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Peptide-Based Switching of Polymer Fluorescence in Aqueous phase

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Phage display[1] is a powerful methodology to obtain peptides that specifically bind to desired targets. This approach has been applied not only to biological molecules, but also to the solid surface of artificial materials such as synthetic polymers.[2] The peptides specific for chlorine-doped polypyrrole,[2a] yohimbine imprinted methacrylate resin,[2b] poly(methyl methacrylate),[2c−e] polystyrene,[2f,g] and polylactic acid[2h] have all been identified thus far. Despite their great potential as peptidyl nanomaterials, their applications are still limited to solid-surface functionalizations,[2a] because the target polymers are all water-insoluble. Therefore, the identification of peptides specific for "water-soluble" targets will widely extend the utility of these polymer-peptide complexes.

Water-soluble conjugated polymers (CPs) have been attracting much attention as fluorescent probes in aqueous solution,[3] as their fluorescence is extremely sensitive because of amplification by a collective response.[3a−d] Changes in the color and/or fluorescence induced by the mainly electrostatic interactions of the analyte molecules with the CPs have successfully detected proteins,[3e] nucleic acids,[3f] and even viruses.[3g] In addition to conventional combinations of CPs and biomolecules, the identifications of the "specific" combinations are attractive candidates for the further utilization of CPs and/or biomolecules in biological applications.

Here, we applied for the first time the phage display method on water-soluble and commonly used poly(2-methoxy-5-propyloxysulfonate-1,4-phenylenevinylene) (mpsPPV) (Figure 1a), and report that the identified peptide binds to mpsPPV, even in aqueous solution. After the complexation of mpsPPV with its specific peptide, the fluorescence of mpsPPV was obviously enhanced at a relatively low concentration of the peptide. This new phenomenon was used to distinguish changes in the chemical structures of peptides, thereby demonstrating the peptide-based switching of the fluorescence in aqueous phase for novel fluorescent biodevices.

In contrast to other water-insoluble polymeric films, mpsPPV is water-soluble, thus is not a facile substrate for the experimental procedure of the phage display method. To overcome this problem, we immobilized mpsPPV using a layer-by-layer (LbL) assembly technique[4] with poly(diallyldimethylammonium chloride) (PDDA) to obtain water-insoluble polyelectrolyte multilayer films, with an outermost surface composed of mpsPPV, on a glass substrate. Because the outermost polyelectrolyte of multilayer films has charged segments free from electrostatic interactions,[4] the target film's surface has the similar segments to water-solubilized mpsPPV. The assembly process and the stability of the obtained LbL films were monitored by quartz-crystal microbalance (QCM) measurements[4b] (Figure 1b). The thickness of the LbL film increased with the number of assembly step. To avoid peeling off the film and exposing the glass substrates during phage display experiments, we used sufficiently thick films (ca. 40 nm, 21 step, the outermost surface is mpsPPV). A linear 12-mer peptide library solution (Ph.D.-12 Phage Display Peptide Library Kit, New England Biolabs) was applied to this LbL film (Figure 1a). Weakly bound phages were washed away and strongly bound phages were collected by elution procedure. These phages were amplified by infecting with *E. coli*, and were subjected to next rounds. The LbL film was considered to be stable throughout the phage display experiment, because the frequency shift was negligible after immersion in ethanol and Tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) (Figure 1b). After 5 rounds

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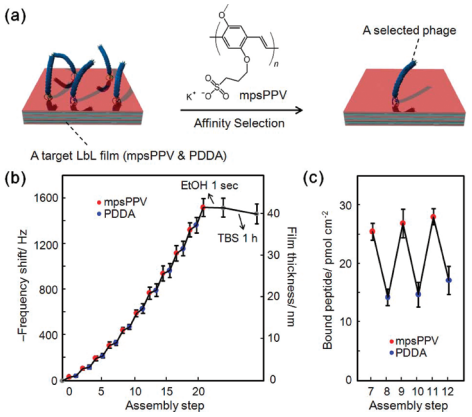

Figure 1. Selection of mpsPPV-binding peptides. (a) Schematic illustration of the selection for mpsPPV using a random phage displayed 12-mer peptide library. (b) QCM analysis of the LbL assembled mpsPPV and PDDA. The LbL procedure was repeated 21 steps. To check the stability of the obtained LbL films, we measured the frequency shift after immersion in ethanol and TBS. (c) Amounts bound of Mps01 on the LbL films, as determined by SPR. The results are averaged values with standard deviations from three independent measurements (mean ± S.D.).

of this biopanning procedure, the phage clone carrying the mpsPPV-binding peptide (Mps01, HNAYWHWPPSMT) was successfully identified (see the Supporting Information, Figure S1).

