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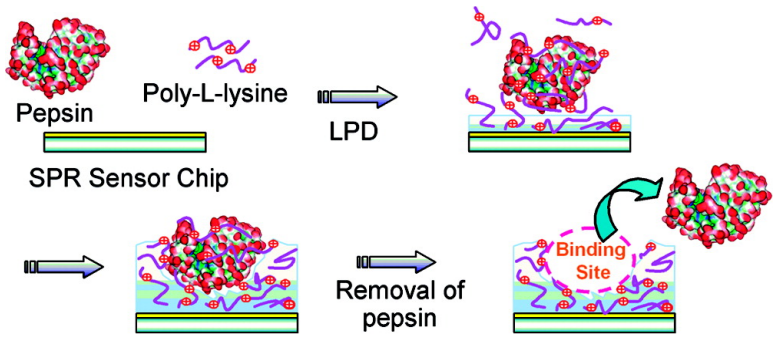

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Protein-Templated Organic/Inorganic Hybrid Materials Prepared by Liquid-Phase Deposition

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Abstract: Organic/inorganic hybrid thin films for protein recognition have been prepared by the liquid-phase deposition (LPD) coupled with template synthesis, i.e., molecular imprinting, where pepsin (Pep) was used as a model protein and titanium oxide was deposited on gold substrates in the presence of Pep-poly-L-lysine (PL) complexes. The complexes remained in the templated film after the deposition, and the binding sites for Pep were constructured after Pep was removed from the film. Surface plasmon resonance signals on the deposited films were measured to examine the binding behaviors toward proteins. The binding of Pep on the templated film was reversible, and the binding isotherm of Pep depicted a saturation curve with a binding constant of 7.3×10^5 M^{-1}, which was 10 times higher than that of albumin. In contrast, titanium oxide films prepared without PL did not show any selectivity; therefore, the hybridization of PL as the organic binder with the inorganic material is necessary to obtain selective binding sites for Pep. It was also shown that the hybridization process should proceed without denaturing the template protein, in order to obtain selective binding sites for the template. The procedure for preparation of the films was simple to perform, and the process for hybridization of the thin films with nanometer-order thickness was easily controlled by changing the LPD reaction time period. Consequently, the proposed LPD coupled with template synthesis is among the most appropriate methods to prepare hybrid materials with protein recognition ability, which proceeds under mild conditions in aqueous solution.

Introduction

Proteomics has become of great interest recently, because it is a potential tool for characterizing a given biological system and discovering biomarkers for particular diseases. There are many proteins in biological systems, and such protein diversity cannot be explained solely by protein-coding genes in the human genome. Thus, protein recognition materials/chips would play an important role, and currently biomacromolecules such as antibodies and enzymes are employed for this purpose. However, such biomacromolecules are sometimes difficult to find and/or produce; therefore, receptor-like synthetic materials for proteins have been intensively studied as substitutes for natural receptors. Template polymerization techniques such as molecular imprinting have attracted attention as a method for the preparation of molecular recognition materials, and synthetic organic polymers capable of molecular recognition have been prepared for many small molecules.[1] Though protein recognition materials have also been proposed,[2] they are often hydrogels having fairly flexible structures, which may not be suitable for precise recognition of target proteins.

Inorganic materials have been frequently used for preparing various particles, thin films, and other structures on chips in the field of nanotechnology. Because the products are stable with rigid structures, templating techniques using inorganic materials have become of interest in the field of molecular recognition and catalysts.[3] Metal oxide thin films have been extensively used in nanotechnology, and a wide variety of deposition techniques have been developed to prepare metal oxide thin films. They can be roughly classified into two main

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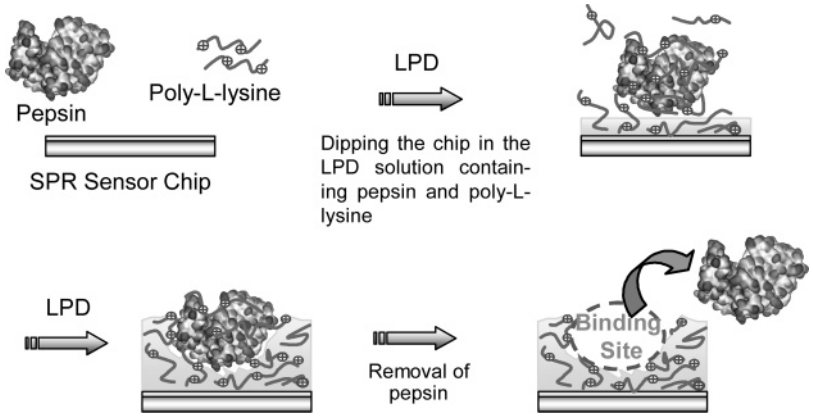

Figure 1. Schematic illustration of protein-templated LPD.

