Superior Activities of Enzymes Physically Immobilized on Structurally Regular Poly(methyl methacrylate) Surfaces

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Ultrathin poly(methyl methacrylate) (PMMA) stereocomplex films with macromolecularly double-stranded regular nanostructures were prepared by layer-by-layer (LbL) assembly of isotactic (*it*) and syndiotactic (*st*) PMMAs on solid substrates, and these films were used for enzyme immobilization supports. Hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside (PNPG) by the immobilized β-galactosidase (β-Gal) on the complex film was 2- and 4-fold faster than those by the enzyme immobilized on single-component *it*-PMMA and atactic (*at*) PMMA films, respectively. Quartz crystal microbalance (QCM) analysis revealed that greater amounts of β-Gal were immobilized on the complex film through physical adsorption than those on the single-component films. Michaelis constants (K_m) of the enzyme were independent of film components; however, catalytic efficiencies (k_{cat}/K_m) were increased by regulation of PMMA conformation at film surfaces. Attenuated total reflection infrared (ATR-IR) analysis revealed that structural denaturation of the enzyme after immobilization processes was well-suppressed on the complex film, although the enzyme on the bare gold or single-component PMMA films were denatured. We propose here that a slight difference of polymer surface structures strongly affects activities of immobilized enzymes, even though polymers have the same chemical component.

Introduction

The immobilization of proteins in biologically active states on solid support surfaces has attracted much attention, not only because of the interesting role of immobilization in pure surface sciences but also because of the important role of immobilization in the development of biosensor chips and their applications in areas such as enzyme reactors,[1] immunoassays,[2] diagnostics,[3] and more recently, protein and peptide microarrays/chips.[4] It is well-known that unlike DNA, the chemical and physical properties of protein, often change upon immobilization. The most serious side effect associated with protein immobilization when compared to native proteins in homogeneous solutions is a loss in original protein activity. Therefore, 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.

Recent strategies have used single covalent linkages between support matrices and proteins to improve active site accessibility as well as overall conformational stability.[5] Site-directed mutagenesis permits the introduction of a single cysteine residue at a position that is known to be surface-exposed in the protein's native conformation. Through the unique SH group on the protein surface, the protein could subsequently be site-directly immobilized on thiol-activated surfaces. The aforementioned method is very practical and attractive for maintaining proteins active on immobilized surfaces; however, the method is restricted to proteins with known amino acid sequences and to proteins lacking cysteine residues.

On the other hand, 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. Therefore, many strategies have also been reported, and in this case, the physical and chemical properties of the support matrix play an important role in the activity of immobilized proteins.[6]

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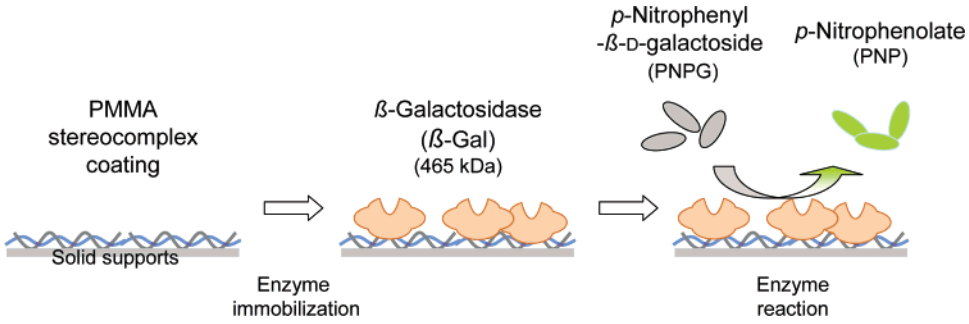

Figure 1. Schematic representation of the enzymatic hydrolysis of PNPG by the immobilized β-Gal on the double-stranded PMMA stereocomplex film.

