analytical chemistry

Technical Note

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Strategy for Sensor Based on Fluorescence Emission Red Shift of Conjugated Polymers: Applications in pH Response and Enzyme Activity Detection

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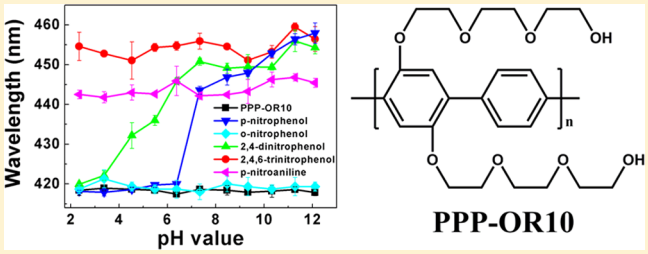
Supporting Information

ABSTRACT: A new strategy was developed and applied in monitoring pH response and enzyme activity based on fluorescence emission red shift (FERS) of the conjugated polymer PPP-OR10 induced by the inner filter effect (IFE) of nitrobenzene derivatives. Neutral poly(*p*-phenylenes) functionalized with oligo(oxyethylene) side chains (PPP-OR10) was designed and synthesized by the Suzuki cross-coupling reaction. Nitrobenzene derivatives display different light absorption activities in the acidic or basic form due to adopting different electron-transition types. When environmental pH is higher than their pK_a values, nitrobenzene derivatives exhibit strong absorbance around 400 nm, which is close to the maximal emission of polymer PPP-OR10. As a result, the maximal emission wavelength of PPP-OR10/nitrobenzene derivatives red shifts with the pH value increasing. Apparently, the IFE plays a very important role in this case. A new method has been designed that takes advantage of this pH-sensitive platform to sensor α-chymotrypsin (ChT) based on the IFE of *p*-nitroaniline, since the absorption spectrum of *p*-nitroaniline, the ChT-hydrolyzed product of *N*-benzoyl-L-tyrosine-*p*-nitroaniline (BTNA), overlaps with the emission spectrum of PPP-OR10. In addition, the present approach can detect α-chymotrypsin with a detection limit of 0.1 μM, which is lower than that of the corresponding absorption spectroscopy method. Furthermore, the pH response and enzyme detections can be carried out in 10% serum, which makes this new FERS-based strategy promising in applications in more complex conditions and a broader field.



In recent years, conjugated polymers (CPs) have attracted much attention owning to their unique photo- and electrocharacteristics. CPs are comprised of a large number of repeated conjugated units.[1,2] The long conjugation within the molecular backbone allows the excitation energy along the main chain transfer to an energy or electron acceptor quickly after excited by UV light, which accounts for the fluorescence signal amplification.[3] In the past few years, we and others have taken advantage of its fluorescence signal amplification to design a variety of biosensors and chemosensors.[4−11] The detections of DNA, RNA, ligand (sugar, lectin, biotin)-protein specific interaction, enzyme (ALP, kinase, protease) activity, protein fibrillation, as well as toxic metal ions, etc.[12−26] have been reported with good specificity and high sensitivity. Recently, water-soluble conjugated polymers present unexpectedly promising applications in antimicrobial susceptibility testing, drug screening, cell imaging, and barcoding.[27−30] It is interesting to note that the sensing systems are designed and realized mainly by means of fluorescence resonance energy transfer (FRET),[31] polymer aggregation or target-induced conformational change,[10,32,33] and photoinduced electron transfer (PET) or charge transfer.[34,35] The electrostatic interactions and hydrophobic interactions play an important part among these techniques. However, some reports have shown that the nonspecific interactions especially electrostatic interactions between conjugated polymers and targets are more difficult to control than anticipated and are inevitably disturbed by experimental conditions such as ionic strength.[3,36]

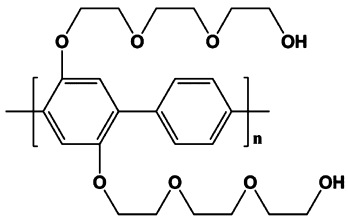
In this study, we present an alternative approach to design sensors based on the fluorescence inner filter effect (IFE), resulting in fluorescence emission red shift of conjugated polymers. The IFE is stemmed from absorption of the incident light or absorption of the emitted light by absorbers in the detection system.[37] At present, some novel fluorescent assays have been reported by researchers based on the IFE, although it is usually thought of as an adverse factor in spectrofluorometry.[38−43] Notably, distance and interactions are dominant in the techniques such as FRET, PET, etc.; however, this approach does not require approximity and strong interactions between the fluorescent probe and the target, which provides considerable flexibility and broad applications in sensors.