Table 1. Recipes for Preparation of SPR Chips

	BF(–)	IF(Pep)	BF(PL1)	IF(Pep–PL1)	BF(PL10)	IF(Pep–PL10)
0.5 M $(NH_4)_2TiF_6$ (mL)	2	2	2	2	2	2
0.5 M H_3BO_3 (mL)	4	3	4	3	4	3
distilled water (mL)	4	4	3.9	3.9	3	3
0.01% poly-L-lysine (mL)	0	0	0.1	0.1	1	1
1 mg/mL pepsin in 0.5 M H_3BO_3 (mL)	0	1	0	1	0	1
total (mL)	10	10	10	10	10	10
reaction time (h)	1.5	1.5	2.5	2.5	2.5	2.5

processes: physical and chemical processes. Physical processes include dry processes such as vacuum evaporation and sputtering.[4] Chemical processes such as chemical vapor deposition,[5] sol−gel methods,[6] and electrodeposition have been reported. Both types of processes are widely applied to the preparation of various metal oxides and have been studied in detail. To prepare templated metal oxide materials, the deposition should proceed in the presence of template molecules; however, it is difficult to use organic compounds, especially biomolecules, as the templates for the preparation of metal oxide composite films due to damage caused by the high-energy process and/or heat treatment during the processes.

Among these techniques, wet processes called "soft-solution processes" are suitable for use with organic templates, because they do not need high energy. Liquid-phase deposition (LPD) has been known as a novel soft-solution process for preparing metal oxide thin films from aqueous solutions.[7] In this process, metal oxide can be deposited onto various kinds of immersed substrates through the chemical equilibrium reaction between a metal fluoro complex and metal oxide. The formation of metal oxides may proceed via the following ligand-exchange (hydrolysis) equilibrium reaction:

$$MF_x^{(x-2n)-} + nH_2O \rightarrow MO_n + xF^- + 2nH^+ \qquad (1)$$

$$H_3BO_3 + 4HF \rightarrow BF^{4-} + H_3O^+ + 2H_2O \qquad (2)$$

Reaction 1 can be shifted to the right by adding boric acid, which can react with F^- ions to form more stable complex ions (reaction 2).[8] F^- ions are being consumed, and the ligand-exchange reaction is accelerated. As a result, thin films are slowly deposited homogeneously on the substrate; the process requires no special equipment.

Herein, we report on protein-templated metal oxide thin films prepared by LPD. Because organic/inorganic hybrid materials can be easily prepared by LPD by adding organic compounds to the LPD solution,[9] we intend to use such organic compounds as binders for target proteins, which are located inside the molecular recognition cavities (Figure 1). In this study, we employed titanium oxide as a base thin-film matrix and poly-L-lysine (PL) as an organic compound/binder that can interact with acidic proteins to form protein−PL complexes. A typical acidic protein, pepsin (Pep), was used as a model template, and the effectiveness of the proposed method was demonstrated by the selective binding of the target protein to the templated organic/inorganic hybrid films.

Results and Discussion

Titanium oxide-deposited thin films were prepared on glass chips with gold-coated surfaces, which were directly attached to a surface plasmon resonance (SPR) sensor to examine their binding properties. During the deposition, the SPR chip was covered with a Teflon cell to deposit titanium oxide on one side of the chip to allow SPR signals to be measured (see Experimental Section). The chip was soaked for an appropriate time to form nanometer-order thin films. We prepared six chips according to the recipe in Table 1, and the binding behaviors of proteins, including Pep, lactalbumin (Lac), albumin (Alb), and chymotrypsin (Chy), on the six chips at pH 3.5 were examined. BF(−) adsorbed all of the proteins tested, and no selective binding was observed (Figure 2a); this may be due to

