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Conjugated Polyelectrolytes for Protein Assays and for the Manipulation of the Catalytic Activity of Enzymes

Lingling An, Shu Wang,* and Daoben Zhu*[a]

Dedicated to Professor Ryoji Noyori on the occasion of his 70th birthday

Abstract: A new method has been developed to discriminate between proteins with different isoelectric points by using fluorescent conjugated polyelectrolytes. Charged water-soluble polyfluorenes that contain 2,1,3-benzothiadiazole (BT) units demonstrate intramolecular energy transfer from the fluorene units to the BT sites when oppositely charged proteins are added to the mixture. This results in a shift in emission color from blue to green and a change in the emission intensity of conjugated polyelectrolytes, which makes it possible to assay proteins. The formation of conjugated polyelectrolyte/enzyme complexes by electrostatic interactions can be utilized to manipulate the activity of enzymes by means of local alteration of enzyme charge density. The oppositely charged substrate binds to conjugated polyelectrolyte and reduces the distance between the enzyme and the substrate, leading to an increase in the cleavage reaction rate. The new method has three important features: 1) it offers a convenient "mix-and-detect" continuous approach for protein assays and rapid detection of enzyme activity; 2) the use of water-soluble conjugated polyelectrolytes imparts the sensor with a high sensitivity; 3) this method does not require fluorescent labels on the targets, which should significantly reduce the cost.

Keywords: absorption · biosensors · electron transfer · fluorescence · proteins

Introduction

In recent years, biodetection technologies have received more and more research and application interest owing to the rapidly increasing demands of genetic analysis, clinical diagnosis, environmental analysis, biowarfare, and homeland defence. Detection sensitivity is one of the most important aspects in biosensor development, with the ultimate goal being trace detection of biological analytes. Water-soluble conjugated polymers have recently attracted much attention as the optical platform in biological sensors.[1–6] In comparison with small-molecule counterparts, the electronic structure of the conjugated polymer (CP) coordinates the action of a large number of absorbing units. The transfer of the excitation energy along the whole backbone of the CP to the energy/electron acceptor attached to the CP results in the amplification of fluorescent signals.[7] The CPs have been used for the detection of inorganic ions, biologically active small molecules, nucleic acids, and proteins.[8–19]

According to recent research, the principal signal-transduction mechanisms of CPs are based on fluorescence resonance energy transfer (FRET), electron transfer, or the conformation transitions of polymer backbones.[1–6] To obtain insight into the additional signal-transduction mechanism by water-soluble conjugated polymers, the aggregations of CPs in solutions have been studied to understand how the interchain arrangement can be optimized for use in biosensors.[20–23] The aggregation induced by the analyte can result in fluorescence self-quenching owing to π stacking of the backbones of the conjugated polymers.[20] Several biological sensors have been developed to take advantage of the optical perturbations of CPs driven by analyte-induced aggregation.[24–27] As was first reported by Bazan and co-workers,[28] the cationic poly{(1,4-phenylene)-2,7-[9,9-bis(6′-*N*,*N*,*N*-trimethylammonium)-hexyl-fluorene] dibromide} containing

[a] L. An, Prof. Dr. S. Wang, Prof. D. Zhu
Beijing National Laboratory for Molecular Sciences
Key Laboratory of Organic Solids
Institute of Chemistry
Chinese Academy of Sciences
Beijing, 100080 (P.R. China)
Fax: (+86) 10-6263-6680
E-mail: wangshu@iccas.ac.cn
zhudb@iccas.ac.cn

