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Enantioselective anion exchange on a positively charged poly(L-lysine) layer assembled on thin TiO_2-gel films†

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Ion exchange between negatively charged fluorescent dyes and chiral glutamic acids was examined on a poly (L-lysine) (PLL) layer assembled on ultrathin TiO_2-gel films. First, a TiO_2-gel film with a thickness of 6–7 nm was prepared on a quartz substrate by means of the surface sol–gel process. A PLL layer of about 2.4 nm was then immobilized by immersing the substrate in the polymer solution at pH 10.7. Subsequently, fluorescent dye (sulforhodamin B or carboxyfluorescein) was electrostatically adsorbed on the PLL layer, and desorption of the dye molecules into dilute D- (or L-) glutamic acid solution was monitored by UV-Vis absorption and fluorescence spectroscopies. The speed and amount of desorption depended highly on the chirality of glutamic acids as well as the molecular structure of fluorescent dyes.

Introduction

In the biosynthetic process of a protein, first a peptide chain with a unique amino acid sequence is produced. The chain forms secondary structures such as α-helix, β-sheet, and β-turn, and then these are regularly folded to give a particular three-dimensional structure. Maintaining the tertiary structures of proteins is indispensable for expressing their specific functions such as enzymatic activity and biological communication. Interaction between biomolecules and surfaces of proteins is increasingly attracting much attention in the areas of proteomics and related biotechnology. Immobilization of proteins without disrupting their tertiary structures is expected to pave the way for the comprehensive understanding of their macromolecular functions.[1–6]

Mild fixation of biomacromolecules has been widely studied. Proteins, DNAs, and sugar chains were immobilized on the surface of self-assembled monolayers (SAMs), for example, by means of amino coupling, avidin–biotin binding, lectin–glycogen interaction, *etc.*[7–9] Usefulness of biospecific interaction at the interface has been frequently stressed in past reports. Alternate adsorption of polyelectrolytes pioneered by Decher *et al.* gave a convenient way for the biomacromolecular fixation.[10,11] Almost all of the water-soluble proteins were electrostatically assembled with oppositely charged polymers.[12] The technique has been extended to other biomaterials such as antibodies and viruses.[13] Their multilayer films were employed for bio-sensing,[14] drug release,[15] and other objectives.[16] The proteins immobilized on electrodes were capable of electrochemically communicating with the substrates.[17] The proteins assembled on nanoparticles are expected to give interesting drug delivery systems.[18]

We have reported that ultrathin metal oxide films prepared by surface sol–gel process were highly adherent for biomolecules.[19] For example, protected amino acids, peptides, and saccharides were incorporated into the oxide gel films in the course of layer-by-layer adsorption of metal alkoxides. After extracting the organic components into alkaline water, the resulted microporous films selectively adsorbed the biomolecules which had been employed as templates.[20] In order to convert inert surfaces to biocompatible ones, the surface sol–gel process was applied to a gold substrate, a silicon wafer, and a Teflon sheet. Acharya and Kunitake used such substrates, and reported that fibrinogen on an ultrathin titania-gel layer maintained its biological activity and produced a fibrin network after treating with thrombin.[21] Furthermore, the surface sol–gel process enabled us to uniformly assemble ultrathin layers of synthetic polymers. One or two nanometers thick poly(vinyl alcohol) layer adsorbed on zirconia-gel films was quite useful for immobilizing cytochrome c without disrupting the tertiary structure.[22]

In the present paper, we demonstrate the immobilization of a molecularly thin poly(L-lysine) (PLL) layer on titania-gel films. Our objective here is to chemically understand the interaction between charged biomolecules at the solid–liquid interface. It is known that PLL has a random coil conformation in neutral solution, which changes to α-helical at high pH because of the deprotonation of amino side chains. We studied the conformation of PLL chains electrostatically fixed on solid substrates, and then examined the enantioselective binding of glutamic acids. The binding was successfully monitored through the anion exchange between the glutamic acids and pre-adsorbed fluorescent dyes. We report for the first time that the release of the fluorescent dyes is strongly coupled with molecular recognition on the PLL chains.