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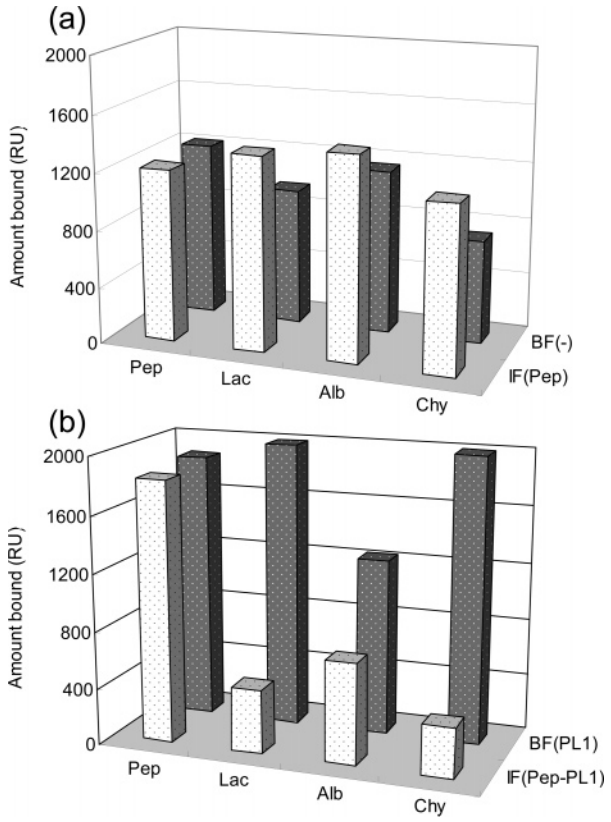

Figure 2. Binding behaviors of proteins on the prepared LPD thin films. (a) Thin films prepared without PL. (b) Thin films prepared with PL. BF(−), titanium oxide film only; IF(Pep), prepared with Pep; BF(PL1), prepared with PL; IF(Pep−PL1), prepared with Pep and PL. The thicknesses of the films were roughly estimated to be 3.2, 1.6, 4.4, and 3.9 nm, respectively, by using quartz crystal microbalance experiments.

the surface charge of titanium oxide on the film. According to the literature,[10] the estimated isoelectric point of titanium oxide is 6.2; thus, the thin film may be positively charged at pH 3.5. Therefore, negatively charged Pep (pI = 1.0) should be more strongly bound to BF(−) than positively charged Chy (pI = 8.4), Alb (pI = 4.8), and Lac (pI = 4.5). However, the apparent order of affinity was different from the order of the proteins' pI values, meaning that the surface of BF(−) may not have simple ion-exchange properties. BF(−) and IF(Pep) showed similar binding behaviors toward proteins (Figure 2a). These results suggest that Pep could not work as a molecular template during the titanium oxide−LPD process to make binding sites under the conditions employed; i.e., binding sites capable of Pep recognition were not created by the simple titanium oxide−LPD, even in the presence of the template Pep. Recently, an L-glutamic acid-recognizable titanium oxide thin film has been prepared by LPD;[11] however, it was prepared using only titanium oxide. Because of the intrinsic binding property of titanium oxide itself, this system may not work for various classes of target compounds and the applicability would be limited; it did not work for protein recognition as presented here.

Because the simple titanium oxide prepared by LPD did not show any specific binding, we tried to prepare titanium oxide thin films fabricated with PL, where PL can interact with Pep by electrostatic interaction;[12] thus, PL is expected to work as a

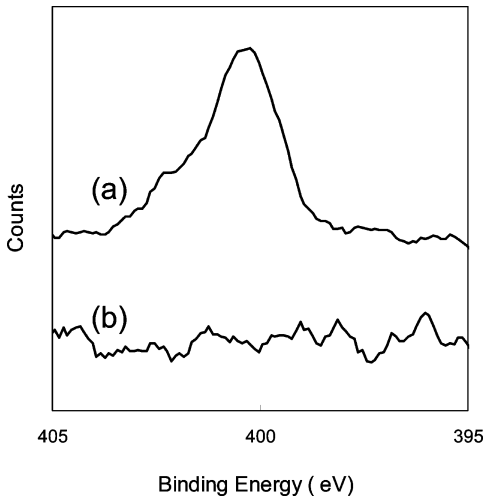

Figure 3. N 1s X-ray photoelectron spectra on the surfaces of (a) BF(PL10) and (b) BF(−).

binder to Pep on the surface after the removal of Pep. In the LPD solution, Pep and PL formed complexes that could be taken into the thin film as the LPD reaction proceeded, yielding Pep−PL−titanium oxide composite thin films. After the film was washed with a basic buffer containing concentrated salt and water, templated cavities complementary in shape to Pep could be generated (Figure 1). At first, we prepared thin films only in the presence of PL, to be sure that organic/inorganic hybrid thin films can be obtained by this LPD procedure. X-ray photoelectron spectroscopy (XPS) data clearly showed that nitrogen atoms remained on the surface of the film even after washing when PL was co-deposited with titanium oxide. As shown in Figure 3a, a peak at a binding energy of 400.3 eV was found for the film prepared with PL, and this can be assigned to N−C groups, in good agreement with the previous XPS measurement.[13] Since no nitrogen peak was observed for titanium oxide itself (Figure 3b), it is confirmed that PL was located on the surface of the deposited titanium oxide film, yielding an organic/inorganic hybrid thin film, and PL still remained after the washing process.