Poly(*p*-phenylenes) substituted with oligo(oxyethylene) side chains (PPP-OR10) (Scheme 1) are designed and synthesized through a palladium-mediated Suzuki cross-coupling reaction

Received: June 12, 2012
Accepted: December 14, 2012
Published: December 14, 2012

   dx.doi.org/10.1021/ac302840t | *Anal. Chem.* 2013, 85, 825−830

Scheme 1. The Chemical Structure of PPP-OR10



PPP-OR10

(Scheme S1 of the Supporting Information), and its monomer can readily be synthesized according to previous literature.[44] The alkoxy side chains significantly improve the water solubility of PPP-OR10, which makes it feasible to develop assays in aqueous media. Because conjugated polymers PPP-OR10 have no charge, the electrostatic interactions will hardly affect the sensor system. In the presence of targets, such as nitrophenol and nitroaniline, even if they present in basic form, there are no obvious electrostatic interactions between PPP-OR10 and the targets. As we know, some compounds such as *p*-nitrophenol and 2,4-dinitrophenol[45] exhibit pH-dependent absorbance. Generally, the maximal absorption of nitrobenzene derivatives is shifted toward longer wavelength with the pH value increasing. Thus, the variable fluorescence emission of PPP-OR10/nitrobenzene derivatives is red-shifted with the pH value increasing, which results from the IFE because of spectral overlap of PPP-OR10 emission with the absorption of nitrobenzene derivatives. Furthermore, a new pH sensor is designed based on the fluorescence emission red shift (FERS) of conjugated polymers, which is independent of electrostatic interactions, and a sensitive method for enzyme detection is developed by taking advantage of the sensor system as described above.

EXPERIMENTAL PROCEDURES

Materials and Measurements. All chemicals were purchased from Sigma-Aldrich or Alfa Aesar. All reagents were of analytical grade and used without further purification. *N*-Benzoyl-L-tyrosine-p-nitroanilide (BTNA) and α-chymotrypsin (from bovine pancreas) (ChT) were obtained from Sigma-Aldrich. Fetal bovine serum was purchased from Sangon Biotech Company, Ltd. (Shanghai, China). UV-vis absorption spectra were taken on a UV-2450 (Shimadzu Company) spectrophotometer. Fluorescence measurements were recorded in 3 mL quartz cuvettes at room temperature using a Hitachi F-7000 spectrofluorometer equipped with a xenon lamp excitation source. All fluorescence spectra were measured at an excitation wavelength of 340 nm. Water was purified by using a Millipore filtration system.

Absorption and Fluorescence Spectra of PPP-OR10. A solution with a total volume of 3.0 mL H_2O containing PPP-OR10 ([PPP-OR10] = 1.0×10^{-6} M in RUs (repeated units) for fluorescence and 5.0×10^{-5} M for absorption, respectively) was prepared at room temperature. Then the absorption and fluorescence emission spectra were measured respectively. The excitation wavelength for the fluorescence emission spectra was 340 nm. The absorption and fluorescence emission spectra of PPP-OR10 in other organic solvents (CH_3OH, $CHCl_3$, THF, and DMSO) were measured according to the same procedure.

Absorbance of Nitrobenzene Derivatives under Different pH. Nitrobenzene derivatives (*o*-nitrophenol, *p*-nitrophenol, 2,4-dinitrophenol, 2,4,6-trinitrophenol, or *p*-nitroaniline) (2.0×10^{-4} M) was added in 5 mM PBS (pH = 3.5) solution, and the absorption spectra were recorded at room temperature. Then, the absorption spectra of nitrobenzene derivatives in 5 mM PBS at pH 8.5 were obtained using a UV-2450 spectrophotometer.