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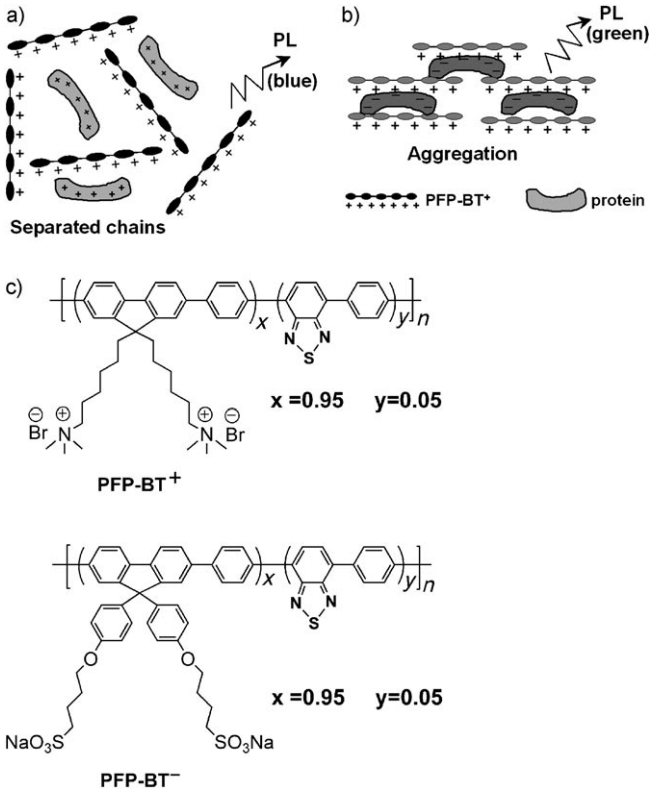
5% benzothiadiazole (BT) units (PFP-BT$^+$; see Scheme 1 for chemical structure) demonstrates intramolecular energy transfer from the fluorene units to the BT sites when a negatively charged DNA molecule is added. The energy transfer results from the increase in the interchain contacts of PFP-BT$^+$ by the formation of an electrostatic complex and aggregations. The energy transfer process triggers a shift in the emission color of PFP-BT$^+$ from blue to green. This ratiometric fluorescence technique exhibits a big shift in emission profiles and provides a measurement to reduce the non-specific interaction events in comparison with those biosensors based on intensity measurements.



Scheme 1. Schematic representation of the protein assay based on analyte-induced aggregation of charged conjugated polymers (a–b), and the chemical structures of PFP-BT$^+$ and PFP-BT$^-$ (c). PL = photoluminescence.

Abstract in Chinese:

通过静电相互作用构建共轭聚合物/蛋白质复合物，利用蛋白质分子作用前后聚合物聚集态变化而导致的荧光信号改变实现了对蛋白质的灵敏分析。共轭聚合物/酶复合物的形成还可用于改变酶活性。这些方法为快速灵敏检测蛋白质提供了简便的混合检测技术。

There is currently a strong appetite to explore new, simple, and efficient protein detection techniques because of their involvement in a wide range of research fields such as proteomics, medical diagnostics, and signal transduction.[29–31] The unchangeable isoelectric point imparts positive or negative protein charges in aqueous solution, which makes characteristic electrostatic interactions play a robust role in the design of protein sensors.[16c,32,33] Our recent work has shown that PFP-BT$^+$ or negatively charged PFP-BT$^-$ (see Scheme 1 for chemical structure) can be used as sensitive probes to assay enzyme-mediated cleavage reactions of the charged substrates, such as adenosine triphosphate and polyarginine peptide.[34] In this case, we take advantage of the analyte-induced aggregations of PFP-BT$^+$ or PFP-BT$^-$ to discriminate proteins with different isoelectric points based on the ratiometric fluorescence measurement. Furthermore, as a member of the giant protein family, in most cases, enzymes also exploit the coulomb attraction to immobilize a substrate to its reaction site, which guarantees that the biocatalytic reaction proceeds well. The local alteration of the enzyme's charge density may speed up or slow down the biocatalytic process. Enzyme modification not only provides a means for the screening of novel biocatalysts but also finds application in sensing and enzyme-related biotechnology.[32,33,35] In this case, the conjugated polyelectrolyte is also utilized to decorate the surface of the enzyme to adjust its catalytic activity.