In random immobilization with physical adsorption, the protein is usually immobilized to the support matrix via multiple amino acid groups on the protein. Therefore, the large number of bonds may result in unfolding of the active site of the protein and hence reduce its activity. In fact, it has been demonstrated that activities of proteins immobilized on solid supports coated with hydrophobic polymers are higher than activities from those that are coated with hydrophilic polymers.[7]

A PMMA stereocomplex, a crystalline-like structure with a high thermal stability composed of stereoregular *it*- and *st*-PMMA chains, adopts a double-stranded helical structure via van der Waals interactions between *it*- and *st*-PMMA chains. In PMMA stereocomplexes, *it*-PMMA is surrounded by *st*-PMMA in the length ratio of one to two.[8] In our previous studies, the alternative layer-by-layer (LbL) assembly method[9] was applied for the stereocomplexation on various solid material surfaces in a stepwise manner, fabricating ultrathin complex films with regular nanostructures at molecular level.[10]

Quantitative analysis of physical adsorption of proteins such as albumin, fibrinogen, lysozyme, and protein A on PMMA films revealed that (1) greater amounts of proteins adsorbed on the stereocomplex film surface than those on conventionally prepared spin-coated homogeneous films composed of a single PMMA component and (2) apparent adsorption constants (K_{app}) of proteins on the complex surface were commonly smaller than those on the conventional film.[11] In addition, interactions of human whole blood with the complex surface supported nondenaturing of serum proteins adsorbed.[12] These observations indicate that the protein adsorption property on polymer films is strongly dependent on surface assembly structures of polymers. To further demonstrate and convince that structurally regular polymer surfaces such as the complex is generally useful for immobilizing greater amounts of active-state proteins, we may choose other proteins with definite functions, such as enzymes. Detailed analysis of enzymatic reactions as well as other quantitative and spectroscopic data would show significant information about the relationship between protein activities and polymer surface structures.

In this study, we report greater activities of enzymes immobilized on supports coated with structurally regular PMMA surfaces by using the stereocomplex, *it*-PMMA, and *at*-PMMA films. *Escherichia coli* β-Gal (EC.3.2.1.23), with its well-characterized its three-dimensional structure,[13] was chosen as a model protein enzyme for precise investigation about not only the immobilized state of enzymes but also its functional activity on PMMA surfaces with different structures. Effect of PMMA conformations at the surface on the enzymatic activity was discussed in detail. A schematic representation of this study is shown in Figure 1.

Experimental Section

Fabrication of PMMA Films. PMMA stereocomplex films were coated on 96-well multiplates (F96 MicroWell Plates, Nunc) by the LbL method. Each well was filled with 200 μL of a 1.7 mg mL^{-1} *it*-PMMA (the starting polymer) solution in acetonitrile (M_n = 19 000, M_w/M_n = 1.10, *mm* > 98%, Polymer Source, Inc.) and incubated for 5 min at room temperature. After being rinsed with acetonitrile and Milli-Q water, *it*-PMMA-adsorbed wells were filled with *st*-PMMA solution (M_n = 18 600, M_w/M_n = 1.23, *rr* > 85%, Polymer Source, Inc.) and incubated (at this step, the complex was completed), followed by rinsing in the same manner. The *it*-PMMA and *st*-PMMA adsorption steps were alternatively repeated 14 times.