Fluorescence Spectra of PPP-OR10/Nitrobenzene Derivatives at Various pH. A series of solutions of PPP-OR10 ([PPP-OR10] = 8.0×10^{-6} M in RUs) and nitrobenzene derivatives (2.0×10^{-4} M) were prepared with the total volume of 3 mL in 5 mM PBS at different pH (from 2.35 to 12.11). Then, the fluorescence spectra were measured at room temperature using a Hitachi F-7000 spectrofluorometer.

Fluorescence Spectra of PPP-OR10/Nitrobenzene Derivatives at Various pH in Serum. A series of solutions of PPP-OR10 ([PPP-OR10] = 8.0×10^{-6} M in RUs) and nitrobenzene derivatives (2.0×10^{-4} M) were prepared with the total volume of 3 mL in 5 mM PBS with 10% serum (volume %) at different pHs (from 2.35 to 12.11). Then, the fluorescence spectra were measured at room temperature.

Reversibility of Maximal Emission Wavelength of PPP-OR10/*p*-Nitrophenol. The solution of PPP-OR10/*p*-nitrophenol ([PPP-OR10] = 8.0×10^{-6} M in RUs, [*p*-nitrophenol] = 2.0×10^{-4} M) was prepared in 5 mM PBS at pH 3. All of the fluorescence spectra were recorded at room temperature, unless otherwise specified. After adjusting the pH value from 3 to 11 with 2 M NaOH, the fluorescence spectrum was obtained. Then, the pH value was adjusted from 11 to 3 with 2 M HCl. The fluorescence spectrum was measured. The measurements were cycled ten times according to the same procedure.

Assay of α-Chymotrypsin by Fluorescence Spectroscopy. A solution of PPP-OR10/BTNA ([PPP-OR10] = 8.0×10^{-6} M in RUs, [*p*-nitrophenol] = 2.0×10^{-4} M) was prepared in 5 mM PBS at pH 8.5. After addition of various amounts of α-chymotrypsin ([ChT] = 0−62 μM), the fluorescence spectra were measured at 30 °C every minute for 15 min. The assay of α-chymotrypsin in the presence of PPP-OR10 in 5 mM PBS with 10% serum (volume %) were carried out using the same procedures described above.

Assay of α-Chymotrypsin by Absorption Spectroscopy. A solution of BTNA ([*p*-nitrophenol] = 2.0×10^{-4} M) was prepared in 5 mM at pH 8.5 at room temperature. After addition of different concentrations of α-chymotrypsin ([ChT] = 0−62 μM), the absorption spectra were measured every minute in the range from 0 to 15 min at 30 °C.

RESULTS AND DISCUSSION

First, the basic photophysical properties of PPP-OR10 were investigated in different solvents. The UV−vis absorption and photoluminescence emission spectra of PPP-OR10 are presented in Figure 1. With the increasing solvent polarity, the maximal UV−vis absorption and photoluminescence emission migrate toward longer wavelengths, which results from the reduced gap between the $\pi-\pi^*$ energy level that is induced by the solvent effect. Also the photoluminescence of polymer is quenched significantly in water compared to the other four organic solvents, which stems from the fact that the fluorescence of PPP-OR10 is self-quenching because of the $\pi-\pi$ stacking effect. The alkoxy side chains were designed for improving the water solubility of PPP-OR10 and making PPP-OR10 neutral, which has some advantages, such as feasibility in aqueous media and immunity to electrostatic interactions.

dx.doi.org/10.1021/ac302840t | *Anal. Chem.* 2013, 85, 825−830

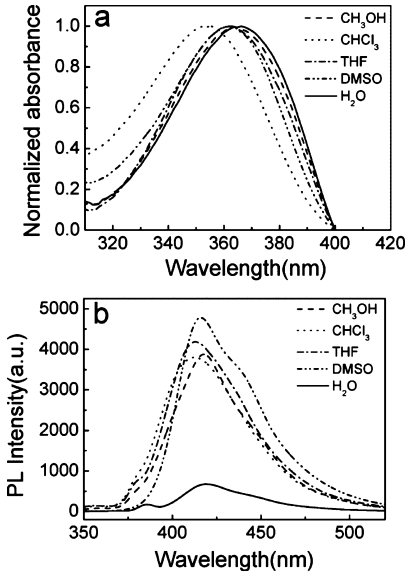


Figure 1. (a) Normalized UV−vis spectra and (b) fluorescence emission spectra of PPP-OR10 in various solvents. The excitation wavelength is 340 nm.

