

Conjugated Polymers as Efficient Fluorescence Quenchers and Their Applications for Bioassays

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We have developed a novel approach for simple and convenient detection of ligand–receptor interactions with high sensitivity and selectivity. The method harnesses a reversible fluorescence quenching between nonfluorescent polyelectrolyte quenchers and fluorescent probes tagged to ligands. Formation of more strongly bound ligand–receptor complexes forces the breakup of nonspecific, electrostatic interaction-based quencher/probe pairs to reverse the fluorescence quenching, triggering a fluorescence increase, and providing an off-to-on detection of the ligand/receptor interaction. Although a conjugated polyelectrolyte was used in the study, other nonconjugated polyelectrolyte quenchers are expected to work as well. The method should potentially find a wide variety of applications in several areas, ranging from drug screening, medical diagnostics, and biosensing to assay development.

1. Introduction

Ligand–receptor interactions including antibody–antigen recognition are a paradigm of specific biological interactions. Assays of these selective interactions are of great importance in many areas, ranging from drug discovery, medical diagnostics, and environmental monitoring to quality control of food. Currently, antibody/antigen interaction-based immunoassays, particularly heterogeneous immunoassays (e.g., enzyme-linked immunosorbant immunoassay), are the most commonly used techniques. In those heterogeneous immunoassays, antibodies (or antigens) are often immobilized on solid surfaces and antigens (or antibodies) are captured on the modified surfaces through either direct or competitive binding, followed by washing and signal generation steps.¹ On one hand, the heterogeneous immunoassay methods are usually quite general, sensitive and selective; on the other hand, they are expensive, labor-intensive, and time-consuming. For many applications such as high throughput drug screening where a large number of assays are carried out daily, homogeneous assays are desired because they are usually simpler, faster, and cheaper. Another advantage of homogeneous detection methods is the possibility for more quantitative and accurate measurements. However, homogeneous assays are often more difficult to develop, particularly for complex samples. Currently, one widely used homogeneous assay method is fluorescence-polarization assay with severely limited detection sensitivity.^{2,3} It has remained a challenge to develop versatile

homogeneous assays with acceptable detection performance for ligand–receptor interactions.

Recently, conjugated polymers have become a subject of extensive research for development of chemosensors and biosensors.^{4–6} Conjugated polymers present molecular wire effect, which has been targeted for potential signal amplification. Different physical properties of conjugated polymers have been utilized, such as absorption, fluorescence, and conductivity. Charych and co-workers^{6–9} applied stress-sensitive chromism of self-assembled polydiacetylene derivatives coupled with recognition molecules on the surfaces to construct biosensors for a variety of biological targets, ranging from protein receptors and enzymes to viruses. LeClerc and co-workers^{10,11} reported a similar method using polythiophene derivatives with covalently tagged biotin as side chains for homogeneous detection of avidin through a binding-induced absorption spectral shift. Chromism of polythiophene derivatives has also been used for detection of ions, including organic and inorganic cations and anions.¹² Swager and co-workers^{4,5} have reported electron-rich, highly fluorescent conjugated polymers for detection of electron-deficient aro-

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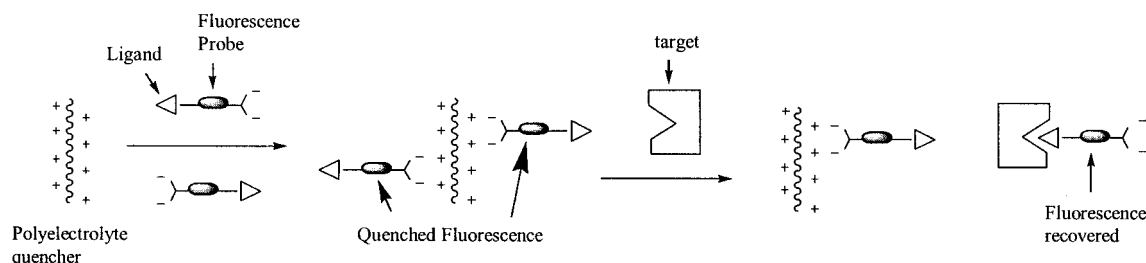
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Scheme 1. Schematic of Binding-Induced Fluorescence Recovery for Biosensing Applications

