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Polyelectrolyte platform for sensitive detection of biological analytes via reversible fluorescence quenching

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Abstract

Non-conjugated polyelectrolytes were used to quench oppositely charged fluorescent dye–ligand conjugate (DLC) molecules by bringing them to the vicinity of the polyelectrolyte backbone to form aggregates and hence quench the dye fluorescence. As target protein molecules are added to the solution, the specific interaction between the DLC and the protein disrupts the aggregate structure, thus recovering the luminescence. The binding of DLC to oppositely charged polyelectrolyte and the disruption of the aggregates are investigated by fluorescence spectroscopy. The static-quenching mechanism is clearly manifested in the Stern–Volmer plots that show the decrease in slope with increasing temperature. This polyelectrolyte-based sensing platform has a sensitivity of <100 pM. We also show the selectivity of this platform by comparing the fluorescence recovery between two proteins (avidin and bovine serum albumin) with similar molecular weight. Our results suggest a highly sensitive approach for detecting biological analytes.
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1. Introduction

The recent rise of terrorism and increased threat of attack by biological and chemical agents have demonstrated the need for quick and facile methods for sensing the presence of hazardous analytes. Polymer-based biosensors [1], especially conducting polymer-based chemical [2,3] and biosensors [4–8], have certain advantages in their low cost and ease of processing. Binding-induced conformational changes and concomitant change in optical properties, fluorescence properties in particular, can be used as the transduction signal [9,10]. The use of polymers in the reversible quenching of fluorescent probes has been examined for applications in bioassays. Recent investigations by Chen et al. [11] demonstrated that the formation of a complex between a fluorescent conjugated polyelectrolyte and a quencher-tethered-ligand (QTL) with built in charge transfer function allows fluorescence quenching. Recovery of the fluorescence occurs upon binding of the specific target and subsequent removal of the quencher from the polyelectrolyte complex. The QTL approach is shown to work for protein recognition in a biotin/avidin pair with nanomolar sensitivity [11]. Another detection platform using a non-fluorescent conjugated polymer as quencher of a fluorophore–ligand conjugate through resonant energy transfer (FRET) also shows nanomolar sensitivity for the avidin–biotin platform [12]. In both platforms, electrostatic interactions between the polyelectrolyte and the ligands drive the quenching, and specific binding of the protein target results in fluorescence recovery. Therefore, binding ionic species with oppositely charged polyelectrolyte in aqueous solution through electrostatic interactions offers a simple means to create functional assemblies in which their electronic and optical properties are switched by reversing the electrostatic interaction between two species. The association of ionic dye molecules with polyelectrolyte often introduces strong variation in

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the absorption, fluorescence, and excitation spectra which would allow us to determine the structure, binding mode, and aggregation pattern [13,14].

In addition to being used in the bulk phase, these polyelectrolyte assemblies have also been demonstrated in a versatility of roles in potential biosensing applications. For example, polyelectrolytes have been shown to serve as bulk matrices for reusable immobilization of enzymes, and can allow for greater control of catalytic activity. Enzyme immobilization can be achieved by various methods, including covalent attachment, intermolecular cross-linking, physical adsorption, and entrapment [15,16]. Caruso et al. immobilized a range of enzymes on micro- and nanospheres using LbL electrostatic self-assembly of oppositely charged polyelectrolyte species [17–19]. Another recent development involves the use of polyelectrolyte encapsulates loaded with fluorescence assay as a biosensor platform [20–22]. These encapsulates entrap enzymes such as glucose oxidase, and can potentially be used as implant devices for monitoring glucose with high sensitivity. These efforts demonstrate the potential to use polyelectrolytes in various sensing platforms for diagnoses and bioassays. In addition, specific interactions among proteins and dye-functionalized ligands (or dye–ligand conjugates: DLCs) offer high sensitivity detection of proteins in solution media. This DLC sensing approach has been successfully demonstrated as a highly versatile and general mode for sensing of proteins, peptides, and antibodies [23–28]. Furthermore, a general strategy for extending this DLC approach to other analytes can be achieved by synthetically modifying the DLC through a simple change of specific receptor group attached to the quencher ligands, which may also be synthetically modified to optimize sensitivity. Implementation of previously demonstrated DLC assays based on conjugated polymers, however, is hampered in that the signal transduction mechanism requires reversal of energy or charge transfer between the DLC and the conjugated polymer [11,29]. This mechanism requires a significant design and synthesis component in incorporating the desired physical and electronic structures (e.g., energetics that favor the energy/charge transfer process) to enhance the required interactions between DLC and conjugated polymer.