Figure S1 of the Supporting Information showed the absorption spectra of nitrobenzene derivatives under different pH values, which present that the absorptions of *p*-nitrophenol, *o*-nitrophenol, and 2,4-dinitrophenol are pH-dependent while those of 2, 4, 6-trinitrophenol and *p*-nitroaniline are pH-independent in the experimental condition. The pK_a values of the five compounds used in the paper were shown in Table S1 of the Supporting Information. Under the detection conditions (pH 2.35−12.11), the former three compounds (2.35 < pK_a < 12.11) will change their forms from acidic to basic, along with increasing pH, meanwhile, the absorptions red shift, due to adopting different electron-transition types ($\pi-\pi^* \rightarrow n-\pi^*$). However, the absorption maximal wavelength of 2,4,6-trinitrophenol and *p*-aniline do not shift because they stay the same basic form at the same condition (pH > pK_a). The difference of absorption spectra will bring the different overlap effect with absorption and fluorescence emission spectra of PPP-OR10. As shown in Figure S2 of the Supporting Information, when pH is far below pK_a (pH 2.35, acidic form), the absorbance of the *p*-nitrophenol compound is relatively low, while no obvious absorbance appears in the polymer emission region (350−500 nm), which overlaps absorption partly but barely overlaps fluorescence spectra of PPP-OR10. When environmental pH value is higher than the pK_a (pH 12.11, basic form), *p*-nitrophenol exhibits a broader overlap band with both absorption and emission of PPP-OR10. The 2,4-dinitrophenol contains a similar overlap effect with PPP-OR10 (Figure S2b of the Supporting Information). Undoubtedly, 2,4,6-trinitrophenol and *p*-nitroaniline possess the unchanged spectral overlap range with PPP-OR10, during the tested pH range. Notably, the absorption spectra of the basic form of *o*-nitrophenol almost overlap with the emission spectra of PPP-OR10 completely. As a result, the emission spectrum of PPP-OR10 is modulated by nitrobenzene derivatives due to IFE, which depends on the spectral overlap effect. Thus, the change in the absorbance of nitrobenzene derivatives can be translated into changes in the maximal fluorescence emission wavelength.

Figure 2a shows the emission spectra of PPP-OR10/*p*-nitrophenol in a 5 mM PBS solution, from which we can see

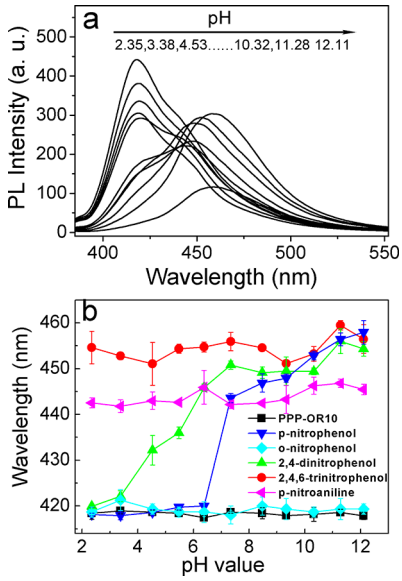


Figure 2. (a) The fluorescence spectra of PPP-OR10/*p*-nitrophenol in 5 mM PBS buffer solution with various pH values. (b) The maximal emission wavelength of PPP-OR10 in the presence of nitrobenzene derivatives as a function of pH value. [PPP-OR10] = 8.0×10^{-6} M, [nitrophenol] = [nitroaniline] = 2.0×10^{-4} M. The error bars represent the standard deviations of three parallel measurements. The excitation wavelength is 340 nm.