matic molecules (e.g., trinitrotoluene: TNT) through efficient fluorescence quenching. A recent example was reported by Chen et al.¹³ to utilize water-soluble, highly fluorescent polyphenylene vinylene (PPV) derivatives for detection of aromatics and proteins through an efficient reversible fluorescence quenching. Binding-induced changes of electrochemical properties have also been utilized for construction of biosensors and chemosensors.¹⁴ Swager and co-workers^{4,5} have extensively investigated application of molecular wire effect of conjugated polymers for detection of different targets using electrochemical techniques. Oligonucleotide-functionalized polypyrroles have been reported as intelligent polymers for fabrication of DNA sensors.¹⁵

Because of the delocalization of the π - π electrons in the conjugated backbones of conjugated polymers, their fluorescence can be efficiently quenched through an exciton migration process. Current research is mainly focused on using small quenchers to quench the fluorescence of conjugated polymers with amplifications.^{13,4,5} However, it is not clear if conjugated polymers can act as efficient quenchers for the fluorescence of either small molecules or macromolecules. Here, we report our studies of using conjugated polymers as efficient quenchers and their applications for design of a general sensing approach using water-soluble nonfluorescent polyelectrolytes as fluorescence quenchers for detection of ligand-receptor interactions. Although we used a conjugated polyelectrolyte as our model system, nonconjugated polyelectrolytes are also expected to be applicable for the sensing approach.

2. General Approach

Our current signal generation method is inspired by the approach reported recently by Chen et al.¹³ The method used reversible fluorescence quenching between a highly fluorescent, water-soluble conjugated PPV derivative and an oppositely charged quencher (methyl viologen, MV) tagged to a ligand (biotin). In aqueous solutions, the PPV derivative and the quencher form a complex through electrostatic interaction to result in an efficient fluorescence quenching of the PPV derivative. A target (avidin) which recognizes and binds with the

tagged ligand (biotin) strips the quencher-tagged ligand off the PPV derivative to reverse the quenching process, triggering a fluorescence recovery and providing a simple, yet sensitive, method for detection of biological macromolecules.

Instead of using highly fluorescent conjugated polyelectrolytes to provide fluorescence and small oppositely charged nonfluorescent molecules as quenchers, the current method reported here utilizes nonfluorescent polyelectrolytes as quenchers and small oppositely charged fluorescent probes tagged to a ligand to provide a fluorescence signal. As shown in Scheme 1, a nonfluorescent polyelectrolyte quencher forms a complex with the oppositely charged ligands tagged with fluorescent probes. Depending upon the molecular structure of the quenchers and fluorescent probes, the fluorescence of the probes can be quenched through either electron transfer or energy transfer. Binding of a target with the tagged ligand can potentially drag the probe-tagged ligands off the polyelectrolyte quencher to recover the quenched fluorescence, providing a reagent-free and off-to-on detection.

3. Experimental Section

Poly(2-ethynylpyridinium tosylate) having a propagyl side chain (PEPT) was prepared by the reported method.¹⁷ Lucifer yellow biocytin, potassium salt (biocytin lucifer yellow, BLY), and lucifer yellow cadaverine biotin-X, dipotassium salt (LY-CBDP) were purchased from Molecular Probes, Inc. (Eugene, Oregon). The molecular structures of PEPT, BLY, and LY-CBDP are shown in Scheme 2. Avidin was purchased from Sigma. Other materials used in this study were obtained from Aldrich and used directly as-received. Milli-Q water was used for all the aqueous solutions. Fluorescence measurements were recorded on either a Spex Fluorolog-2.0 or QuantaMaster spectrofluorometer (Photon Technology International, Lawrenceville, NJ). UV-vis absorption spectra were obtained on a Hewlett-Packard 8452A diode array spectrophotometer. The concentrations of both BLY and LYCBDP in aqueous solutions are estimated based on their extinction coefficient constants¹⁸ of 11×10^3 at 428 nm. All the experiments for absorption and fluorescence spectra were collected at ambient temperature.