In this study, we use *unconjugated*, *non-fluorescent polyelectrolytes* to quench oppositely charged fluorescent dye–ligand conjugates (DLCs) by driving DLC aggregate formation at the polyelectrolyte backbone to form a self-quenched polymer–dye complex [30]. The temperature-dependent Stern–Volmer plots clearly show a static-quenching model dominated mechanism. The DLCs consist of a fluorescent dye functionality that is covalently bound to a bioreceptor ligand. In our studies, the ligand is biotin. Specific binding of avidin to the DLC (ligand = biotin) pulls the complex away from polyelectrolyte and results in fluorescence recovery (Scheme 1). We present two systems: one with a negatively charged dye and positively charged polyelectrolyte quencher and the other with a positively charged dye and negatively charged quencher. These two systems clearly show the fluorescence recovery that is directly related to the specific binding of protein and capable of detecting protein (avidin) with subnanomolar sensitivity. The unique mechanism for signal transduction presented by this new polyelectrolyte platform is in clear contrast with the previously demonstrated DLC-based sensing platforms. The template that polyelectrolyte provides for facilitating the self-quenching (via aggregate formation) of the DLCs is built into the polyelectrolyte structure. Thus, another important advantage is that these polyelectrolytes are readily available from several commercial sources without the need for further modification, in contrast to the involved synthesis required to produce the previously demonstrated conjugated systems. The polyelectrolyte assay method we present here is therefore potentially cheaper and easier to implement than the conjugated polymer-based biosensors.