the maximal fluorescence emission wavelength around 418 nm in a relatively acidic environment (pH < 6) because the spectral overlap mainly comes from the absorption of *p*-nitrophenol and that of PPP-OR10. The IFE causes the fluorescence intensity attenuation; however, the maximal emission wavelength is affected a little. As the pH value continues to increase, the maximal emission peaks of PPP-OR10/*p*-nitrophenol exhibit gradual red shifts until the final emission wavelengths appear at 460 nm when the pH value increased to 12,[46] which is mainly attributed to the *p*-nitrophenol absorbing the partial emission light of PPP-OR10 from the spectral-overlap band. With the increasing of pH value from 2 to 12, the absorbance of *p*-nitrophenol at 317 nm decreases while the peak at 400 nm increases gradually (Figure S1 of the Supporting Information). Thus, the spectral overlap of the absorption band of *p*-nitrophenol with the emission spectra of PPP-OR10 changes to the longer wavelength range. Therefore, the emission peak wavelength of PPP-OR10 red-shifted due to the IFE. The extreme change of the pH value indeed has a certain impact on the fluorescence intensity of PPP-OR10, which leads to the fluctuation in irradiation (Figure 2a) but will not affect the maximal emission wavelength of PPP-OR10. From Figure 2b, we can see that 2,4-dinitrophenol had a similar maximal emission wavelength changing curve as *p*-nitrophenol, while 2,4,6-trinitrophenol and *p*-nitroaniline just kept nearly the same maximal emission wavelength due to their constant basic forms in our experimental conditions. If the assay was carried out in a more acidic buffer (pH value lower than pK_a of 2,4,6-trinitrophenol and *p*-nitroaniline), an upward plot can also be found for 2,4,6-trinitrophenol and *p*-nitroaniline, just like the other two nitrobenzene derivatives (data not showed here). However, the maximal emission wavelength of PPP-OR10

dx.doi.org/10.1021/ac302840t | *Anal. Chem.* 2013, 85, 825−830

keeps around 419 nm in the presence of *o*-nitrophenol, even if the pH increases from 2 to 12 because of the almost complete spectral overlap of absorption of the basic form of *o*-nitrophenol with emission of PPP-OR10. The corresponding results are that the fluorescence intensity decreases while the maximal emission wavelength remains unchanged due to the IFE. These results demonstrated that the red shift of the maximal emission wavelength depends on the spectral overlap effect. In addition, some research groups have reported that the IFE can be corrected mathematically.[37,47−50] The corrected emission wavelengths and intensities through the IFE equation were compared with those observed from experiments. (see the Supporting Information). All results indicated that the IFE caused the fluorescence quenching and wavelength red shift. To investigate the effect of the solution ionic strength on the emission red shift, we carried out the experiments in 50 mM PBS, as the same procedure. The results we obtained present the same fluorescence emission red-shift trend (data not showed here), which show the strategy based on the IFE could avoid the interference to some extent from the ionic strength that generally affects FRET or PET techniques.

To detect the reversibility of this system, the maximal emission wavelength of PPP-OR10/*p*-nitrophenol can be switched back and forth by adjusting the pH value between 3 and 11. As shown in Figure 3, after experiencing a shuttling

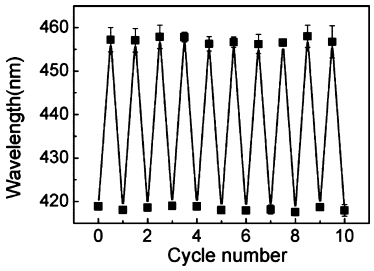
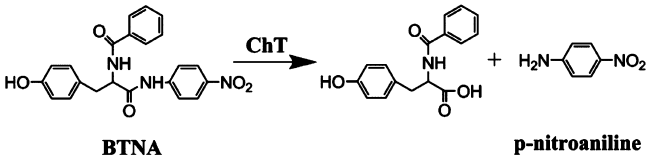


Figure 3. The maximal emission wavelength of PPP-OR10/*p*-nitrophenol upon cycling the pH value between 3 and 11 in a 5 mM PBS buffer solution. [PPP-OR10] = 8.0×10^{-6} M and [*p*-nitrophenol] = 2.0×10^{-4} M. The error bars represent the standard deviations of three parallel measurements. The excitation wavelength is 340 nm.

between 3 and 11 (pH value) ten times, the system still exhibits stable results: the maximal emission wavelength is situated around 418 nm at pH 3 and 458 nm at pH 11, respectively. It can work very well as long as the emission of the two compounds partly overlapped without additional requirements, such as distance and interactions, so a better cycling result can be obtained.

