13 ng cm^{-2} for the complex, it-PMMA, and at-PMMA surfaces, respectively. One reason for the greater rate obtained on the complex surface was attributed to the greater amount of ALP immobilized.

V_0 values estimated from the maximum slope of time dependences from Fig. 1a were plotted against substrate concentrations as shown in Fig. 1b. In all cases, V_0 was saturated at high concentrations of 4-MUP. Michaelis-Menten parameters after standardization with ALP amounts are summarized in Table I. The large K_m values for it- and at-PMMA single-component surfaces suggested that the substrate binding ability of enzymes decreased at equilibrium state. One of the reasons might be due to orientation of enzymes on the surfaces. Turnover numbers (k_{cat}) significantly depended on the PMMA conformation at the surface, followed by changing total enzymatic activities. On the complex surface, the k_{cat} value was greatest (1.5 s^{-1}); however, on the single-component films composed of it-PMMA and at-PMMA chains, the k_{cat} values were 1.2 s^{-1} and 1.0 s^{-1}, respectively. Catalytic efficiency (k_{cat}/K_m) of ALP immobilized on the complex was greater than those on the single-component PMMAs, and these observations agreed with previous data obtained from β-galactosidase analyses [11].

3.2 Hydrolysis reaction of cellulose by immobilized cellulase

Activities of cellulase enzyme complex were also investigated using fluorescence-labeled soluble cellulose with similar manner. This substrate is heavily labeled, resulting in almost total quenching of the conjugate's fluorescence. Cellulose-catalysed degradation relieves this quenching. Degradation rates of cellulose by immobilized enzymes on PMMA films were shown in

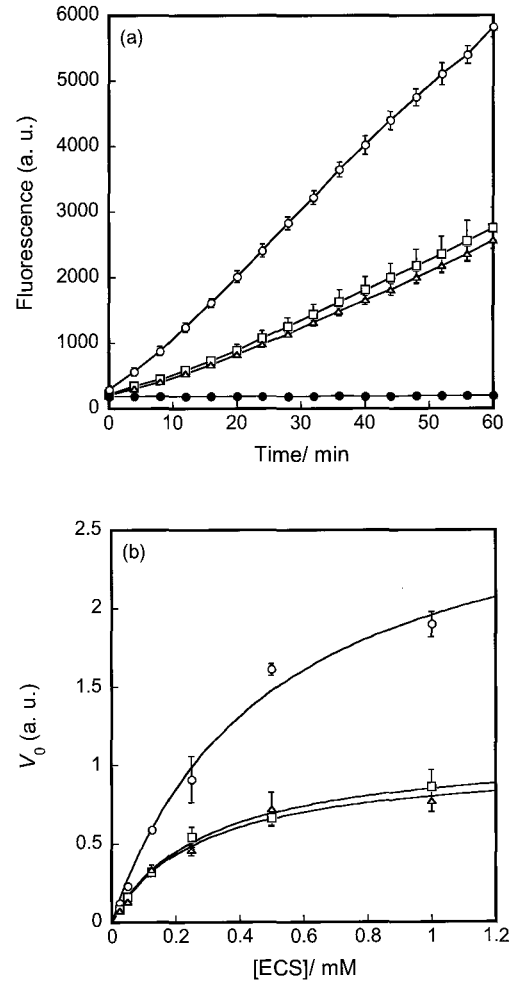

Fig. 2. (a) Enzymatic activity of cellulase immobilized on PMMA-coated surfaces as a function of time; stereocomplex (open circle), it-PMMA (open triangle), and at-PMMA (open square), spontaneous on stereocomplex (closed circle). Before hydrolysis, enzyme was immobilized onto PMMA-coated surfaces at 10 mg mL^{-1} of cellulase. Hydrolysis conditions: 100 mM acetate buffer, pH 5.0, [ECS] = 500 μM. Samples were excited at 360 ± 17.5 nm, and the fluorescence emission intensity was measured at 465 nm ± 17.5 nm. (b) Effect of substrate concentration on the initial rate of the enzyme-catalyzed reaction; stereocomplex (circle), it-PMMA (triangle), and at-PMMA (square). Lined curves are the fitting to the Michaelis-Menten equation. Plots show the mean values (n = 3) ± standard deviation.

Table II Reaction kinetics of the immobilized cellulase

coating	K_m [a] (mM)	V_{0max} [b] (a. u.)	E [c] (a. u.)	R^2 [d]
complex[e]	0.49	2.9	1.8	0.99
it-PMMA[e]	0.29	1.1	1.6	0.99
at-PMMA[e]	0.28	1.0	1.6	0.99

[a] K_m, Michaelis constant, the substrate concentration at which the reaction rate is half-maximal. [b] V_{0max}, the maximum initial velocity. [c] E, Apparent enzymatic reaction efficiency standardized with enzyme amounts. [d] R^2, coefficient of determination. [e] Enzyme was immobilized onto PMMA-coated surfaces at 1.0 mg mL^{-1}

Fig. 2a, and concentration dependencies of V_0 were shown in Fig. 2b. Kinetic parameters are summarized in Table II. Reaction rate on the complex surface was approximately 2-fold faster than those on it- and at-PMMA surfaces. Cellulase amounts were estimated by using the QCM substrate to be 324 ± 13, 236 ± 16, and 223 ± 19 ng cm^{-2} for the complex, it-PMMA, and at-PMMA surfaces, respectively. Apparent catalytic efficiency (E) after standardization with adsorbed molecules of cellulase complex on the PMMA stereocomplex was slightly greater those on it- and at-PMMA. However, in terms of apparent efficiency per area of solid supports, greater amounts of cellulase complex can adsorbed on the complex films, resulting in high degradation efficient. These results show advantage for industrial cellulose degradation by immobilized cellulose on the complex PMMA.

3.3 ATR-IR analysis of the adsorbed ALP

It is known that the amide I band of proteins (1600-1700 cm^{-1}) is sensitive to different secondary structures, where α-helices, β-sheets, β-turns, and extended coil structures absorb at different frequencies [22]. Peak frequencies of ALP immobilized on various PMMA films were observed by ATR-IR. The peak frequency of the amide I band of the free ALP powder was 1634.9 ± 0.4 cm^{-1}. The peak frequency of immobilized enzymes on it- and at-PMMA surfaces shifted to 1673.1 ± 1.2 and 1666.7 ± 0.9 cm^{-1}, respectively. These aforementioned shifts from lower wavenumber to higher wavenumbers on the surface are due to change of the protein hydration state on the surface after immobilization compared to the free protein, and unfolding of the protein on the surface took place. Therefore, the enzymes on it- and at-PMMA partially denatured following immobilization. On the other hand, the peak frequency of the enzyme on the complex also shifted (1656.9 ± 1.0 cm^{-1}); however, the degree of the shift was smaller than that observed on other surfaces. The aforementioned results also agree with previous data, suggesting that unfolding of the protein on the complex could be suppressed.

4. CONCLUSIONS

Using two kinds of enzymes, ALP and cellulase complex, as a model of functional proteins, immobilization and activities of the enzymes on PMMA ultrathin films were investigated. We demonstrated the generality that denaturing of proteins immobilized on structurally well-defined PMMA films composed of stereocomplex were suppressed. PMMA complex is acceptable for wide range of pH condition, and it is very attractive in various industrial applications. We believe that PMMA stereocomplexes could be used wide range areas in polymer surface modification and might be helpful in developing a new category of polymer bionanomaterials.

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