2. Experimental

2.1. Materials

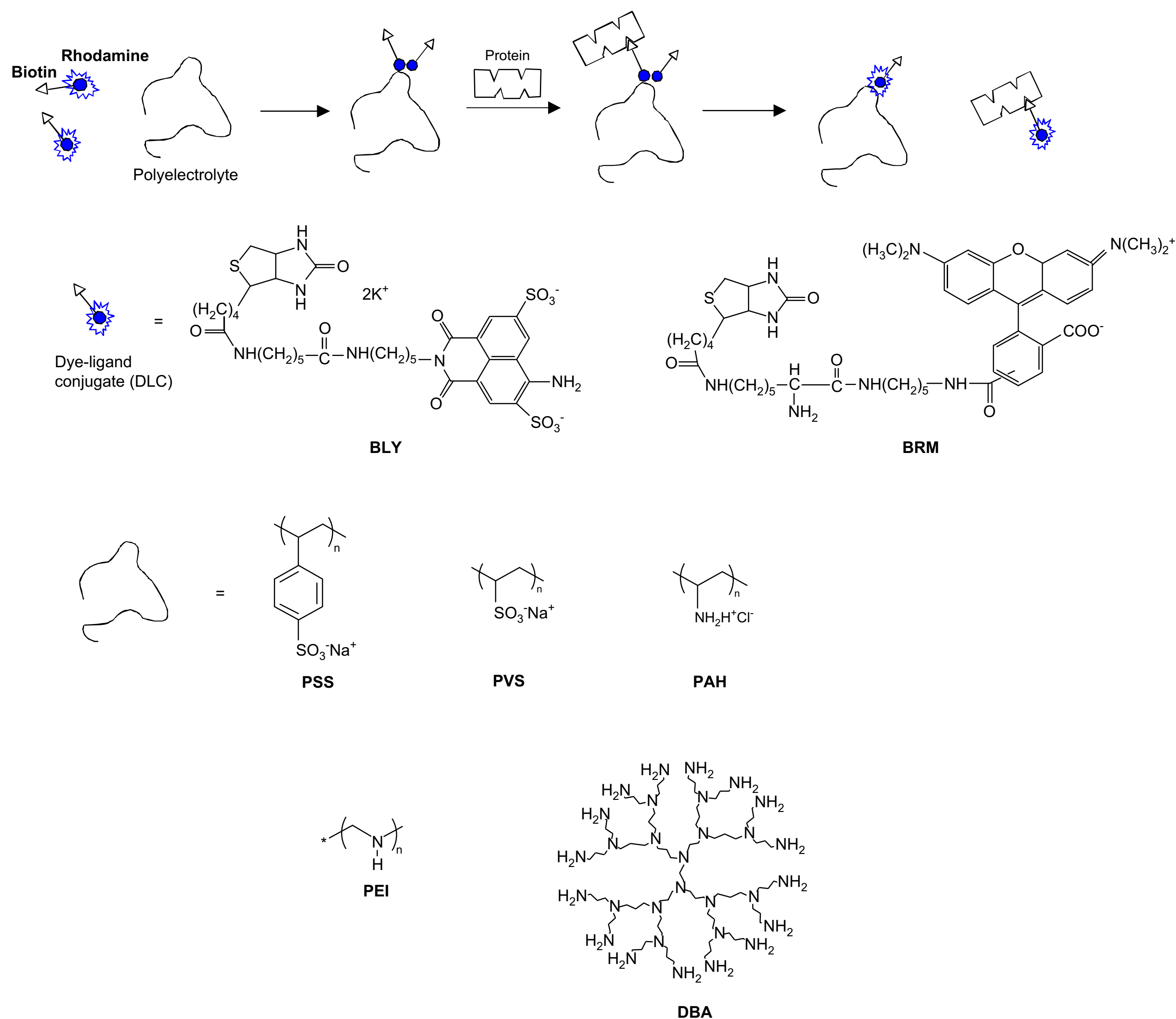
The anionic dyes lucifer yellow cadaverine biotin-X, dipotassium salt (BLY), and the positively charged dye, 5-(and-6)-((*N*-(5-aminopentyl)amino) carbonyl) tetramethylrhodamine (RM) and a biocytin tagged version (BRM) were purchased from Molecular Probes, Inc (Eugene, OR). A variety of positively charged and negatively charged polyelectrolytes were used to quench these dyes, including poly(allylamine hydrochloride) (PAH, Mw 15 000 and 70 000), poly(propyleneimine) (DBA, 3rd generation), polyethylenimine (PEI, Mw 14 000), poly(vinyl sulfonic acid sodium salt) (PVS, 25 wt% solution in water), and poly(sodium 4-styrene sulfonate) (PSS) (Mw 70 000). Polyelectrolytes were purchased from Sigma–Aldrich and used without further purification. Avidin (98%) is purchased from Sigma–Aldrich and used as it is.

2.2. Instruments

Nanopure water was used for all aqueous solutions at room temperature. UV–vis–NIR spectra are obtained using a Varian Cary 500 spectrometer. Fluorescence measurements were recorded on a Jobin Yvon Fluorolog3 spectrometer.

2.3. Sample preparation

All fluorophores were measured in 1–2 mg amounts and dissolved in water, except for the rhodamine dyes, which were first dissolved in 0.5 mL ethanol and then mixed in a large amount (1.5–5 mL) of water. The concentrations of the fluorophores were determined using their extinction coefficients given by Molecular Probes, Inc (Table 1). Polymer solutions were made by dissolving 1–10 mg of polymer in 10 mL water and vortexing. Protein solutions were made similarly, and dilutions were made to obtain the desired concentrations. Experiments were done in a 1 cm path-length quartz cell by adding 3 μL of fluorophore solution to 3 mL of water. The concentrations of dye and polymer in the cell were in the nanomolar range. To minimize volume changes during the experiments, small amounts (microliters) of quenchers, dyes, and avidin



Scheme 1. Representation of hypothesized mechanism for fluorescence recovery and the molecular structures of dye–ligand conjugates (DLCs) and polyelectrolytes.

were added. To observe incremental quenching, fluorophores were quenched with aliquots of 10^{-7} M polymer solutions. To quench immediately (i.e., with the smallest volume possible), more concentrated (10^{-4}–10^{-5} M) polymer solutions were used. In a typical experiment, there was a volume change of no more than 100 μL. Unless specifically identified, all experiments were carried out in pure aqueous solution (deionized water) with a pH value of ~6.9.