Experimental section

Structures of organic compounds used are shown in Fig. 1. Poly(L-lysine hydrobromide) (PLL) (Mw > 70 000) was obtained from Kanto Chemical. D- and L-glutamic acids and sulforhodamin B (SRB) were purchased from Aldrich. 2′,7′-Bis(carboxyethyl)-4 or 5-carboxyfluorescein (BCECF) and titanium *n*-butoxide were obtained from Dojindo and Gelest. All these chemicals were used as received without further purification. Ultrapure water of 18.2 MΩ produced by Millipore direct-Q system was used throughout the experiments.

UV-Vis absorption and fluorescence spectra were obtained by using a Shimadzu UV-3150 spectrophotometer and a

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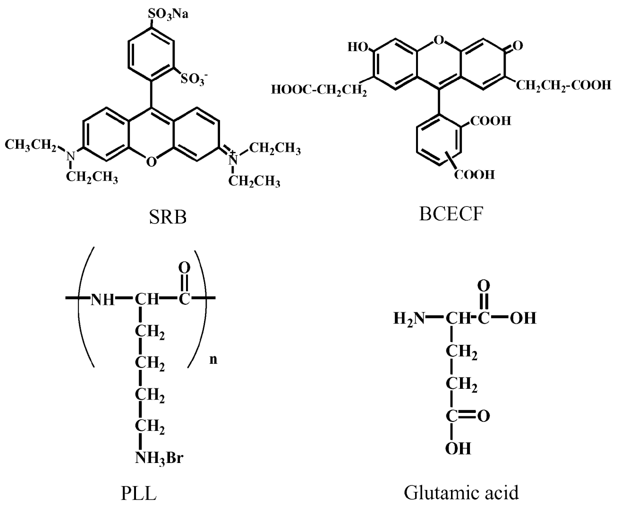

Fig. 1 Structures of sulforhodamin B (SRB), 2′,7′-bis(carboxyethyl)-4 or 5-carboxyfluorescein (BCECF), poly(L-lysine hydrobromide), and glutamic acid.

JASCO FP-6500 spectrofluorometer, respectively. Layer-by-layer deposition of TiO_2-gel films and the following immobilization of PLL layer were monitored by a 9-MHz quartz crystal microbalance (QCM) using an instrument GCS-300 produced by USI system, Japan. The frequency shifts were converted into mass changes by using the Sauerbrey equation.[23] The relationship between QCM frequency shift (ΔF) and adsorbed mass (ΔM) is as follows:

$$\Delta F\ (\mathrm{Hz}) = -1.832 \times 10^8\ \Delta M\ (\mathrm{g})/A\ (\mathrm{cm}^2)$$

In our system, the surface area of the QCM resonator (A) is $0.17 \pm 0.01\ \mathrm{cm}^2$, and the frequency decrease of 1 Hz corresponds to a mass increase of 0.9 ng. Film thickness (d) was estimated from the frequency shift and density (ρ) by using the following equation:

$$2d\ (\text{Å}) = -\Delta F\ (\mathrm{Hz})/1.832\ \rho\ (\mathrm{g\ cm}^{-3})$$

The details of QCM experiments have been reported in the literature.[19,24] Fourier transform infrared (FTIR) spectra were obtained using a Nicolet Nexus-670 attached with an attenuated total reflection (ATR) unit. pH was measured by an IQ 240 pH meter. For FTIR measurements, a gold coated glass substrate ($2.5 \times 1.2\ \mathrm{cm}^2$) purchased from Nicolet was immersed in 20 mL of piranha solution (3 : 1 mixture of conc. H_2SO_4 and 30% H_2O_2) for 30 min and thoroughly washed with pure water. The substrate was then immersed in 10 mM ethanolic solution of 3-mercaptopropionic acid for 12 h, rinsed with pure water, dried in the air, and used for the surface sol–gel process. The surfaces of QCM electrode were quickly treated with the piranha solution and modified with 3-mercaptopropionic acid in the same way to the gold coated glass substrate. For UV-Vis absorption experiments, a quartz substrate ($4.0 \times 1.0\ \mathrm{cm}^2$) was immersed in 1 M aqueous NaOH for 12 h, washed thoroughly in ethanol and pure water, dried, and used for the surface sol–gel process.