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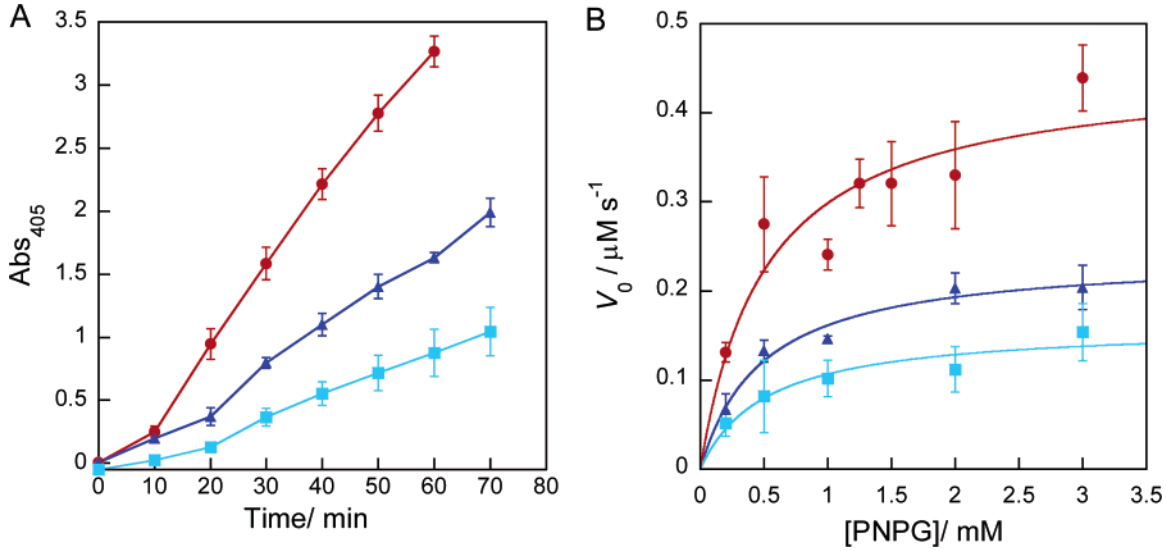

Figure 2. (A) Enzymatic activity of β-Gal immobilized on PMMA-coated surfaces as a function of time; stereocomplex (red circle), *it*-PMMA (blue triangle), and *at*-PMMA (light blue square). Before hydrolysis, enzyme was immobilized onto PMMA-coated surfaces at 1.2 μM of β-Gal. Hydrolysis conditions: [PNPG] = 2 mM, 86 mM sodium phosphate, 116 mM β-mercaptoethanol, 1 mM $MgCl_2$, pH 7.3. (B) Effect of substrate concentration on the initial rate of the enzyme-catalyzed reaction; stereocomplex (red circle), *it*-PMMA (blue triangle), and *at*-PMMA (light blue square). Lined curves are the fitting to the Michaelis−Menten equation. Plots show the mean values ($n = 3$) ± standard deviation.

In the case of single-component film coating, each well was filled with 200 μL of a 0.17 mg mL^{-1} *it*- or *at*-PMMA (M_n = 22 200, M_w/M_n = 1.03, Polysciences, Inc.) and left at room temperature until acetonitrile had completely evaporated, followed by rinsing with Milli-Q water. For quantitative measurements of PMMA film thickness, PMMA coating was also performed on a 9-MHz QCM substrate with gold electrodes (4.5 mm diameter, USI) as described in a previous paper,[10a] which estimates the mass of adsorbent (Δm) from frequency decreases ($-\Delta F$) as follows: $-\Delta m$ (ng) = 0.87 × ΔF (Hz).[14]

Immobilization and Activity Analysis of β-Gal on PMMA Films. For physisorption of β-Gal from *Escherichia coli* (465 kDa, lot CEH0261, Wako), the PMMA-coated wells were filled with 1.2 μM enzyme solution in buffer (50 mM sodium phosphate, 1 mM $MgCl_2$, pH 7.3) and incubated for 1 h at 37 °C, followed by rinsing with the buffer solution. Amounts of adsorbed enzymes on film surfaces were also estimated by QCM. The enzymatic activity of physisorbed β-Gal was determined by following *O*-glycosyl bond cleavage of PNPG. The solution of PNPG at various concentrations was put into the well, and absorbance of a product *p*-nitrophenolate (PNP) at 405 nm was followed as a function of time using a microplate reader (model 680 microplate reader, BIO-RAD). Experiments were repeated three times.

ATR-IR Analysis of β-Gal on PMMA Films. ATR spectra of β-Gal adsorbed on PMMA films were obtained 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.