Table 1
The efficacy of polyelectrolyte used to quench the DLC fluorescence

Polyelectrolyte	PEI	PEI–HCl	DBA	DBA–HCl	PAH–HCl
I_0	139 000	137 000	145 700	140 000	134 000
n	7.8	4.8	6.0	3.5	2.6

I_0: initial fluorescence intensity, n: number of repeat units needed to quench the fluorescence of the DLC; PEI: polyethylenimine, DBA: poly(propyleneimine, dendrimer 3rd generation); PAH: poly(allylamine).

3. Results and discussions

The quenching of the dye luminescence is caused by self-quenching due to the formation of DLC aggregates as the oppositely charged polyelectrolyte brings the DLC molecules into close proximity. Polyelectrolyte is a quencher only in the sense that it serves the role of facilitating the formation of the DLC aggregates. Optimizing detection sensitivity requires first choosing the most efficient polyelectrolyte quenchers for the DLC fluorescence. The most efficient quenchers demonstrated an ability to quench the fluorescence by at least 90%. This maximum quenching suggests that the number of free dyes not associated with polyelectrolyte is minimized. Polyelectrolytes

used to quench anionic lucifer yellow cadaverine biotin-X (BLY) were polycations such as PAH, PEI, and DBA (Scheme 1). PAH was determined to be the most efficient cationic quencher and was subsequently used for the preparation of stock solution of quenched DLC for use in the detection of avidin (Table 1). The cationic 5-(and-6)-((*N*-(5-aminopentylamino)carbonyl) tetramethylrhodamine (RM) and 5-(and-6)-tetramethylrhodamine biocytin (BRM) were quenched well by the polyanions including poly(sodium 4-styrene sulfonate) (PSS) and poly(vinylsulfonate) (PVS). The biotinylated fluorophores BLY and BRM were quenched in a salt-free environment at neutral pH using oppositely charged polyelectrolyte. For the studies shown here, optimally quenched DLC stock solution is prepared by adding 4 μL of 0.38 mM PAH solution to 3 mL of 93.8 nM BLY aqueous solution. In this case, 93.8 nM of dye molecules is quenched by 506 nM of polyelectrolyte (calculated by Mw of repeat unit). More than five polyelectrolyte repeat units are required to quench one fluorescent BLY molecule. Fig. 1 shows the monotonic decrease of the fluorescence intensity with increasing polyelectrolyte concentration. Quenching of BLY fluorescence reaches more than 94% and the quenching of BRM by PSS is greater than 90%. Further increase of polyelectrolyte results in fluorescence recovery due to redistribution of dye molecules to well separated binding sites (see inset of Fig. 1B), consistent with previous studies using rhodamine derivative (R6G) [31]. In addition, nonbiotinylated RM can be quenched by more than 93% using PAH; the contrast between using BRM and RM is small and this difference is believed to be due to the presence of additional biotin, which sterically inhibits the formation of quenched BRM aggregates. These results suggest that polyelectrolyte facilitates DLC self-quenching through aggregate formation and excess polyelectrolyte could untangle the aggregate and hence recover the dye fluorescence.

To better understand the optimized conditions for preparing quenched DLC/polyelectrolyte solution, a series of polyelectrolytes were used to determine the efficacy of forming quenched lucifer yellow solution. The results are shown in Table 1. We start with the DLC solutions with similar initial fluorescence intensity. Once the maximized quenching is achieved (>90%), we can then calculate the molar ratio between the DLC and polyelectrolytes (by the molecular weight of the repeat unit). The PAH/BLY system is chosen for the obvious reason. It requires only 2.6 protonated PAH repeat units to quench one BLY, whereas 7.8 PEI repeat units are required to quench one BLY. The protonated polyelectrolyte solutions are more effective than their unprotonated counterparts, due to the enhanced charge density on the polyelectrolyte backbone.