Results and discussion

Immobilization of PLL on TiO_2-gel films

The surface sol–gel process is a preparation method of ultrathin metal oxide films with thickness control to nanometer precision. In this process, metal alkoxides are chemisorbed on a solid substrate, and the substrate is washed in adequate organic solvents. After hydrolysis of the chemisorbed alkoxides, an ultrathin metal oxide gel film is produced (see Supplementary Information†). In sharp contrast to crystalline metal oxides, the amorphous gel film produced contains many hydroxyl groups at the surface, and these are available for further adsorption of alkoxides to make the oxide gel film grow. These hydroxyl groups readily dissociate in alkaline solution and give negative charges on the substrate.[19,22] For example, TiO_2-gel and ZrO_2-gel films are known to be able to strongly adsorb positively charged polymers and proteins. Based on these experimental results, we immobilized poly(L-lysine) (PLL) layer on ultrathin TiO_2-gel films.

The film deposition process was first monitored by QCM technique. A gold coated QCM electrode modified with 3-mercaptopropionic acid was immersed in titanium *n*-butoxide solution (100 mM in 1 : 1 mixture of toluene and ethanol) for 1 min and rinsed in ethanol. The resonator was then immersed in pure water for 1 min, and dried in the air. The amount of TiO_2-gel adsorbed in each cycle was constant for at least the first six cycles. The average frequency change was 68 ± 44 Hz. Thickness increase for each TiO_2-gel layer was estimated to be 1.1 nm supposing that the density was same as that of bulk TiO_2-gel ($1.7\ \mathrm{g\ cm}^{-3}$). We also monitored the film growth by UV-Vis absorption spectroscopy. As shown in Fig. 2, the peak intensity at 240 nm which was attributed to the absorption of amorphous TiO_2-gel film regularly increased.

Then, the substrate was immersed in PLL solution (1.5 mM in water) for 30 min, followed by rinsing in adequate water. For the PLL solution, we didn't use any salts or buffers in order to minimize the ion effect, and the pH was adjusted to 10.7 by using 10 mM NaOH solution. PLL has right handed helical conformation in aqueous solution above pH 9 because of the decrease of electrostatic repulsion between amino side chains, where the side chains are oriented outwards from the helical peptide chain. It has been reported that all the secondary structures of PLL gave an absorption peak near 190–197 nm in the dilute aqueous solution.[25] Therefore, increase in absorbance at 200 nm indicates the adsorption of PLL layer (Fig. 2). There was no further change in absorbance with increasing the immersion time. QCM frequency change due to PLL adsorption was 112 Hz. The thickness of the PLL layer was estimated to be 2.4 nm from the frequency shift (112 Hz) and the supposed density of $1.3\ \mathrm{g\ cm}^{-3}$ for PLL. This value is slightly larger than that expected for a PLL monolayer, because the diameter of α-helical PLL is in a range from 1.2 to 1.8 nm depending on the orientation of amino side chains. Therefore, we concluded that some part of PLL chains overlapped each other on the surface of TiO_2-gel. The roughness of the TiO_2-gel film might contribute to the overestimation in part. As mentioned before, hydroxyl groups of TiO_2-gel readily