BF(−), IF(Pep), BF(PL1), and IF(Pep−PL1) were prepared on quartz crystal microbalance (QCM) sensor chips to monitor the deposition process. Although the growth of BF(−) was faster than that of other thin films, all films were grown continuously, and the growth rate was about 1.5−2 nm/h (Figure 4). The estimated thickness of each film used in this study is shown in the captions of Figures 2 and 7. The QCM studies confirmed that the organic/inorganic hybrid films can be prepared in a nanoscale thickness and easily controlled by changing the time of the LPD process.

IP(Pep−PL1) was prepared on the SPR sensor chip in the presence of both Pep and PL during the deposition. In this case, selective binding for Pep was observed, and the binding behaviors of proteins were very different from those in BF(PL1) (Figure 2b). Binding isotherms of the proteins are shown in Figure 5, and the binding constant of Pep was approximately 10 times higher than that of Alb: the binding constants are estimated to be 7.3×10^5 and 5.7×10^4 M^{-1}, respectively. The binding profile of Pep was saturable, meaning that the

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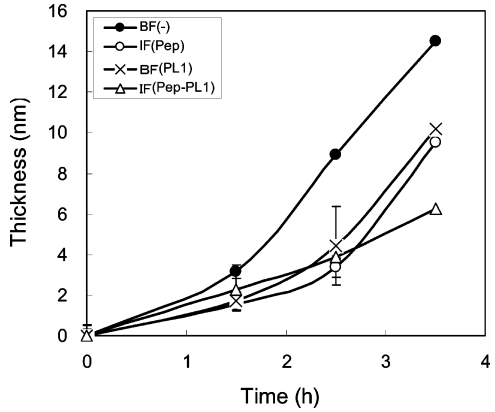

Figure 4. Time course of titanium oxide deposition on the QCM sensors.

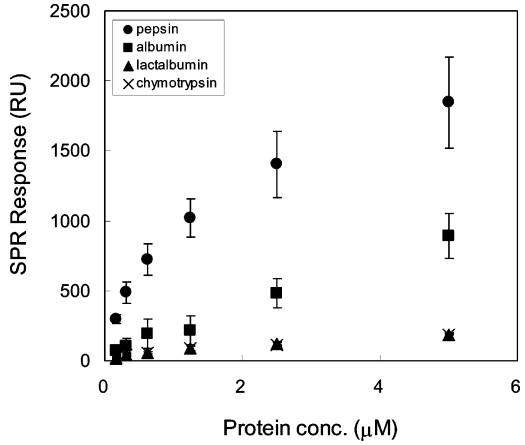

Figure 5. Binding isotherms of the tested proteins on IF(Pep−PL1).

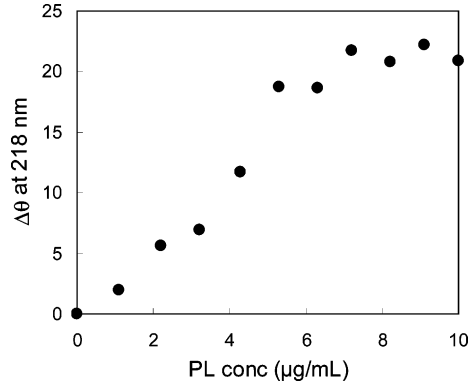

Figure 6. Circular dichroism change of Pep dissolved in a citrate buffer (pH 3.5) containing various concentrations of PL at 218 nm. $\Delta\theta$ = observed θ − initial θ.