Results and Discussion

Hydrolysis Reaction of PNPG by Immobilized β-Gal. Ultrathin PMMA films were coated on solid supports, and enzymatic activities of β-Gal adsorbed on PMMA films were investigated quantitatively. By QCM analysis, the mean thickness of the stereocomplex film prepared by the 14-step LbL method was estimated to be 4.8 nm, and that of single-component (*it*- or *at*-PMMA) casting films were estimated to be 180 nm, assuming the film density to be 1.188 g cm^{-3}. The thickness of the complex film is reasonable compared with previous films.[10a] In this paper, we have compared the potential of the complex surface with those of single-component *it*- PMMA or *at*-PMMA surfaces, to understand effects of surface polymer chains with regular conformation on activities of immobilized enzymes.

Table 1. Reaction Kinetics of the Immobilized and Free β-Gal

coating	K_m[a] (mM)	k_{cat}[b] (s^{-1})	k_{cat}/K_m[c] (mM^{-1} s^{-1})	R^2 [d]
stereocomplex[e]	0.51	44[g]	86	0.81
it-PMMA[e]	0.49	30[g]	61	0.96
at-PMMA[e]	0.51	19[g]	37	0.90
free[f]	0.33	130	390	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 for the Michaelis−Menten fitting (Figure 2B). [e] Enzyme was immobilized onto PMMA-coated surfaces at 1.2 μM. [f] Hydrolysis was performed in solution with 0.013 μM of enzyme. [g] The amount of enzyme was estimated by QCM.

Enzymatic activities on PMMA films were estimated by following initial rates (V_0) of the hydrolytic reaction of PNPG (note that the practical area for enzyme immobilization was 0.94 cm^2). As shown in Figure 2A, PNPG hydrolyzes by immobilized enzymes were observed successfully. V_0 was estimated by the maximum slope of time dependences within 10 min. Relatively slower hydrolysis at initial 10 min must be due to the fact that substrate diffusion was incomplete. V_0 apparently depended on species of PMMA surfaces. V_0 on the complex surface was greatest and approximately 2- and 4-fold faster than those on *it*-PMMA and *at*-PMMA surfaces. Because V_0 depends on total amounts of β-Gal immobilized, we estimated β-Gal amounts by using the QCM substrate to be 512 ± 14, 392 ± 16, and 430 ± 16 ng cm^{-2} for the complex, *it*-PMMA, and *at*-PMMA surfaces, respectively. Therefore, one of the reasons for the greatest

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Table 2. Adsorption Parameters of β-Gal on Stereocomplex and Single-Component PMMA Films

coating	K_{app}[a] (M^{-1})	A_{max}[b] (ng cm^{-2})	R^2 c
stereocomplex	4.87	508	0.98
It-PMMA	6.42	388	0.95
At-PMMA	8.31	406	0.94

[a] k_{app}, apparent adsorption constant. [b] A_{max}, maximum adsorption amount. K_{app} and A_{max} were obtained from the Langmuirian adsorption fitting. [c] R^2, coefficient of determination for the Langmuirian adsorption fitting.

V_0 measured on the complex surface is attributed to the greatest amount of β-Gal. β-Gal amounts were then considered to estimate Michaelis−Menten parameters. The effect of substrate concentrations on V_0 is shown in Figure 2B. In all cases, V_0 was saturated at high concentrations of PNPG, suggesting enzymatic reactions occurred by immobilized β-Gal. PNPG autohydrolysis on PMMA-coated surfaces lacking β-Gal was not observed, also suggesting that products were produced by reactions of immobilized enzymes.

Michaelis−Menten parameters after standardization with β-Gal amounts are summarized in Table 1. The k_{cat}/K_m values for the immobilized enzyme were unfortunately lower than those for the free enzyme in aqueous phase, possibly due to changes in protein structures after immobilization even on the complex surface. However, the enzyme on the complex surface composed of structurally well-ordered polymer chains with double-stranded helical conformation was more active than those on the single-component surface with *it*- or *at*-PMMA. The difference in K_m values was small or the same for PMMA surfaces, suggesting that the substrate binding ability of enzymes at equilibrium state is almost the same in all surfaces. On the other hand, 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 (44 s^{-1}); however, on the single-component films composed of *it*-PMMA and *at*-PMMA chains, the k_{cat} values were 30 s^{-1} and 19 s^{-1}, respectively. It is noted that the order of hydrophobicity of aforementioned PMMA film surfaces[10a,15] is not the same as that of k_{cat}, indicating that hydrophobicity does not determine activities of immobilized enzymes (see mechanistic speculation).