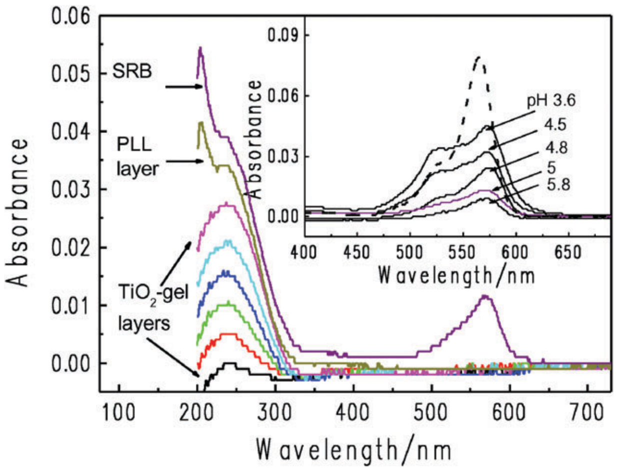

Fig. 2 UV-Vis absorption changes during stepwise deposition of TiO_2-gel layers, PLL layer, and fluorescent dye (SRB). Inset shows absorption spectra of SRB deposited on PLL layer at different pH. Dashed line shows absorption spectrum of 0.07 mM aqueous solution of SRB.

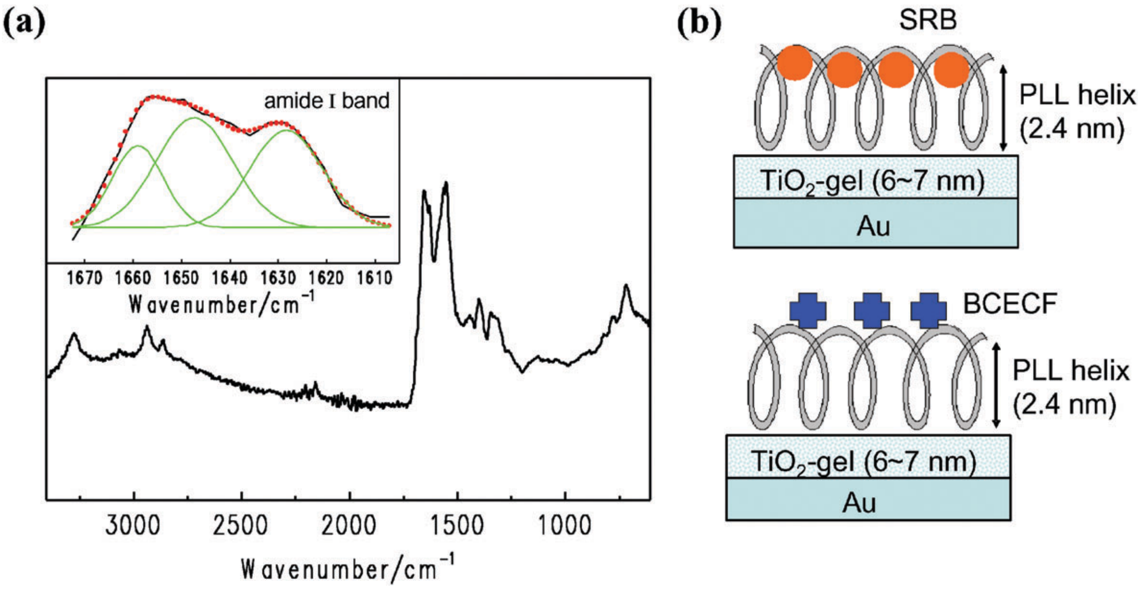

Fig. 3 (a) FTIR-ATR spectrum of the PLL layer assembled on a TiO$_2$-gel film. Inset shows an enlarged view of the spectrum near 1640 cm^{-1} and decomposition of amide I band. The red dots are the result of the curve-fitting with three component Gaussian bands. The PLL layer was adsorbed from the solution at pH 10.7, thoroughly washed in pure water, and dried by flushing with N$_2$ gas. (b) Schematic illustration of plausible binding modes of two fluorescent dyes.

dissociate in alkaline solution. Therefore, they should be deprotonated by the amine groups of PLL at the boundary between the PLL layer and the TiO$_2$-gel film. The resulted electrostatic interaction firmly fixes the PLL chains even at high pH. This interaction is so strong that the PLL is never desorbed by rinsing with water.[26]

Next, negatively charged fluorescent dye, SRB (or BCECF) was adsorbed from 1 mM aqueous solution. SRB is an anionic dye with two sulfonate groups and one diethyliminium group, and has some hydrophobic moiety. On the other hand, BCECF has four carboxyl groups which can interact with the amino groups of PLL. These molecules readily aggregated in the solutions at low pH and the aggregation was expected to be strongly induced by the positively charged PLL layer. For these reasons, we studied their adsorption in detail by changing the pH from 3.6 to 5.8 for SRB and 4.4 to 7.7 for BCECF.