It was reported that *p*-nitrophenol and *p*-nitroaniline have been utilized to covalently attach to substrates as tags to monitor enzymatic activity through a signal change of the UV−vis spectra.[51,52] However, when large amounts of samples are required, high-throughput analysis is necessary and convenient, which can be achieved mainly by fluorescence spectroscopy. So, it is interesting to design a new assay method involving the utility of *p*-nitrophenol and *p*-nitroaniline in the fluorescence field. Here, we also investigate the feasibility of applying our system to detect an enzyme. *α*-Chymotrypsin (from bovine pancreas), a kind of protease, selectively catalyzes the hydrolysis of peptide bonds on the C-terminal side of tyrosine, phenylalanine, trytophan, and leucine. *N*-Benzoyl-L-tyrosine-*p*-nitroanilide (BTNA) is used here as a substrate to assay the *α*-chymotrypsin in our system. From Scheme 2, we can see after

Scheme 2. Schematic Demonstration of *α*-Chymotrypsin Hydrolysis Process



BTNA being hydrolyzed by ChT, *p*-nitroaniline is released in our testing environment (pH = 8.5). It is undisputed that the absorption spectrum of *p*-nitroaniline overlaps with the emission spectrum of PPP-OR10, which leads to the maximal emission wavelength of the PPP-OR10 red shift, as a result of IFE. Thus, different fluorescence emission signals can be obtained to monitor the enzyme activity before and after the hydrolysis of BTNA by ChT.

After the addition of ChT (31.0 μM) to a working solution containing PPP-OR10/BTNA ([PPP-OR10] = 8.0×10^{-6} M in RUs, [BTNA] = 2.0×10^{-4} M) in 5 mM PBS at pH 8.5, Figure 4a was obtained by monitoring the enzymatic reaction time at

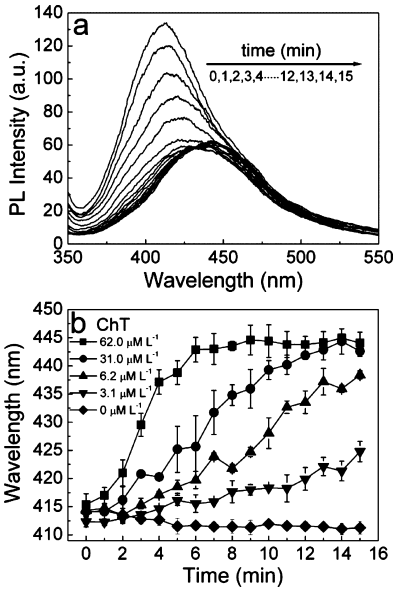


Figure 4. (a) Fluorescence emission spectra of polymer/BTNA as a function of *α*-chymotrypsin (ChT) incubation time ([*α*-chymotrypsin] = 31 μM). (b) The maximal emission wavelength as a function of *α*-chymotrypsin incubation time ([*α*-chymotrypsin] = 0−62 μM). The measurements were performed in a PBS buffer solution (5 mM, pH = 8.5) at 30 °C, [PPP-OR10] = 8.0×10^{-6} M, and [BTNA] = 2.0×10^{-4} M. The error bars represent the standard deviations of three parallel measurements. The excitation wavelength is 340 nm.

30 °C for an interval of 1 min for 15 min. The maximal emission red-shifted about 30 nm, while fluorescence was quenched gradually with the incubating time from 0 to 15 min. Figure 4b illustrates the maximal emission wavelength of PPP-OR10 in different concentrations of *α*-chymotrypsin as a function of incubation time. In these experiments, the BTNA concentration was fixed (2.0×10^{-4} M) and the ChT concentration was varied over a range of 0−62.0 μM. Control tests show that BTNA itself has little effect on our detection

dx.doi.org/10.1021/ac302840t | *Anal. Chem.* 2013, 85, 825−830

system in limited time. It is noted that the maximal emission wavelength of PPP-OR10 is obviously concentration-dependent. When the concentration of α-chymotrypsin is 3.1 μM, the increased maximal emission wavelength is around 12 nm after incubation for 15 min. Interestingly, when the concentration of α-chymotrypsin is 62.0 μM, the maximal fluorescence emission wavelength red shifts 30 nm and it reaches the platform after an incubation time of only 6 min. The limit of detection (LOD) of this system is obtained as 0.1 μM. In comparison with the LOD (0.3 μM) obtained by measuring the absorbance of *p*-nitroaniline (Figure S3 of the Supporting Information), this method is more sensitive than UV−vis absorption spectroscopy. The results in Figure 4b show that the maximal emission wavelength is time- and enzyme concentration-dependent, which indicates that the sensor is available in real-time detection and the enzyme reactions are the rate-limiting process in the assay.