4. Results

Although conjugated polyelectrolytes are not required (e.g., polyelectrolytes with pendent nonfluorescent dyes should work as well), a simple conjugated positively charged PEPT was used as an efficient quencher for the fluorescence of lucifer yellow. As shown in Figure 1,

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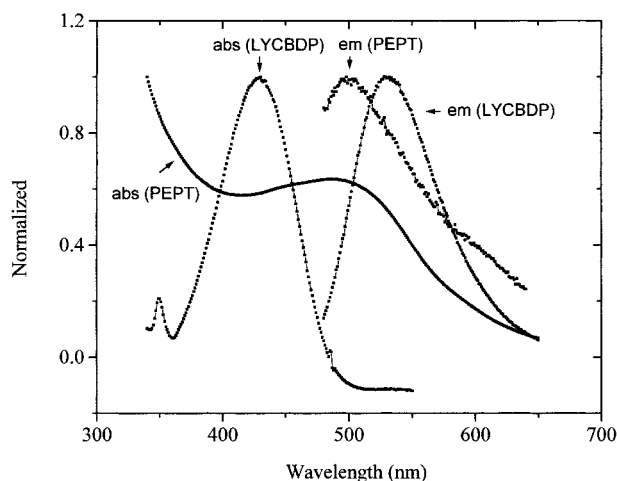
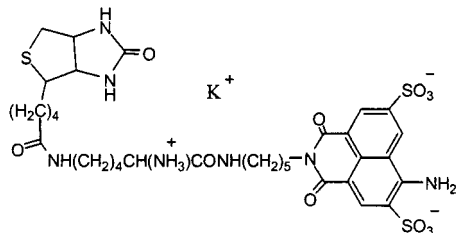
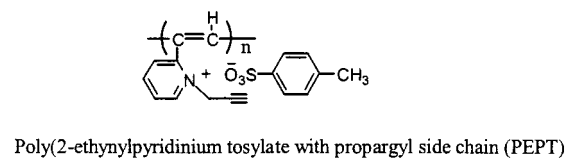
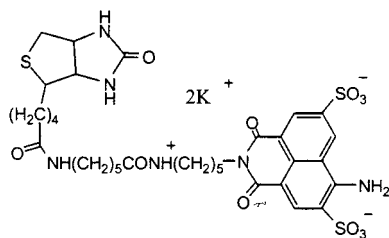


Figure 1. Absorption and emission spectra of LYCBDP and PEPT in water.

Scheme 2. Molecular Structures of PEPT, BLY, and LYCBDP



Lucifer yellow biocytin, potassium salt (biocytin lucifer yellow, BLY)



Lucifer yellow cadaverine biotin-X, dipotassium salt (LYCBDP)

PEPT has a broad absorption spectrum from 300 to 650 nm, which makes it an ideal quencher for the fluorescence of many fluorescent probes (including lucifer yellow dyes) through spectrum-overlap-dependent energy transfer.

A biotin-avidin binding pair was selected as a ligand-receptor model system for the demonstration of the direct signal generation approach because of their high affinity ($K_d \sim 10^{-15}$ M).¹⁶ As shown in Scheme 2, lucifer yellow biocytin, potassium salt (biocytin lucifer yellow, BLY) and lucifer yellow cadaverine biotin-X, dipotassium salt (LYCBDP) have one and two net negative charges, respectively. The fluorescence spectrum of lucifer dye has a large overlap with the absorption spectrum of PEPT, which is essential for an efficient quenching through a resonant energy-transfer

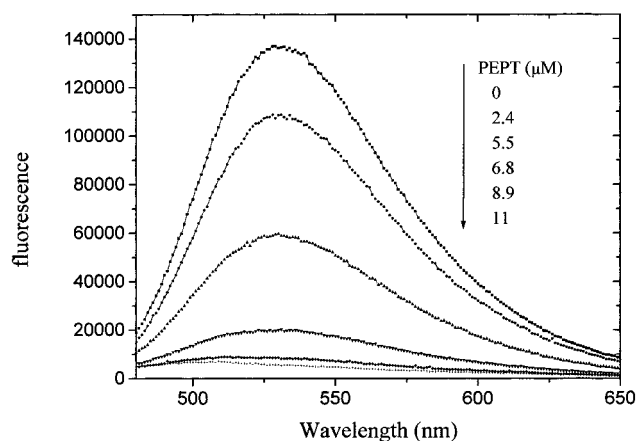


Figure 2. Fluorescence spectra of LYCBDP (0.26 μ M) in water in the presence of different concentrations of PEPT.