The UV-Vis absorption spectrum of 0.07 mM SRB solution is shown by a dashed line in the inset of Fig. 2. At this concentration, SRB gave a monomer peak at 565 nm and a shoulder at 527 nm. As shown in the same inset, the amount of SRB deposited on PLL layers decreased with the increase of pH from 3.6 to 5.8. The dye deposited at low pH gave a clear shoulder near 527 nm. The enhancement of this shoulder is due to the aggregation of SRB.[27] The shoulder became unclear at pH 5.0 or higher. When focused on the monomer peak, the absorption maximum at 573 nm was red-shifted by 8 nm in comparison with that of the solution. This indicates that hydrophobic environment surrounding the dye molecule is enhanced on the PLL layer. In order to figure out the binding mode, we conducted molecular model calculation using Spartan'04. As a result, we revealed that SRB had a suitable size to be inserted into the hydrophobic cavity of the helical PLL chain. In the following experiments, we adsorbed SRB from the solution at pH 5.0. The spectra indicated by a purple line in Fig. 2 and in the inset do not show any enhancement of the shoulder near 527 nm. The peak intensity at 573 nm is just one-fifth of that of the spectrum obtained at pH 3.6. This supports that the dye molecules adsorbed at pH 5.0 are isolated from each other probably due to the intercalation. Similarly, the adsorption condition for BCECF was decided as pH 7.0. This hydrophilic dye adsorbs in isolation above this pH, showing a monomer peak near 500 nm (see Supplementary Information†).

The conformation of PLL chains adsorbed on TiO$_2$-gel films was examined by FTIR-ATR measurements. The spectrum is shown in Fig. 3a. The broad peaks near 700 cm^{-1} are attributed to the vibrations of Ti–O bonding in TiO$_2$-gel. The secondary structures of PLL are usually characterized by amide I and amide II bands corresponding to C=O stretching and N–H bending vibrations, respectively. The observed spectrum gave two amide I peaks at 1628 cm^{-1} and 1656 cm^{-1} together with an amide II peak at 1548 cm^{-1}. This indicates that α-helix and other conformations (random coil and β-turn) co-exist in the PLL layer.[28,29] The amide I peaks of the above three conformations are very close, as described in literature. So, we carried out the decomposition into component Gaussian bands in the range of 1610–1670 cm^{-1}. The results are shown in the inset of Fig. 3a. Similarly to the reported process, the wavenumbers of individual bands were first determined by means of second derivative of the smoothed spectrum.[28] After defining the number of the component bands, the spectrum was curve-fitted by using width and intensity as fitting parameters. The contribution of each component was estimated from the peak area. The percentages were 36% for α-helix (1628 cm^{-1}), 43% for random coil (1647 cm^{-1}), and 21% for β-turn (1659 cm^{-1}). Even though the PLL in the alkaline solution mainly has α-helical structure, two-thirds of the peptide chains were adsorbed in other conformations.[30] Probably, strong electrostatic attraction from TiO$_2$-gel loosens the tight helical conformation of PLL. We observed that all the helical conformation was slowly transformed to other conformations during one month if allowed to stand in the air. However, the formation of β-sheet structure which should have peaks near 1611 cm^{-1} was not observed in any time.[31]

Fig. 3b shows schematic illustration of the binding modes of two fluorescent dyes. Both of SRB and BCECF bind on PLL layers with a thickness of 2.4 nm formed on the TiO$_2$-gel films of 6–7 nm. SRB molecules are adsorbed as a monomeric species probably in the hydrophobic cavities of PLL chains, showing slightly red-shifted UV-Vis absorption spectra. In contrast, BCECF is supposed to exist in hydrophilic environment at the surface of PLL layer at least in our experimental condition near pH 7.