Results and Discussion

Mechanism for Protein Assays

The overall strategy for protein assays based on analyte-induced aggregation of charged conjugated polymers is illustrated in Scheme 1. The isoelectric point imparts positive, neutral, or negative protein charges in aqueous solution at certain pH values. The neutral or identically charged protein to that of PFP-BT$^+$ or PFP-BT$^-$ prohibits tight aggregation and keeps their main chains separated, and FRET from the fluorene units to the BT sites is inefficient. A protein that contains multiple charges can form complexes with oppositely charged polymer PFP-BT$^+$ or PFP-BT$^-$ through electrostatic interactions. The protein-induced aggregation of PFP-BT$^+$ or PFP-BT$^-$ keeps the isolated main chains in close proximity, which allows for efficient FRET from the fluorene units to the BT sites. As a result, the emission color of the polymer shifts from blue (420 nm) to green (530 nm). The mechanism of the intrachain energy transfer was supported by measuring absorption and excitation spectra of the PFP-BT$^+$ and PFP-BT$^-$ before and after adding oppositely charged proteins (see Figure S1 in the Supporting Information). Thus, by triggering the shift in emission color and the change of emission intensity of PFP-BT$^+$ or PFP-BT$^-$, it is possible to discriminate proteins with different isoelectric points.

CHEMISTRY
AN ASIAN JOURNAL

Conjugated Polyelectrolyte/Protein Complex for Protein Assays

Six proteins are chosen as our targets, of which the isoelectric points (pI) extend from 4.2 to 11.35 in the testing phosphate-buffered saline solution (PBS, 1 mM; pH 7.4).[16c] They can be classified into three categories: positively charged lysozyme (pI 11.35) and avidin (pI 10.4); nearly neutral streptavidin (pI 6.4); and negatively charged bovine serum albumin (BSA, pI 4.8), concanavalin A (Con A, pI 4.5–5.5), and glucose oxidase (pI 4.2).

To support the mechanism of our new protein assay, the fluorescence responses of PFP-BT$^+$ to the six proteins were examined. Figure 1a shows the emission spectra observed upon addition of negatively charged BSA ([BSA] = 0.22–440 nM) to the solution of PFP-BT$^+$ ([PFP-BT$^+$] = 5.0×10^{-7} M in repeat units (RUs)) with an excitation wavelength of 370 nm. The emission maximum of PFP-BT$^+$ itself in PBS buffer solution appeared at around 420 nm and no emission of the BT unit at 530 nm was observed. Adding BSA led to a significant quenching of fluorine-unit emission at 420 nm and the appearance of the emission of the BT unit at 530 nm. Similar results were observed when negatively charged Con A and glucose oxidase were used. There was less quenching of the emission of PFP-BT$^+$ and no emission of the BT unit at 530 nm was observed upon the addition of non-negatively charged proteins, lysozyme, avidin, or streptavidin. This shows that non-negatively charged proteins do not induce the aggregation of PFP-BT$^+$. Figure 1b shows the dependence of the ratio of emission intensity at 530 and 420 nm (I_{530nm}/I_{420nm}) of PFP-BT$^+$ on the concentrations of proteins. In the case of negatively charged proteins, the values of I_{530nm}/I_{420nm} increase gradually with the protein concentration from 0.22 to 30 nM and reach a plateau after 40 nM. In the case of the other three proteins, almost no increase in the I_{530nm}/I_{420nm}value was observed for PFP-BT$^+$, demonstrating that the cationic water-soluble PFP-BT$^+$ is selectively responsive to negatively charged proteins.