3.1. Salt effects

If the DLC self-quenching is brought about through direct electrostatic interactions with polyelectrolyte, then the quenching is expected to show sensitivity to the ionic strength of the solutions. In fact, we find that the presence of salt in solution affects the quenching efficiency by electrostatic screening of polyelectrolyte that inhibits complex formation with DLC molecules. Adding greater than 100 mM of NaCl salt to a quenched DLC/polyelectrolyte complex causes complete recovery of fluorescence. Additionally, less quenching was observed in solutions of $MgSO_4$ than NaCl due to the increased ionic charges, as observed in other systems that used conjugated polymers as quenchers. Song et al. found that salts reduced the quenching efficiency of poly(2-ethynylpyridinium tosylate) with a propargyl side chain (PEPT) of LY by 10–30%, to still have >70% quenching [12]. The markedly lower percentage of fluorescence quenching in our experiments is indicative of a different quenching mechanism, one that relies on electrostatic interactions to cause self-quenching rather than energy transfer to a conjugated or fluorescent polymer. These results are consistent with our hypothesis that electrostatic interactions between polyelectrolyte and the

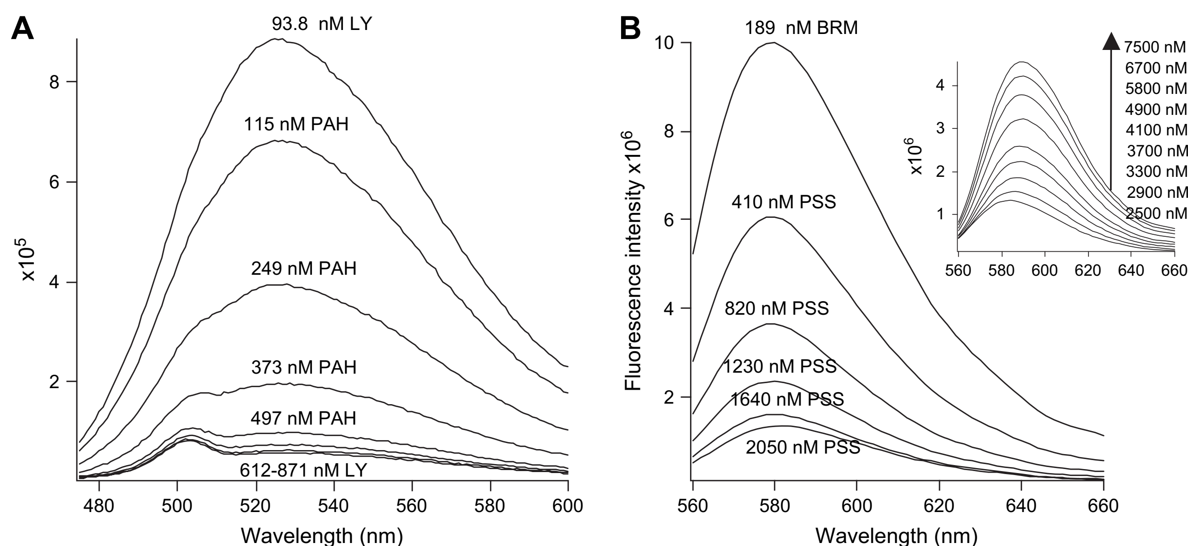


Fig. 1. Quenching of fluorescence of BLY with increasing [PAH] (A) and BRM with increasing [PSS] (B); inset shows excess polyelectrolyte results in redistribution of DLCs and recovery of fluorescence.

DLCs are the main driving force for DLC aggregate formation and self-quenching (Scheme 1).