Enantioselective release of pre-adsorbed dye molecules

Molecular recognition at the air–water interface and solid surfaces has been extensively studied in the last decade.[32–34] In the present paper, we aimed at coupling such molecular

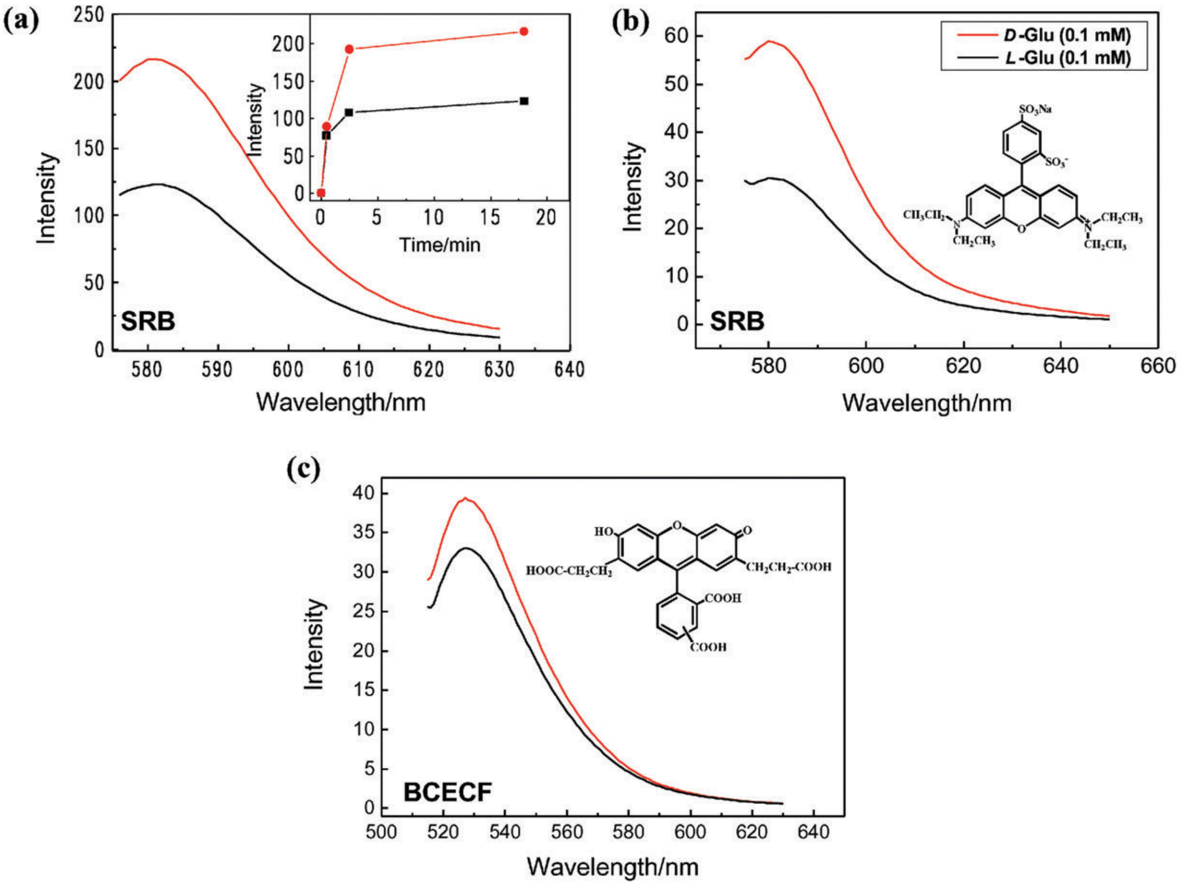

Fig. 4 Fluorescence spectra of dye molecules released in D-(red) and L-(black) glutamic acid solutions. (a) SRB released in 0.01 mM glutamic acid solutions after 18 min. Inset shows increases in fluorescence intensity at 581 nm. (b) SRB released in 0.1 mM glutamic acid solutions after 5 min. (c) BCECF released in 0.01 mM glutamic acid solutions after 18 min. Excitation wavelengths are 565 nm for SRB and 495 nm for BCECF, respectively. Fluorescence intensities of (b) cannot be directly compared with those of (a) and (c) due to the difference of slit width.

recognition with the release of fluorescent dyes. The question is whether the exchange occurs in a realistic way at the surfaces of solid substrates. We monitored the release of fluorescent dyes into the solutions of chiral glutamic acids.