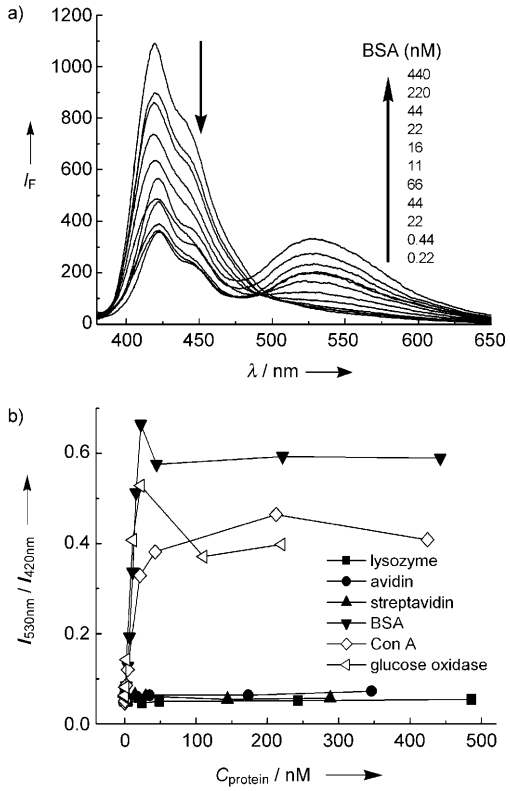


Figure 1. a) Emission spectra of PFP-BT$^+$ as a function of the concentration of BSA in PBS buffer solution (1 mM; pH 7.4). [PFP-BT$^+$] = 5.0×10^{-7} M in RUs, [BSA] = 0.22–440 nM. b) The ratio of the emission intensity of PFP-BT$^+$ at wavelengths 530 and 420 nm (I_{530nm}/I_{420nm}) versus the concentrations of proteins. [PFP-BT$^+$] = 5.0×10^{-7} M in RUs, [protein] = 0.22–490 nM. The excitation wavelength is 370 nm.

When PFP-BT$^-$ is used as a fluorescent probe, adding positively charged lysozyme led to a significant quenching of the fluorene unit emission at 420 nm, and the appearance of the BT unit emission at 530 nm (Figure 2a). As shown in Figure 2b, the values of I_{530nm}/I_{420nm} level off at around 0.2 or below for non-positively charged proteins, and 0.6 for positively charged ones; the large gap between the two plateaus can be exploited to help us discriminate different proteins. Note that the lysozyme could even be discriminated from a mixed solution containing the other five proteins (see Fig-

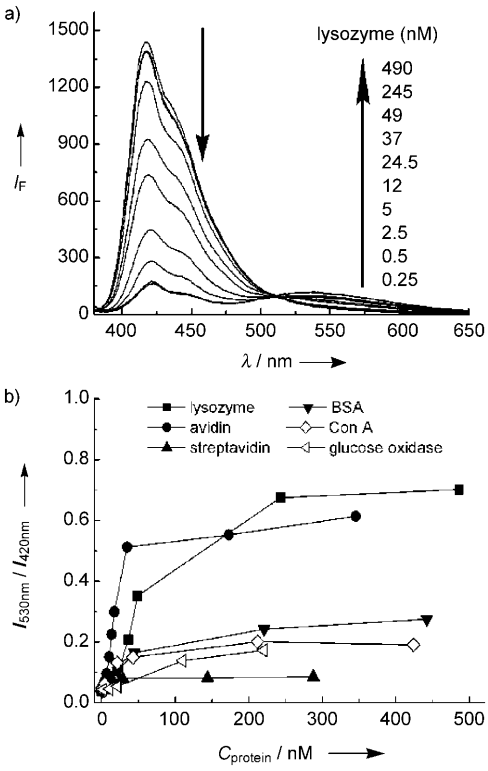
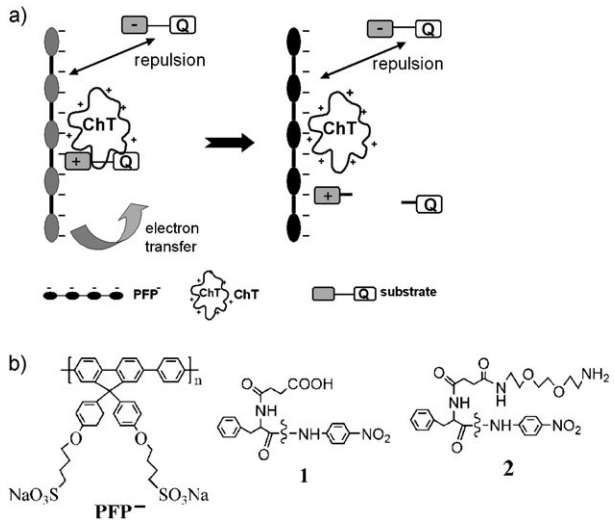