3.2. Temperature effects and quenching mechanism

The stability of the quenched DLC/polyelectrolyte complex is also affected by the temperature. Quenched PAH/BLY undergoes temperature responsive aggregation in aqueous solutions within the temperature range of 4–29 °C, as shown in Fig. 2. In a 93% quenched BLY/PAH solution, the DLC fluorescence exhibits a monotonic increase with increasing temperature. This temperature responsive luminescence is fully reversible. A separate control experiment shows that DLC (BLY) luminescence is independent of the temperature up to 35 °C. This difference can be rationalized by the dye–polymer association constant being temperature dependent and thus the fraction of free dye increases with increasing temperature. In addition, higher temperature prompts disassociation of BLY/PAH complex bound by electrostatic interaction. This thermally induced disassociation is further validated by the temperature-dependent quenching experiments, in which PAH is added to the BLY solution at different temperatures, and the results are shown in Fig. 2b. This figure represents a Stern–Volmer plot [32], in which $I_0/I-1$ (where I_0 is the initial BLY fluorescence intensity, I is the fluorescence intensity in the presence of PAH), vs. increasing PAH concentration, is plotted. At lower temperatures (<25 °C), the Stern–Volmer plot is not linear, suggesting that more than one quenching mechanisms are involved in overall quenching. At higher temperatures (>50 °C), we observe linear Stern–Volmer plots and the slopes of these plots decrease with increasing temperature, indicative of reduced quenching dominated by a static-quenching model [33]. The above results clearly point to a static-quenching model because an increase in temperature leads to a decrease in the association constant between BLY and PAH, and hence results in a decrease in fluorescence quenching. In contrast, the collision model will lead to an increase in quenching. The salt effect and temperature effect studies indicate a static dominated quenching mechanism that results in electrostatic-based aggregation of "nearest neighbors" that is apparently further enhanced by further coiling of the polyelectrolyte conformation.

3.3. Enhanced interaction

In addition to electrostatic interactions, π–π interactions may have an impact on the quenching of the dyes. For example, poly(vinylsulfonate) (PVS) and poly(styrene sulfonate) (PSS) are both negatively charged polyelectrolytes, but PSS, which contains an aromatic ring in the repeat unit, quenches >90% of the RM fluorescence vs. only 33% quenching by PVS. We attribute this difference to the preferential π–π and hydrophobic interactions between planar conjugated chromophores, a common structural feature of dye molecules in DLC, and polyelectrolyte. A similar study by Zimerman et al. showed that aromatic polyelectrolytes strongly quench the fluorescence of indole derivatives of opposite charge by an electrostatic mechanism [34]. This result suggests that we can potentially fine-tune the π–π interaction between polyelectrolyte and DLC by way of varying the molecular structure. This may be important for overcoming ionic strength effects in real biosensing applications.

3.4. Detection of avidin

Fig. 3 shows that addition of nM avidin to this quenched solution yields an increase in fluorescence intensity. We observed the recovery of the fluorescence upon adding 1.0 nM increments of avidin to the PAH-quenched BLY solution. Similarly, fluorescence recovery is also seen by adding 0.3 nM of avidin to a PSS quenched BRM solution. Although avidin itself has been found to quench a biotinylated dye's fluorescence due to a proximity-induced self-quenching [12], the sensitivity and selectivity of this sensing platform are not significantly influenced by this effect. Fig. 4 shows detection of avidin with 100 pM (picomolar) sensitivity. This subnanomolar sensitivity

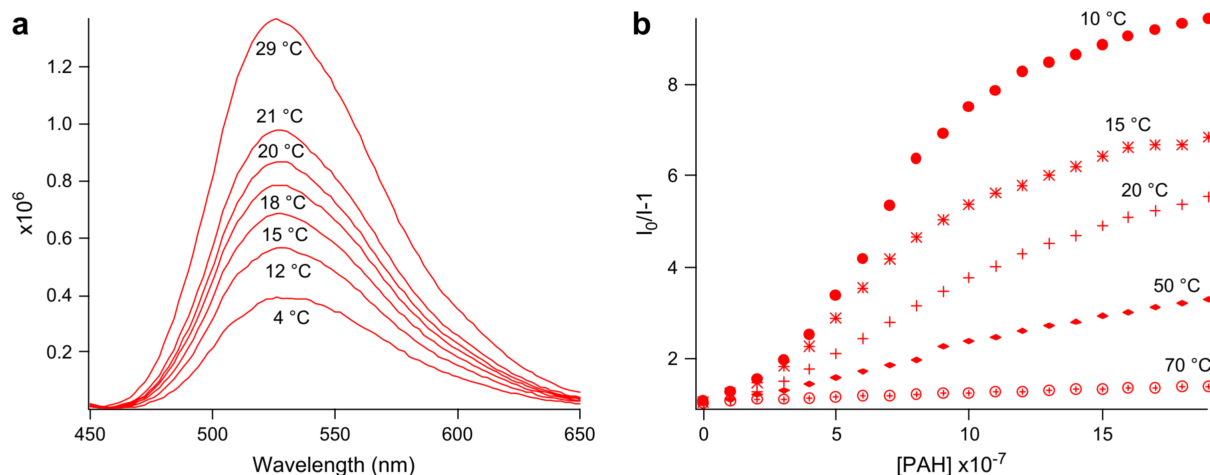


Fig. 2. Fluorescence of quenched BLY/PAH solution as a function of temperature (a) and the quenching of BLY fluorescence in different temperatures with increasing [PAH] (b).