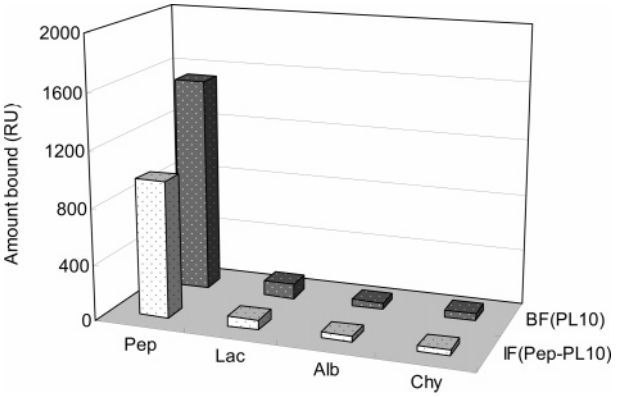

Figure 7. Binding behaviors of proteins on the LPD films prepared with 10 times higher concentration of PL. The thicknesses of the films were roughly estimated to be 6.9 nm for BF(PL10) and 2.8 nm for IF(Pep−PL10) by using quartz crystal microbalance.

binding is not likely to be nonspecific on the surface; i.e., a finite number of binding sites can be created by the templated LPD process. It could be explained that polyion complexes of Pep and PL were formed in the LPD solution and taken into the titanium oxide thin film, and then binding sites complementary to Pep were produced after the removal of Pep, revealing that a templated organic/inorganic hybrid thin film was prepared. In BF(PL1), PL may be homogeneously distributed, while the presence of Pep makes PL localize on the surface of IP(Pep−PL1). This specific localization of PL as a print may enhance the selective binding of Pep. The selectivity appeared to be higher at lower concentration ranges. This means that the binding sites may be heterogeneous, as is often the case with noncovalent molecular imprinting, where at higher concentration ranges nonspecific binding would occur more, and at lower concentration ranges only high-affinity binding sites bearing high selectivity could work.

Because the complexation with PL may affect the secondary structure of Pep, circular dichroism was measured for Pep dissolved in a citrate buffer (pH 3.5) containing various concentration of PL (Figure 6). A standard Pep solution without PL showed a typical band at around 218 nm, derived from β-sheet structures. This CD signal became smaller as the PL concentration was increased. This means that most of the Pep in the LPD solution keeps its native structure at the PL concentration of 1 μg/mL, which was the concentration used in the preparation of IP(Pep−PL1). Assuming that the average molecular weight of PL is 100 000, this concentration can be converted to ca. 10 pmol/mL. About 29 nmol/mL of Pep exists in the solution (molecular weight of Pep is ca. 35 000); thus, the molar ratio of Pep to PL can be approximately 300:1.

In order to examine whether the native structure of Pep is necessary for the present templating technique, the binding behaviors of IP(Pep−PL10) prepared with 10 μg/mL PL were examined, in which Pep was denatured by PL during the LPD process. There was no difference in binding behaviors between IP(Pep−PL10) and BF(PL10), although Pep-selective binding was observed (Figure 7). This suggests that denatured Pep may not work properly as a molecular template to create specific binding sites, and furthermore, overloaded PL on the surface may increase ion-exchange-based nonspecific binding to IP(Pep−PL10), resulting in the similar binding behaviors toward BF(PL10) shown in Figure 7.

Conclusion

We have successfully developed novel organic/inorganic hybrid materials with protein recognition ability, prepared by the templated LPD method using protein−polyion complexes as the templates. The films were obtained easily by mixing Pep and PL in the LPD solution and showed selectivity for the target protein. These films were stable enough to allow repetitive measurements to be conducted for several weeks. In conventional soft-solution processes, such as the sol−gel process, the deposition species are formed anywhere in the solution, and it is difficult to control the reaction process. In the case of LPD, the metal oxide species grow on the chips during the equilibrium

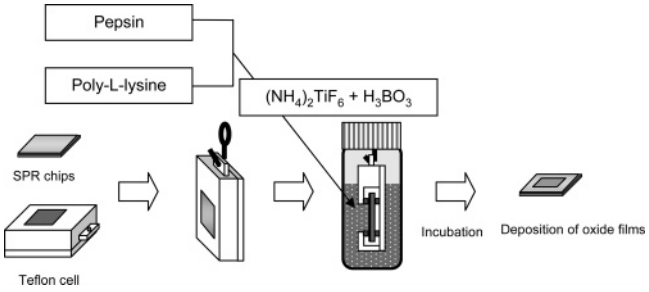

Figure 8. Schematic illustration of the templated LPD process.

reaction of the metal fluoro complex due to the small chemical potential difference between the reaction species on the chip and those in the bulk solution of LPD. As a result, the metal oxides were deposited only on the substrate surface, without precipitation from the bulk solution. The density of thin films prepared by LPD was higher than that of films prepared by other methods, such as vapor deposition and sol−gel methods.[6a,14] Such high density would promote high step coverage on any shapes of substrates, suggesting that LPD has great advantages for the preparation of templated materials, which requires molecular-scale step coverage during the deposition of metal oxides. Not only PL but also various polyion compounds can be used with LPD of various metal oxides to construct specific binding cavities. Consequently, the proposed technique could provide a wide variety of metal oxide-based molecular recognition materials for a diverse range of target compounds from small molecules to biomacromolecules.