When comparing single-component film surfaces, an approximately 1.6 times difference in k_{cat} values between *it*- and *at*-PMMA surfaces may also result from the conformation of each PMMA chain, because it is known that *it*-PMMA partially forms a double-stranded 10_1 helix or a single-stranded 5_1 helix.[16] In our previous study,[11c] various antibodies were immobilized through the physically adsorbed protein A on PMMA surfaces and used for antigen detection. However, we could not observe the significant advantage of *it*-PMMA surfaces, compared with *at*-PMMA surfaces. Because all immobilized protein A with a smaller molecular size than antibodies are not used for antibody immobilization, we did not observe total protein A activities. On the other hand, because total enzymatic activities could be analyzed here, we successfully demonstrated the slight difference between *it*- and *at*-PMMA surfaces. As a consequence, it was found that activities of enzymes immobilized on PMMA films were dependent on the complex formation as well as polymer stereoregularities, which determine regular chain conformation.

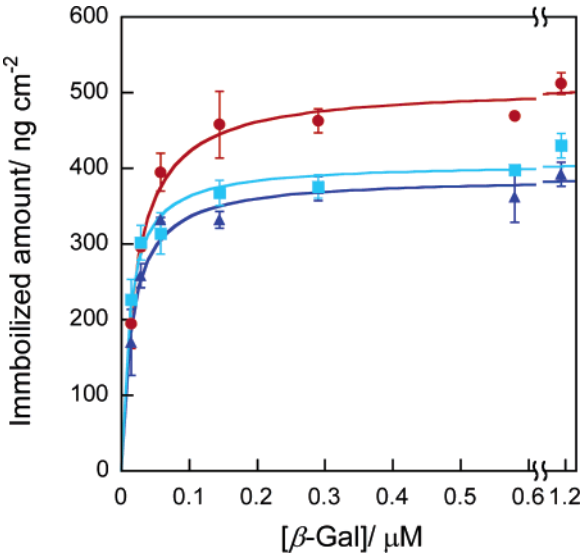

Figure 3. Langmuir isothermal plots of β-Gal for the surfaces coated with PMMA stereocomplex (red circle), *it*-PMMA (blue triangle), or *at*-PMMA (light blue square) obtained by 9 MHz QCM. Conditions: 50 mM sodium phosphate containing 1 mM $MgCl_2$, pH 7.3. Plots show the mean values ($n = 3$) ± standard deviation.

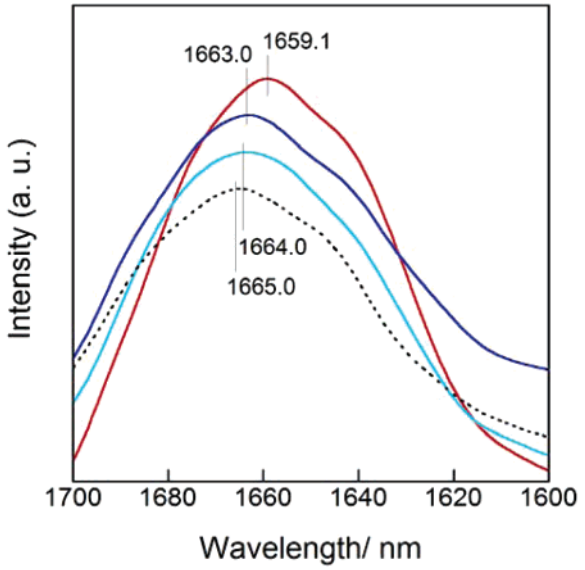

Figure 4. ATR-IR spectra of the immobilized β-Gal on various PMMA-coated or gold surfaces in the range 1600−1700 cm^{-1} (amide I). Stereocomplex-coated (red), *it*-PMMA-coated (blue), *at*-PMMA-coated (light blue), and bare gold (black, dashed).