A quartz substrate coated with a PLL layer containing fluorescent dye molecules (4.0×1.0 cm^2) was immersed in 0.01 mM aqueous solution of D- (or L-) glutamic acid (8 mL, pH 7.0). The anionic dye molecules were slowly replaced by glutamic acids, and released into the solutions. Fig. 4a shows the fluorescence spectra after 18-min immersion of SRB-coated substrate. As shown in the inset, the fluorescence intensity at 581 nm was increased with immersion time. The intensity of D-glutamic acid solution was 1.7 times higher than that of L-glutamic acid solution after the initial period of release. Similar exchange behavior was observed for 0.1 mM solutions of D- and L-glutamic acids (Fig. 4b). The enantioselectivity became unclear at concentrations higher than 1 mM. As for BCECF, the fluorescence intensity of D-glutamic acid solution was higher than that of L-glutamic acid solution (Fig. 4c). However, the difference was not as much as that of SRB. We also monitored the amounts of released dye molecules by UV-Vis absorption measurements. When the concentration of D- and L-glutamic acids was 0.01 mM (Fig. 4a and 4c), no more than 50% of pre-adsorbed SRB and 10–20% of BCECF were released from substrates during the first 18 min. That is to say, in our experimental conditions, enantioselective anion exchange was observed before less than half of dye molecules were released into glutamic acid solutions.

Since our experiments were carried out in heterogeneous system, it was difficult to estimate the rate constant of anion exchange. However, we observed that the exchange of SRB was more rapid than that of BCECF and more sensitive for the chirality of glutamic acids. The binding mode of hydrophilic BCECF appears to be not very effective for the enantioselective anion exchange. In contrast, SRB with hydrophobic moiety has better configuration for such exchange. In Fig. 5a, we schematically illustrated the anion exchange process of SRB. The dye molecules usually locate in the hydrophobic cavities of PLL. However, an ion-pair between one dye molecule and protonated amino side chain is accidentally formed. This ion-pair can be replaced with glutamic acid, giving a new ion-pair. The replaced dye molecule diffuses into the glutamic acid solution, and the substituted glutamic acid binds strongly to the PLL chain. The exchange of SRB and D-glutamic acid was verified by FTIR measurements. A sharp peak at 1595 cm^{-1} indicated by an arrow in Fig. 5b is corresponding to the C=O vibration of deprotonated glutamic acid. There was no sign of free and dimerized carboxyl groups. Therefore, we concluded that two carboxyl groups of glutamic acid were dissociated and both of them were electrostatically bound to the amino side chains. The peak intensity of D-glutamic acid at 1595 cm^{-1} was about two times higher than that of L-glutamic acid, as averaged over three measurements. This value is almost consistent with the difference of fluorescence intensity in Fig. 4a. It is apparent that PLL chains bind D-glutamic acid enantiomerically. The percentage of α-helical PLL chains is nothing but one-third of total PLL chains. Therefore, other conformation must contribute to the selective binding.

Anion exchange on PLL chains

The enantioselective binding of amino acid derivatives and PLL has been studied in solution.[35] For example, Ihara and his co-workers reported that right-handed α-helical PLL selectively bound carbobenzyloxy-L-phenylalanine (Cbz-L-Phe) against the enantiomer (Cbz-D-Phe) in methanol. The binding