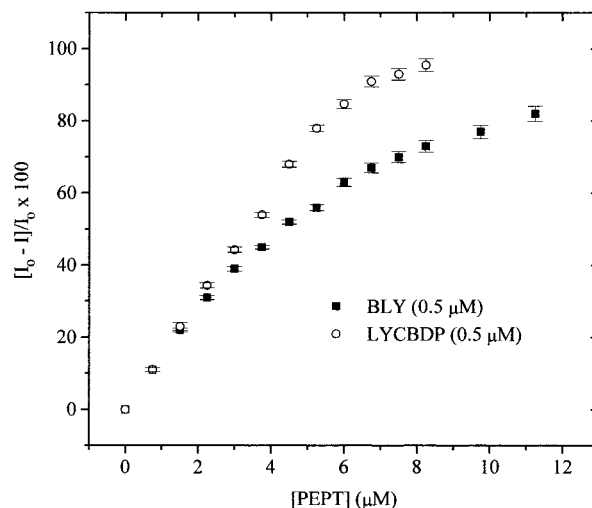


Figure 3. The quenched fluorescence of BLY (0.5 μ M) and LYCBDP (0.5 μ M) as a function of [PEPT] (μ M) in water. I_0 : fluorescence intensity without PEPT; I : fluorescence intensity in the presence of PEPT. The error bars are based on measurements of four duplicates.

mechanism. Another reason to choose biotin/avidin as a model recognition pair is that biotin is small compared with avidin so that the formation of the biotin-avidin complex can overcome the complexation between the probe-tagged biotin and polyelectrolytes to trigger a change of the quenching process.

A. Fluorescence Quenching of Lucifer Yellow Probes Tagged with Biotin by PEPT. PEPT samples prepared for the spectral measurements were found to exhibit very weak photoluminescence from 400 to 700 nm when it was excited at 428 nm. The weak fluorescence is attributed to a small amount of impurity as confirmed by their excitation spectra, which lie around 420 nm and are different from the absorption spectra of the samples. However, compared with the strong fluorescence of lucifer yellow dyes, the interference from the background fluorescence is negligible. Figure 2 shows the fluorescence spectra of LYCBDP in the presence of different concentrations of PEPT. Figure 3 compares the fluorescence quenching efficiencies of BLY and LYCBDP by PEPT. Without PEPT, both BLY and LYCBDP exhibit strong fluorescence from 450 to 650 nm and their strong fluorescence are significantly quenched in the presence of PEPT. More than 50%

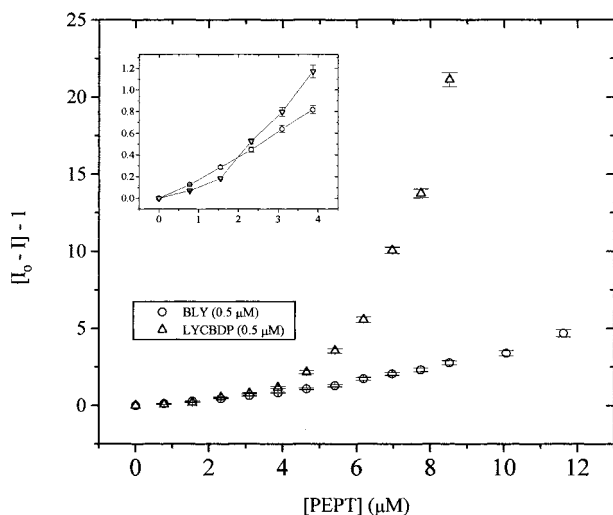


Figure 4. Stern–Volmer plot for the fluorescence quenching of BLY (0.5 μM) and LYCBDP (0.5 μM) by PEPT in water. I_0 : fluorescence intensity without PEPT; I : fluorescence intensity with PEPT. Inset: zoom-out of the low-concentration region of PEPT. The error bars are based on the measurements of four duplicates.