QCM Analysis of β-Gal Adsorption. If proteins adsorb onto the flexible polymer surface, a large number of interactions between the polymer surface and the protein's amino acids should lead to the formation of a stable attachment. The folded structure of the protein may unfold to expose its inner hydrophobic core, so that protein activity may subsequently decrease. For further discussion on the number of interactions (contacting points) between

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proteins and PMMA surfaces, apparent adsorption constants (K_{app}) of β-Gal for PMMA surfaces were determined from a Langmuir adsorption isotherm with the following equation:

$$A = \frac{K_{app}A_{max}}{1 + K_{app}[\beta\text{-Gal}]}[\beta\text{-Gal}]$$

where A is the amount of β-Gal adsorbed, [β-Gal] is the β-Gal concentration, A_{max} is the maximum A. Obtained K_{app} and A_{max} values are summarized in Table 2. The K_{app} values were dependent on PMMA film species. The K_{app} for the complex was smaller than those for *it*- and *at*-PMMAs. These observations are similar to our data previously obtained from the characterization of other proteins such as bovine serum albumin, human serum albumin, fibrinogen, lysozyme, and protein A,[11] suggesting that more multiple bonds were formed between single-component PMMA surfaces and proteins. When comparing single-component film surfaces, the K_{app} for *it*-PMMA was smaller than that for *at*-PMMA. The difference in single-component film surfaces may also result from the deference between partially helical structures of *it*-PMMA[16] and nonregular structure of *at*-PMMA.

The A_{max} for the complex was larger than that obtained for *it*- and *at*-PMMAs, indicating that the complex surface is suitable for immobilizing greater amounts of enzymes. The functional form of β-Gal is a tetramer (465 kDa) of four identical subunits and is roughly ellipsoidal with dimensions $175 \times 135 \times 90$ Å^3 along the 2-fold axes.[13] Assuming that β-Gal adsorbs on the surface at monolayer coverage, ideal adsorption amounts range from 330 to 640 ng cm^{-2}. Therefore, enzymes might adsorb on the *it*-PMMA and *at*-PMMA surface with relatively large contact area (or point); however, on the complex surface with relatively small contact area (or point), possibly resulting in less denaturing of proteins on the complex surface.

The mean square roughness (R_a) of the complex and *it*-PMMA films were slightly different;[11a] adsorption amounts of β-Gal should not be affected by greater β-Gal sizes.

ATR-IR Analysis of the Adsorbed β-Gal. ATR-IR study also supported that structural denaturation of physically adsorbed enzymes might be suppressed on the complex surface (Figure 4). 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.[17] The peak frequency of the amide I band of the free β-Gal powder was 1632.7 ± 0.6 cm^{-1} (spectrum not shown). The peak frequency of immobilized enzymes on *it*- and *at*-PMMA surfaces shifted to 1663.1 ± 0.7 and 1665.3 ± 0.9 cm^{-1}, respectively, approximately equivalent to the value obtained on a bare gold surface (1664.4 ± 1.1 cm^{-1}). It is noted that the difference of β-Gal structures on *it*- and *at*-PMMA surfaces were unfortunately identified by ATR-IR. These aforementioned shifts from lower wavenumber to higher wavenumbers on the surface are due to mainly two reasons. First, the protein hydration state changed on the surface after immobilization compared to the free protein.[18] Second, unfolding of the protein on the surface took place, because peak positions for an ordered α-helix could be observed at lower wavenumbers than those for unordered α-helices and random coils.[19] Therefore, the enzymes on *it*-, *at*-PMMA, and gold surfaces partially denatured following immobilization. On the other hand, the peak frequency of the enzyme on the complex also shifted (1659.1 ± 1.0 cm^{-1}); however, the degree of the shift was smaller than that observed on other surfaces. The aforementioned results suggest that unfolding of the protein on the complex could be suppressed, although it is difficult to discuss the change in each component secondary structure because of the low intensity of the IR spectra.