To investigate the application of the new method in complex condition, the measurements of pH response and α-chymotrypsin analysis based on the IFE were carried out in 10% serum. Figure 5a showed results similar to those observed

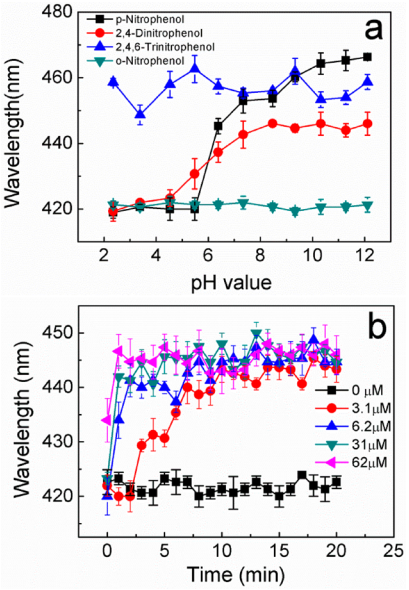


Figure 5. (a) The maximal emission wavelength of PPP-OR10 in the presence of nitrobenzene derivatives as a function of pH value in 10% serum. [PPP-OR10] = 8.0 × 10^{-6} M, [nitrophenol] = [nitroaniline] = 2.0 × 10^{-4} M. (b) The maximal emission wavelength as a function of the α-chymotrypsin incubation time in 10% serum. [α-chymotrypsin] = 0−62 μM, [PPP-OR10] = 8.0 x10^{-6} M, [BTNA] = 2.0 × 10^{-4} M. The error bars represent the standard deviations of three parallel measurements. The excitation wavelength is 340 nm.

in 5 mM PBS solution. For example, the maximal emission wavelength of *p*-nitrophenol/PPP-OR10 in 10% serum red-shifted from 419 to 466 nm, along with the pH increasing from 2 to 12. The maximal emission wavelength of 2,4,6-trinitrophenol stayed around 457 nm and that of *o*-nitrophenol stayed at 420 nm in the detection pH range. Furthermore, the BTNA hydrolysis by α-chymotrypsin was successfully realized in 10% serum. As shown in Figure 5b, the maximal emission wavelength of PPP-OR10 red shifts with increasing the incubation time and concentration of α-chymotrypsin. The enzyme reaction rate is higher to some extent in 10% serum than in 5 mM PBS alone, which may come from the effect of unknown compounds in serum on α-chymotrypsin. In conclusion, the method is feasible in the more complex detection environment that the other techniques are usually limited to.

CONCLUSIONS

In summary, we have developed a new strategy for pH response and enzyme activity detection based on the inner filter effect (IFE). The detection was based on the fluorescence emission red shift that results from spectral overlap of neutral conjugated polymer and nitrobenzene derivatives. Our detection is not just confined to the polymers or absorbers we employed in this contribution but can be applied to any system that satisfies the basic requirements: the absorption of the target must overlap with the emission of probe to a degree, and the target displays a different absorption when the external environment changes. In comparison with previous techniques, the IFE-based approach yields greater flexibility and a larger breadth of applications in sensors because no interactions between conjugated polymers and targets were needed, and it worked very well in a more complex environment, such as 10% serum. In addition, the limit of detection of α-chymotrypsin obtained via this method was lower than that obtained by UV−vis absorption spectrometry. The system brings enzymatic analysis based on the *p*-nitrophenol- and *p*-nitroaniline-tagged substrates to a new level of field-fluorescence detection, which makes high-throughput assays possible.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are grateful for the financial support from the National Natural Science Foundation of China (Grants 21222509 and 21105061), Natural Science Basic Research Plan in Shaanxi Province of China (Grant 2011JQ2008), Fundamental Research Funds for the Central Universities (Grant GK201103002), Specialized Research Fund for the Doctoral Program of Higher Education of China (Grant 20110202120006), and the Program for Changjiang Scholars and Innovative Research Team in University (IRT 1070).

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