fluorescence of 0.5 μM LYCBDP in water was found to be quenched by 3 μM PEPT (concentration in monomer repeating units), and more than 90% fluorescence was quenched by 7 μM PEPT. It was estimated that ≈ 11 monomer repeating units in PEPT can fully quench the fluorescence of LYCBDP (3 $\mu\text{M}/0.5 \mu\text{M} \times 2 \sim 11$). When excess PEPT was added, the residue fluorescence spectrum of the sample starts to shift slightly to blue region, which matches the fluorescence spectrum of PEPT. The quenching behavior of BLY by PEPT is similar to that of LYCBDP with slightly less effective quenching efficiency, particularly at higher concentration, possibly because of less strong electrostatic interaction.

Figure 4 shows the Stern–Volmer plot for the fluorescence quenching for both LYCBDP and BLY in water by PEPT. In contrast to the linear Stern–Volmer plots for the fluorescence quenching of a fluorescent conjugated poly(2-methoxy-5-propyloxy sulfonate phenylene vinylene) by methyl viologen as reported previously,¹³ the Stern–Volmer plot for the fluorescence quenching of LYCBDP in water by conjugated PEPT deviates significantly from linearity even under low PEPT concentrations. This suggests that the quenching process is not a simple diffusion-controlled collisional process by PEPT, but possibly a combination of static and dynamic quenching processes. However, the fluorescence quenching of BLY by PEPT exhibits a linear Stern–Volmer plot under low concentration, and the Stern–Volmer plot deviates from linearity when PEPT concentration increases. This can be understood by the anticipation that PEPT can form relatively tight ion pairs with LYCBDP (with two net negative charges) even under low concentrations, while BLY with only one net negative charge may be loosely bound with PEPT under low concentrations. The charge effect is clearly demonstrated by the data in Figure 4 that the tagged ligands (LYCBDP) with more net opposite charges than counterions of the polyelectrolytes bind more strongly with the polyelectrolytes and are more efficiently quenched by PEPT than those with only one net negative charge (BLY). This observation is consistent

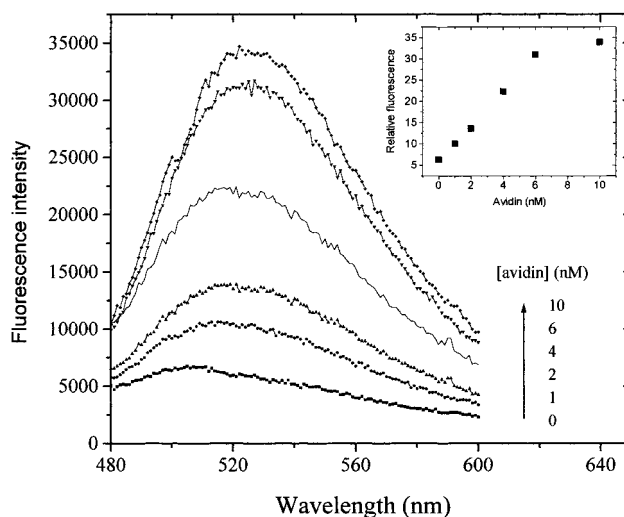


Figure 5. Fluorescence spectra of a sample containing 20 nM LYCBDP and 4 μM PEPT in the presence of different concentrations of avidin. Inset: plot of relative fluorescence intensities of the sample as a function of [avidin] (nM). The standard deviation of the measurements on five duplicate samples ranges from 5% to 8%.

with the reported results for photoluminescence quenching of conjugated PPV derivatives by bipyridinium derivatives with different charges.²⁰

B. Detection of Avidin/Biotin Interaction through Fluorescence Quenching Recovery. LYCBDP and BLY were found to bind with avidin in homogeneous aqueous solutions, resulting in a significant loss ($\approx 55\%$) of their fluorescence, possibly due to a proximity-induced fluorescence self-quenching since each avidin can bind up to four probe-tagged biotins. This aggregation-induced fluorescence self-quenching has been exploited by us^{21,22} to provide reagent-free, sensitive, and selective detections of multivalent interactions. The fluorescence loss may also be attributable to the quenching by proteins through either energy transfer or electron transfer. For the method discussed in the present paper, the fluorescence quenching either through self-quenching or by proteins is not desirable because it reduces the overall fluorescence recovery after the binding of targets with the probe-tagged ligands complexed with polyelectrolyte quenchers. Nevertheless, the pairs are still useful for demonstration of the detection principle.