Figure 2. a) Emission spectra of PFP-BT$^-$ as a function of the concentration of lysozyme in PBS buffer solution (1 mM; pH 7.4). [PFP-BT$^-$] = 5.0×10^{-7} M in RUs, [lysozyme] = 0.25–490 nM. b) The ratio of emission intensity of PFP-BT$^-$ at wavelengths 530 and 420 nm (I_{530nm}/I_{420nm}) versus concentrations of proteins. [PFP-BT$^-$] = 5.0×10^{-7} M in RUs, [protein] = 0.22–490 nM. The excitation wavelength is 370 nm.

ure S2 in the Supporting Information). These results turn out to be perfectly consistent with the assay mechanism shown in Scheme 1.

Manipulation of the Catalytic Activity of Enzymes

Some enzymes also exploit the Coulomb attraction to immobilize substrates to reaction sites. We speculate that the strong electrostatic interaction between oppositely charged CP and an enzyme is likely to be utilized to modify the activity of the enzyme. The local alteration of the enzyme's charge density may speed up or slow down the biocatalytic process.[16c,32,33] Chymotrypsin (ChT) and negatively charged PFP^- are chosen as the basic components of this working system (see Scheme 2 for chemical structures). The pI of



Scheme 2. Schematic representation of the enzymatic adjustment for the positively charged enzyme (ChT) with negatively charged PFP^- (a) and the chemical structures of PFP^- and substrates **1–2** (b). Q = quencher.

ChT is 8.75,[32] which imparts to this enzyme a positive charge density in the phosphate buffer assay solution (5 mM, pH 7.4). Thus, the complex of PFP^- and ChT can form through electrostatic interactions. As shown in Scheme 2, the PFP^- molecule can change the local charge density of ChT, making it more attractive to binding by positively charged substrates and repelling negatively charged ones. Two oligopeptides that contain a quencher (nitro group) were tested as substrates for ChT, including negatively charged **1** and positively charged **2**. When substrates bind to PFP^-/ChT, the fluorescence of PFP^- is quenched by an electron-transfer process. The cleavage reactions of substrates can be traced by monitoring the fluorescence recovery of PFP^- or the absorption change owing to the formation of *p*-nitroaniline.

We first studied the effect of PFP^- on the initial rate of the cleavage reaction of substrates **1–2** catalyzed by ChT. The initial reaction rate (ΔA/min) was calculated as the slope of the plot of the absorbance change at 380 nm of formed *p*-nitroaniline versus reaction time.[36] As shown in Figure 3, upon the addition of PFP^-, the initial reaction rate declines by about 40% in comparison with that in the absence of PFP^- owing to a repulsive interaction between

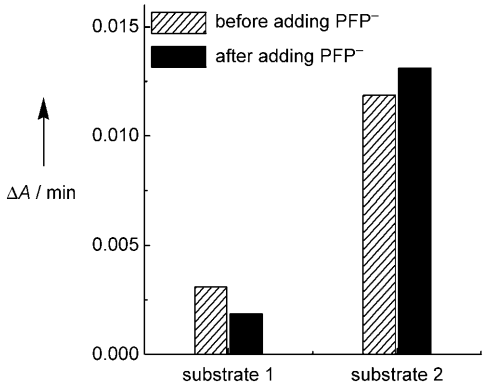


Figure 3. Initial cleavage reaction rate of substrates **1–2** by ChT before and after adding negatively charged PFP^-.