Experimental Section

Preparation of Pepsin-Templated Films by LPD. As shown in Figure 8, SPR chips (Sensor Chip Au, Biacore AB, Sweden) were covered by a Teflon holder in order to deposit titanium oxide only on one side of the chip (gold-coated surface side) for SPR measurements. They were immersed into the LPD treatment solutions prepared according to Table 1, where the molecular weight of poly-L-lysine used was 70 000−150 000. After appropriate reaction periods, the chips were withdrawn from the treatment solutions and then washed with 10 mM borate buffer (pH 8.5) and water. The removal ratios of pepsin from the chips were not estimated, but in each SPR measurement the chips were cleaned by the washing solutions, which should remove most of the bound proteins from the films.

X-ray Photoelectron Spectroscopy. XPS measurements were performed using a JPS-9010MC (JEOL, Japan), employing an Mg Kα X-ray source operated at 100 W (10 kV, 10 mA). Survey and high-resolution spectra of the N 1s binding energy region were collected at 90° to the detector, with pass energy resolutions of 100 and 10 eV, respectively. The binding energy scale was calibrated against the Au 4f7/2 photoemission line at 83.8 eV.

Measurement of the Thickness of the Films Prepared on the QCM Sensor Chips. The thicknesses of the films were measured by using a quartz crystal microbalance (QCM) sensing system (QCA917, SEIKO EG&G, Japan) with 9 MHz AT-cut QCM sensors (QA-A9M-AU, SEIKO EG&G, Japan). The QCM chip, covered by a Teflon holder (QA-CL3, SEIKO EG&G, Japan), was immersed into the corresponding LPD treatment solution to deposit titanium oxide only on one side of the chip. Three sensors were prepared for three samples each of BF(−), IF(Pep), BF(PL1), IF(Pep−PL1), BF(PL10), and IF(Pep−PL10) to check the reproducibility of the LPD process. Frequency changes were measured before and after the LPD process at 25 °C, and each thickness was calculated by using the Sauerbrey equation[15] with the following parameters: density of TiO_2 (anatase), 3.9 g/cm^3; electrode surface area, 0.2 cm^2; and $\Delta 1$ Hz = 1 ng.

Binding Experiments Using the SPR Sensor. The selectivity experiments for the prepared thin films, BF(−), IF(Pep), BF(PL1), IF(Pep−PL1), BF(PL10), and IF(Pep−PL10), were performed with a surface plasmon resonance sensing system (BIACORE Q, Biacore AB, Sweden) using four proteins (chymotrypsin, albumin, lactalbumin, and pepsin, 10 μM) dissolved in 10 mM citrate buffer (pH 3.5). The measurement conditions were as follows: running buffer, 10 mM citrate buffer (pH 3.5, 10 μL/min); injection volume, 30 μL; washing solution, 10 mM borate buffer containing 1 M NaCl (pH 8.5) or 10 mM glycine buffer containing 1 M NaCl (pH 9.5). The amounts of proteins bound were calculated by measuring the signal intensity 60 s after the end of each sample introduction period. The washing step was continued until the baseline returned to the initial level. Each sample was measured in triplicate. For drawing the binding isotherms, protein concentrations from 0.16 to 5.0 μM were used.

Circular Dichroism Measurements. Circular dichroism measurements were carried out with a J-725K instrument (JASCO, Japan) at 25 °C. Pep (1 mg/mL) dissolved in 10 mM citrate buffer (pH 3.5, 300 μL) was placed in a quartz cell and titrated with 0.01% (w/w) poly-L-lysine (30 μL each). Total volumes were adjusted to 3 mL by addition of the citrate buffer. $\Delta\theta$ values were calculated by subtracting the observed θ from the initial θ.

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Supporting Information Available: CD spectra and fluorescence spectra of the titration experiments of pepsin with poly-L-lysine. This material is available free of charge via the Internet at http://pubs.acs.org.

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