The fluorescence of LYCBDP quenched by PEPT in water was found to recover up to 45% of the original fluorescence of LYCBDP in water upon addition of avidin as shown in Figure 5. The fluorescence recovery of LYCBDP is believed to result from the biotin–avidin complexation and the dissociation of the complexes from PEPT into the homogeneous bulk solution. The fluorescence recovery is linearly proportional to the concentration of avidin in the sample prior to saturation (inset in Figure 5). The plot starts to deviate from linearity at around 6 nM of avidin for a sample containing 20 nM LYCBDP, suggesting that each avidin binds with

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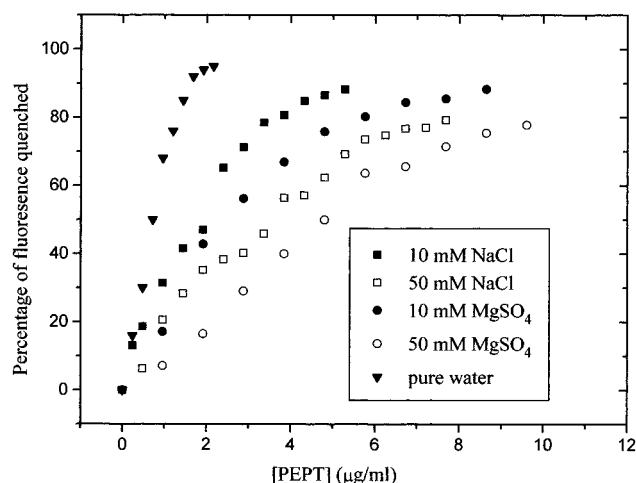


Figure 6. Percentage of fluorescence quenched ($(I_0 - I)/I_0 \times 100$) for LYCBDP ($0.5 \mu\text{M}$) in the presence of PEPT in aqueous solutions with different concentrations of salts. The definitions of I_0 and I are identical to those in Figure 4.

three to four tagged biotin molecules as anticipated. The partial recovery ($\approx 45\%$ recovery) is due to the fluorescence self-quenching by multivalent binding of avidin with LYCBDP and BLY or fluorescence quenching by avidin as discussed above. The fluorescence recovers almost instantaneously upon addition of avidin. Subnanomolar avidin ($<1 \text{ nM}$ avidin) can be readily detected. The method not only provides remarkably simple, rapid, and sensitive detection of avidin (and potentially other macromolecules and micro-organisms), it also provides significant discrimination against other potential interferents through selective recognition of the ligands. Only a negligible fluorescence increase was observed for the same sample upon addition of cholera toxin under identical conditions. Similar fluorescence recovery was also observed for the BLY system.

The method is also useful for detection of ligands (nonlabeled ligands) using a competitive format. In the format, ligands in a sample compete with a known amount of labeled ligands for a limited number of receptors to inhibit the formation of the complexes between the receptors and labeled ligands, resulting in a less efficient fluorescence recovery. For example, the fluorescence quenching efficiency (percentage of fluorescence quenched) that resulted from an addition of a mixture of 10 nM biotin and 20 nM LYCBDP is $\approx 37\%$ lower than that from the addition of only 20 nM LYCBDP for a sample containing $8 \mu\text{M}$ PEPT and 6 nM avidin in water.