PFP^- and substrate **1**. In the case of substrate **2**, a totally different picture appears. The attraction between PFP^- and **2** plays an active role as a result of the reduction in the distance between ChT and **2**, which leads to a 10% increase in the rate ratio in comparison with that observed in the absence of PFP^-. Obviously, conjugated polyelectrolytes indeed influence the catalytic activity of enzymes to different levels depending upon the substrates' charge type. Notably, despite the clear evidence for the modification of enzyme activity by electrostatic interactions, other weak forces, such as hydrophobic interactions, could also play a role in the manipulation of catalytic activity of the enzyme.

The cleavage reactions of substrates **1–2** were also studied by monitoring the fluorescence recovery of PFP^-. After mixing ChT with PFP^-, the solution of the substrate in PBS (5 mM, pH 7.4) was added to initiate the cleavage reaction while simultaneously recording the emission spectra. The initial solution of PFP^-/ChT/substrate **2** showed a quenched fluorescence of PFP^-. The emission intensity of PFP^- at 424 nm gradually increased with the incubation time from 0 to 42 min (Figure 4a). A threefold fluorescence increase of PFP^- resulted from the cleavage of substrate **2** by ChT, which demonstrates the fluorescence "turn-on" response of the PFP^-/ChT/substrate **2** complex for ChT activity. The fluorescence recovery of PFP^- exhibits dependence upon the incubation time of the enzyme (Figure 4b). For negatively charged substrate **1**, the emission intensity of PFP^- at 424 nm changed little at incubation times between 0 to 33 min (Figures 5a and b). The inevitable hydrophobic interaction possibly results in the reduced decrease in the fluorescence intensity. These results show that PFP^- prohibits substrate **1** from sufficiently binding to ChT.

CHEMISTRY
AN ASIAN JOURNAL

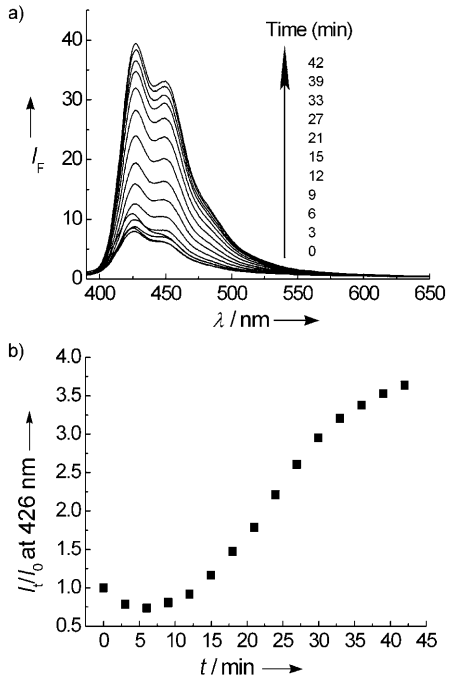


Figure 4. a) Emission spectra of PFP^-/ChT/substrate **2** in phosphate buffer solution (5 mM, pH 7.4) as a function of reaction time for ChT-catalyzed cleavage of substrate **2**. b) The dependence of the emission intensity of PFP^-/ChT/substrate **2** at 424 nm as a function of reaction time for ChT-catalyzed cleavage of substrate **2**. $[ChT]=4.0\times10^{-6}$ M, $[PFP^-]=6.0\times10^{-6}$ M, [substrate **2**] $=4.0\times10^{-5}$ M. The excitation wavelength is 380 nm.

Conclusions

In summary, we have studied the interaction between water-soluble conjugated polyelectrolytes and proteins. The conjugated polyelectrolytes can be utilized as probes for discriminating proteins with different isoelectric points based on the ratiometric fluorescence measurement, which interfaces with the aggregation and light-harvesting properties of conjugated polyelectrolytes. The strong electrostatic interaction between oppositely charged conjugated polyelectrolyte and enzyme can be utilized to modify the activity of enzymes by local alteration of the charge density. Our new method has several important features: 1) it offers a convenient "mix-and-detect" continuous approach for protein assays and rapid detection of enzyme activity; 2) the use of water-soluble conjugated polyelectrolytes imparts the sensor with a high sensitivity; 3) this method does not require fluorescent labels on the targets, which should significantly reduce the cost.