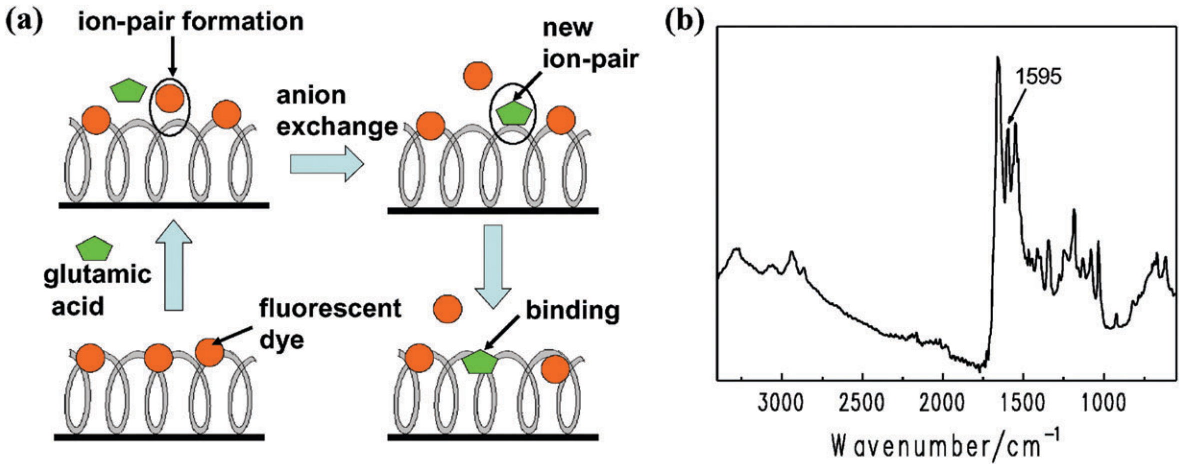

Fig. 5 (a) Schematic illustration of anion exchange on a PLL chain and (b) FTIR-ATR spectrum of the PLL layer after immersing into D-glutamic acid solution.

was characterized by UV-Vis and circular dichroism spectroscopies. They explained the main reason for the selectivity to be hydrophobic interaction between the carbonyl group of PLL and the phenyl group of Cbz-L-Phe as well as steric hindrance in chiral cavity of the amino side chains. In our case, glutamic acid has no hydrophobic moiety. However, it showed high enantioselectivity. The binding is started by electrostatic interaction between two carboxylate groups of glutamic acid and protonated amino side chains of PLL. The glutamic acid is then inserted into the hydrophobic cavity of PLL. At this moment, an amino group of D-glutamic acid should have the third interaction in order to ensure the chiral recognition. We presumed that the amino group formed hydrogen bonding with a carbonyl group of PLL, because a broad FTIR peak near 3300 cm^{-1} attributed to the weakened N–H vibration of primary amine was observed. The important thing is the fact that this enantioselective binding is related to the release of dye molecules. Ion pair formation between glutamic acid and PLL is not the rate-determining step of anion exchange. In this step, the binding of glutamic acid is reversible and in equilibrium with that of dye molecule. However, once the glutamic acid strongly binds to the PLL chain, probably by directing the amino group inside, the dye molecule is released from the surface of PLL layer.

We have also examined the anion exchange of SRB with peptides and polypeptides (see Supplementary Information†). The results are still preliminary, but they support that the anion exchange is available to evaluate the extent of interaction between macromolecules. Furthermore, this technique should be applicable for the separation of enantiomers of peptides and polypeptides.

Conclusion

The chemical communication of proteins is usually conducted through the recognition of helical peptide chains. Therefore, the immobilization of peptide chains is still attracting much attention.[36] In the present paper, electrostatic immobilization of molecularly thin PLL layer was successfully achieved. The immobilized PLL distinguished the chirality of glutamic acids, and the enantioselective binding was monitored by the release of pre-adsorbed fluorescent dyes. The anion-exchange is strongly interlocked with molecular recognition, if the pre-adsorbed molecules bind tightly to the peptide chains. In other words, the molecules are not easily released from the surface until much stronger bindings are formed on the peptide chains. This simple but significant conclusion will contribute to understanding the complicated molecular recognition at the surfaces of proteins and other biomacromolecules. High-throughput detection of proteins has been achieved by ELISA (enzyme-linked immunosorbent assay).[37] Antigen–antibody complexation has a great advantage in terms of specific sensing. However, using such a system it is difficult to understand the chemical interactions between biomolecules. Anion exchange coupled with molecular recognition will contribute to the investigation of such interactions.

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