C. Effect of Ions. Since the charge–charge interaction between the polyelectrolytes and the oppositely charged probe-tagged ligands are mainly responsible for the complexation to result in the fluorescence quenching, ions present in the solution are expected to influence the binding strength between the quencher and the oppositely charged probe-tagged ligands. Figure 6 compares the fluorescence quenching of LYCBDP by PEPT under different concentrations of NaCl and MgSO_4 . As anticipated from the competition such as PEPT counterions from negatively charged chloride ions and sulfate ions with the probe-tagged biotin, the quenching efficiency (and binding strength) is the highest in pure water and decreases as the ion concen-

tration increases. Sulfate ions with two net negative charges are more effective in inhibiting the binding of the tagged ligands with PEPT than chloride with only one negative charge. Apparently, significant variation in ion concentration and composition will cause significant detection error. Fortunately, small variation (e.g., $<1 \text{ mM}$) in ionic concentration has been found to have only a negligible effect. When the ion concentration remains the same, their quenching behaviors are found to be identical from samples to samples. Since the ions and their concentrations influence the binding strength between LYCBDP and PEPT, they will also influence the fluorescence background and detection sensitivity. For detection of a series of samples, the ion concentrations and composition remain constant to avoid false detection.

5. Discussion and Conclusions

Several factors such as fluorescence background, binding affinity between ligand and target, and binding strength of quenchers and probe-tagged ligands are expected to play critical roles in determining detection performance for the method, particularly detection sensitivity. Since the detection is based on a fluorescence increase, low fluorescence background (ideally zero background) is desired to improve detection sensitivity. Several factors can influence background fluorescence: (a) quencher's residue fluorescence which may interfere with the probe's fluorescence; (b) fluorescence quenching efficiency of the fluorescent probes by polyelectrolyte quenchers; (c) binding strength between probe-tagged ligands and polyelectrolyte quenchers. To minimize the background fluorescence, the selected polyelectrolyte quencher should have little or no fluorescence in the fluorescence collection region of the fluorescent probe. The quencher's absorption spectrum should have as much overlap as possible with the fluorescence spectra of the fluorescent probes to maximize the quenching efficiency if a fluorescence resonant energy-transfer mechanism is used for fluorescence quenching. High binding strength of the probe-tagged ligands with the polyelectrolyte quenchers are also desirable to have all the probes bound to the quenchers to eliminate the fluorescent nonbound probes. However, the high binding strength is not desirable for the binding ligand/receptor pairs of low affinity because the high binding strength may hinder the dissociation of the ligand–receptor complexes from the polyelectrolyte quenchers, particularly for weak ligand–receptor pairs. In this case, usage of a large excess of quenchers can compensate the low binding strength between the probe-tagged ligand and quencher, still allowing most of the probes to be bound to the quenchers and providing a low fluorescence background. The large excess of quenchers may not significantly increase the fluorescence background, particularly for quenchers with no or little fluorescence.

The affinity of the ligand–receptor binding pairs is also vital for the ligand–receptor complexes to be stripped off from the polyelectrolyte quenchers. The ligand–receptor pair with higher affinity than the quencher/probe pair is desired so that low concentration of the receptor can still result in the probe–ligand–receptor complexes being stripped away. However, the method should also be useful for weak interaction pairs

through avidity effect of the multivalent ligands since each polyelectrolyte can have multiple probe-tagged ligands bound to increase their local concentrations and increase their effective binding affinity. Since a large concentration of quencher and probe can be used to still provide a low fluorescence background, a low concentration of receptor can still be detected since much excess of the pre-organized ligands are used to promote the ligand–receptor binding.

Although the method described here provides a simple, direct, and sensitive homogeneous detection of ligand–receptor bindings, there are some limitations associated with the method. The limitations include the effect of ions on the signal generations, preferably small ligands with large targets, and ligand–target pairs of high affinity.

The method discussed above should provide a possibility of simultaneous detection of multiple targets in a single sample on a single experiment. For such an application, polyelectrolytes that are capable of simultaneously quenching the fluorescence of a number of fluorescent probes (at different fluorescence regions) are

needed. Conjugated polyelectrolytes with broad absorption spectra should meet the requirements to efficiently quench fluorescence of multiple probes in a broad spectral range through overlap-dependent energy transfer. Polyelectrolytes that can quench the fluorescence of different probes through different mechanisms such as energy transfer or electron transfer may also be feasible for such an application. Another example of such a polyelectrolyte would be nonconjugated polyelectrolytes with different pendent nonfluorescent dyes as side chains to provide a wide range of absorption spectrum for energy transfer or as an electron donor/acceptor for electron-transfer-based quenching. We are currently investigating the feasibility of such a method for simultaneous detection of multiple ligand/receptor interactions.

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