Mechanistic Speculation of Protein Adsorption on Various PMMA Film Surfaces. From the hydrolytic reaction followed by QCM and ATR-IR analyses, we propose a mechanism about the relationship between the PMMA conformation at the surface and the immobilized enzyme conformation, as shown in Figure 5. In our previous studies,[11] adsorption constants of various proteins for the stereocomplex were ordinarily smaller than those for single-component films. In addition, monolayer adsorption amounts of nine kinds of proteins with various molecular weights (12−69 kDa), 3D structures, and surface charges (isoelectric points) for the complex were greater than those for single-component films.[11c] These results intended that protein immobilization on PMMA films was generally independent of a kind of the functional groups at the protein surfaces. Furthermore, the hydrophobicity of the complex surface ranges between *it*- and *at*-PMMAs,[10a,15] so that the functional groups of PMMAs also do not affect protein immobilization. Therefore, state of proteins after immobilization on the PMMA-coated solid supports was affected by not so much the functional groups but by the conformation of PMMAs at the surface. At the complex and the *it*-PMMA surfaces, mobility of polymer chains may be suppressed to some extent, but at the *at*-PMMA surface, polymers are random conformation and their mobility would be very large (disordered). Mobility of polymers at the surfaces affects the enthalpy gain during protein immobilization process. Because adsorption constants are composed of the sum of individual interactions such as hydrogen bonding, hydrophobic, and van der Waals interactions between proteins and PMMAs, the greater numbers of contacting points are necessary for greater adsorption constants. Therefore, entropic disadvantages of the surface polymer for protein adsorption have to be compensated by enthalpy gain through the multiple interactions. The greater numbers of the interaction between proteins and the surface should result in denaturing of proteins from native forms, followed by a loss of activities of proteins.

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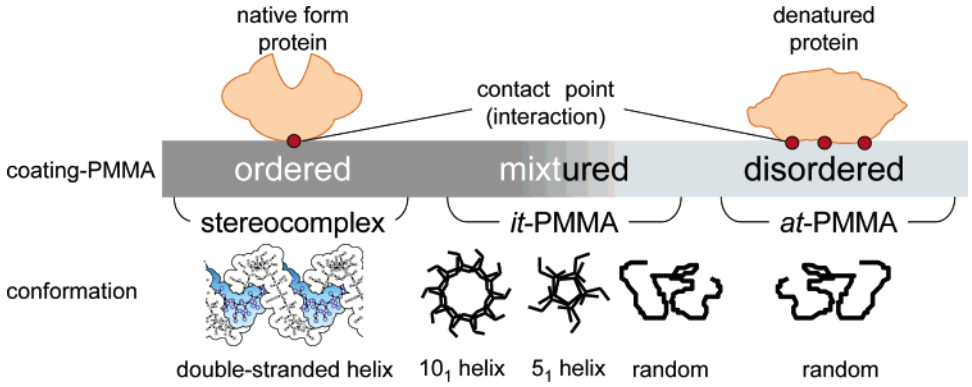

Figure 5. Schematic representation of enzyme immobilized on PMMA surfaces with different ordered levels. Each PMMA conformation referred from refs 8b and 16 is also depicted below. Red circles represent interactions between the enzyme and the PMMA surface.

Because β-Gal can be adsorbed onto the complex surface with weak interactions, protein denaturation following immobilization may be suppressed and the protein activity may remain high on the complex.

Conclusion

The surface modification of substrates for physical protein immobilization is important for a wide range of biomedical and bioengineering applications. Herein, the effect of the surface PMMA conformation on enzymatic activity has been characterized. We demonstrated that a more ordered conformation on the coating surface such as stereocomplex is more effective for maintaining the native activity of the immobilized enzyme. The former holds especially true when coating with PMMA complex, the most ordered conformation, which effectively suppresses protein denaturation; the complex film could be easily fabricated by the simple LbL method on any solid support. We believe that PMMA stereocomplexes used in polymer surface modification might be helpful in developing a new category of polymer bionanomaterials. We proposed that regulation of surface polymer conformation is an essential factor for maintaining activities of physically immobilized enzymes on polymer materials.

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