We investigated the bound amounts of the synthetic Mps01 toward the LbL films, of which the assembly steps were changed from 7 to 12. Figure 1c clearly showed that the amounts bound of Mps01 on mpsPPV surfaces (odd steps) were larger than that on PDDA surfaces (even steps). Although the peptide bound even on PDDA surface, we considered that some peptides could access the underneath mpsPPV, because of the interpenetrated loose nature of the LbL film.[4c] The amounts bound of Mps01 toward PDDA surfaces reflect this possibility in addition to nonspecific binding. Next, the surface plasmon resonance (SPR) measurements were performed in order to quantitatively assess the binding ability of Mps01 toward the target LbL film (9 step, the outermost surface was mpsPPV). The adsorption kinetics of Mps01 toward the LbL film was successfully monitored in real time by SPR detection. Assuming a Langmuir adsorption model,[5] the binding constant (K_a) was determined to be 1.3×10^5 M^{-1} (see the Supporting Information, Figure S2). This value was comparable with that of hyperbranched or linear PPV-binding peptides.[6] Therefore, Mps01 recognized and specifically bound to the mpsPPV surface of the LbL films.

To investigate the interaction between mpsPPV and Mps01 in aqueous solution, the fluorescence spectra in which the amount of added peptide was varied while the amount of mpsPPV was kept fixed at 20 μM (monomer basis) were measured (Figure 2a). The complexation in aqueous buffered solution (10 mM Tris-acetate, pH 7.5) resulted in changes in the fluorescence of mpsPPV (Figure 2a). The fluorescence intensity at 580 nm first

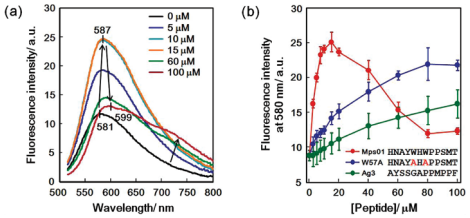

Figure 2. Fluorescence modulation by Mps01. (a) Fluorescence spectra of mpsPPV/Mps01 complexes excited at 500 nm. (b) Plots of the fluorescence intensities at 580 nm against the concentration of the peptides. The results are averaged values with standard deviations from three independent measurements (mean ± S.D.).

obviously enhanced with increasing concentrations of Mps01, reaching a maximum (2.8-fold) at a concentration of around 15 μM (Figure 2b). This fluorescence enhancement is attributed to the increased dispersion of mpsPPV chains.[7] In other words, the peptide seems to untangle the mpsPPV chains, which have a relatively low quantum yield (ca. 5%)[8] due to the nonradiative de-excitation occurring because of the entanglement and aggregation. At a peptide concentration of 15 μM, both the emission and absorption maxima were red-shifted (581 nm → 587 nm and 438 nm → 444 nm, respectively) (Figure 2a and the Supporting Information, Figure S3). These observations suggest that polymer backbone adopts a more planar conformation in the complex.

At higher concentrations (> 20 μM), the fluorescence intensity started decreasing (Figure 2b), indicating that Mps01 induced an aggregation of mpsPPV.[9] In the fluorescence spectra (Figure 2a), the shoulder peak appeared in the longer wavelength region (ca. 730 nm), which can also be attributed to the aggregation of the polymer.[9] In fact, we confirmed that mixing a much more concentrated solution of Mps01 (1 mM) and mpsPPV (2 mM) resulted in the immediate precipitation of the polymer. In a polyelectrolyte−surfactant system, the surfactants disperse the polymer in a low concentration regime, but in a higher concentration regime, fully templated surfactants on the polyelectrolyte backbone chain induce an aggregation of the polymer.[10] A similar mechanism would be present in our system.

On the other hand, in the case of Ag3 (AYSSGAPPMPPF) that has been identified as a silver-binding peptide,[11] the fluorescence intensity monotonically increased and saturated (Figure 2b). Ag3 increases the fluorescence only 1.2-fold at a peptide concentration of 15 μM (cf. 2.8-fold by Mps01). Thus, only the specific interaction of Mps01 with mpsPPV generated a keen fluorescence enhancement.

Next, the Mps01 mutant was used, which has two tryptophan residues mutated into alanine (W57A). Significantly, the increase in fluorescence intensity was delayed at

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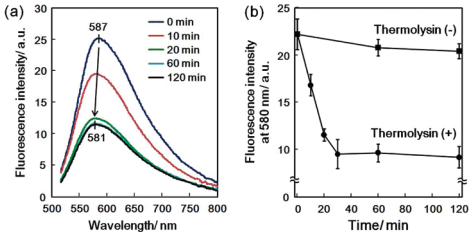

Figure 3. Enzyme-triggered fluorescence quenching. (a) Fluorescence spectra of the mpsPPV/Mps01 complexes after the reaction with thermolysin for the indicated times. (b) Plots of the fluorescence intensities of the mpsPPV/Mps01 complexes against the duration of the hydrolytic reaction. The results are averaged values with standard deviations from three independent measurements (mean ± S.D.).