Experimental Section

Materials and Methods

Avidin and streptavidin were purchased form Amresco. Glucose oxidase and lysozyme were obtained from Biozyme. BSA was received from SABC. Con A, substrates **1–2**, and ChT (α-chymotrypsin from bovine pancreas) were obtained from Sigma. PFP-BT^+,[28a] PFP-BT^-,[34] and PFP^- [37] were synthesized according to the procedures in the literatures.

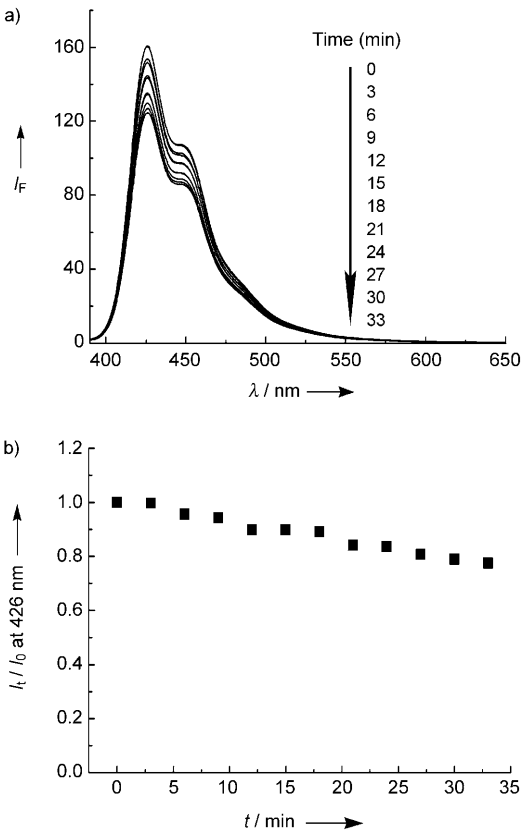


Figure 5. a) Emission spectra of PFP^-/ChT/substrate **1** in phosphate buffer solution (5 mM; pH 7.4) as a function of reaction time for ChT-catalyzed cleavage of substrate **1**. b) The dependence of the emission intensity of PFP^-/ChT/substrate **1** at 424 nm as a function of reaction time for ChT-catalyzed cleavage of substrate **1**. $[ChT]=4.0\times10^{-6}$ M, $[PFP^-]=6.0\times10^{-6}$ M, [substrate **1**] $=4.0\times10^{-5}$ M. The excitation wavelength is 380 nm.

The concentrations of all proteins used herein were determined from their UV spectra. UV/Vis absorption spectra were taken on a JASCO V-550 spectrometer. The fluorescence measurements were recorded on a Hitachi F-4500 spectrophotometer equipped with a Xenon lamp excitation source.

Protein Assays

PBS buffer solution (2 mL, 1 mM; pH 7.4) containing 5.0×10^{-7} M PFP-BT^+, then respectively, lysozyme, avidin, streptavidin, BSA, Con A, and glucose oxidase were successively added to six 3 mL polystyrene cuvettes and the fluorescence emission spectra were measured immediately at room temperature with an excitation wavelength of 370 nm. The assay procedures with PFP-BT^- are the same as that for PFP-BT^+, except for the case of using PFP-BT^- instead of PFP-BT^+. The plots of the emission intensity ratio between wavelengths 530 and 420 nm (I_{530nm}/I_{420nm}) versus protein concentration were obtained.

Manipulation of the Catalytic Activity of ChT

PBS buffer solution (2 mL, 5 mM; pH 7.4) and 4.0×10^{-6} M ChT and 6.0×10^{-6} M PFP^-, were added to two 3 mL polystyrene cuvettes. After incubation for 30 min, a substrate, either **1** or **2**, was added into one of the two cuvettes, to initiate the cleavage reaction. The emission spectra were then recorded at 3 min intervals over 30 min at room temperature with an excitation wavelength of 380 nm. The plots of the fluorescence intensity at 424 nm versus the ChT incubating time were obtained.

Acknowledgements

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