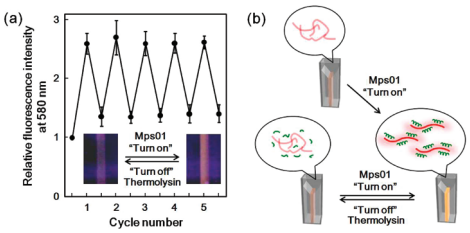

Figure 4. Fluorescence-switching biodevice (on/off). (a) Reversibility of the fluorescence change. The results are averaged values with standard deviations from three independent measurements (mean ± S.D.). (b) Schematic illustration of the fluorescence-switching biodevice. mpsPPV, red lines; Mps01, green lines.

higher concentrations than the original Mps01 (Figure 2b). The fluorescence enhancement at a lower concentration of Mps01 means that the W → A substitutions obviously reduced the binding ability of Mps01. Thus, the tryptophan residues play an important role in forming the complex, possibly due to $\pi-\pi$ interactions, similar to hyperbranched or linear PPV-binding peptides.[6] Circular dichroism (CD) spectral analysis revealed that Mps01 partly adopts a polyproline II helical conformation[12] in the presence and absence of mpsPPV (see the Supporting Information, Figure S4a). The positive peak at around 228 nm, which corresponds to the aforementioned conformation,[12] completely disappeared after the W → A mutations (see the Supporting Information, Figure S4b), implying the importance of the conformation for efficient complexation.

Since Mps01 enhanced the fluorescence of mpsPPV, the intensity might be decreased upon degradation of the peptide. To test this idea, we employed thermolysin to degrade Mps01. Thermolysin is a thermostable endopeptidase which is widely distributed in nature, and the detection of its activity is an important issue.[13] Before the complexation of mpsPPV with peptide, Msp01 was treated with thermolysin. As expected, the enhanced fluorescence of the complex was successfully quenched to its original magnitude upon the enzymatic hydrolysis of Mps01 (Figure 3). In fact, high-performance liquid chromatography confirmed that the hydrolysis of Mps01 by thermolysin was completed within 30 min (data not shown), which is consistent with this fluorescence experiment. The emission maximum was also returned to the same position as that of the original mpsPPV (Figure 3a), suggesting that generated peptide fragments barely interacted with mpsPPV. In contrast, the fluorescence quenching was hardly observed without thermolysin (Figure 3b). These results clearly demonstrated that the fluorescence quenching was actually caused by the hydrolysis of Mps01 by thermolysin.

Importantly, direct hydrolysis of Mps01 in the complex and the subsequent in situ disassembly was feasible. In conjunction with the repetitive addition of Mps01, the fluorescence was switched between enhanced and quenched states, reproducibly and reversibly (Figure 4a). First, the fluorescence emission from mpsPPV was enhanced via the complexation with Mps01. Second, this fluorescently enhanced complex was disrupted by the thermolysin treatment for 30 min. After the hydrolytic reaction, only weak fluorescence was observed. Third, an addition of the fresh Mps01 resulted in reformation of the fluorescently enhanced complex (Figure 4b). Because the restored fluorescence can be quenched again as long as thermolysin turns over Mps01, this cycle can be repeated many times. The dynamic assembly/disassembly of the supramolecular complex enabled us to develop a reversible fluorescence-switching biodevice.

In conclusion, we applied the phage display method to a water-soluble polymer for the first time. The most representative CP, mpsPPV, was selected as the peptide's target. The LbL technique was successfully utilized to prepare the target film's surface. The obtained 12-mer peptide bound to mpsPPV even in aqueous solution, and specifically enhanced the polymer's fluorescence at a relatively low peptide concentration (~15 μM). It was suggested that $\pi-\pi$ interactions were predominant between the peptide and mpsPPV. A detailed point mutation study of the peptide to further elucidate the binding mechanism is now underway. The complex may be useful as a novel biodevice that reversibly responds to bioactive components, because the present method can be principally applied to a wide variety of water-soluble polymers. Moreover, the present enzyme-responsive and fluorescence-variable complex well-dispersed in aqueous solution may have further potential applications in drug delivery and bioimaging.

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Supporting Information Available: Detailed experimental methods and supporting figures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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