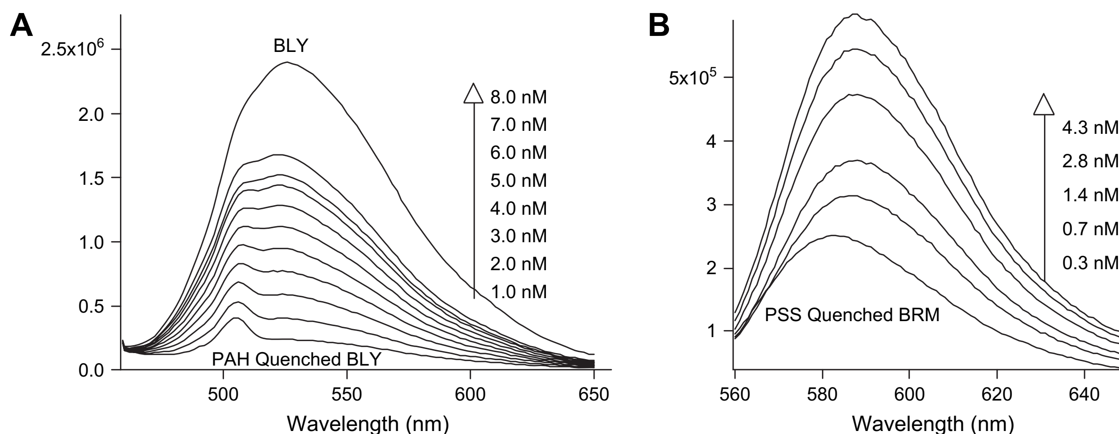


Fig. 3. Fluorescence recovery of quenched BLY (A) and BRM (B) solutions upon addition of nM avidin.

appears to be dominated by the initial dye concentration as the initial dye concentration is in the nanomolar range. The above results suggest amplification of the sensitivity and the potential to further enhance the sensitivity by lowering the initial DLC concentration. Based on a strict definition of the analytical detection limit (three times the standard deviation of blank measurements) we arrive at a detection limit of ~120 pM. The addition of subsequent avidin results in a linear recovery of fluorescence until saturation is reached at about 70% recovery (Fig. 5). Total recovery is not possible, mainly due to quenching between BLYs residing on neighboring avidin binding sites. This proximity-induced self-quenching is further confirmed as we add avidin to the biotinylated dye solution in the absence of polyelectrolyte. Our results show fluorescence quenching of the biotinylated dye solution with increasing avidin concentration. The avidin quenching reaches saturation at ~30% of the total fluorescence intensity as the molar ratio between BLY and avidin reaches 0.25, i.e., one avidin vs. four BLY molecules. This corresponds to the four binding sites available for biotin in the avidin structure. This result also agrees nicely with previous data, which shows 70% recovery of the total fluorescence by saturating the solution with avidin molecules (Fig. 3A).

The selectivity of the fluorescence recovery was investigated by adding bovine serum albumin (BSA, a protein with similar molecular weight to avidin) to the quenched dye solution. Recovery data were normalized relative to the most-quenched solution. The equation describing recovery is

$$\% \text{ recovery} = (\text{protein}-\text{PAH}/\text{BLY}-\text{PAH})100$$

where "PAH" is the fluorescence intensity yielded by the most-quenched solution. "BLY" is the fluorescence intensity of the initial unquenched BLY solution, while "Protein" represents the fluorescence intensity after addition of BSA or avidin. Fig. 5 shows the fluorescence recovery as avidin and BSA are added to the PAH-quenched BLY solution. We observe a linear increase of fluorescence intensity as the molar ratio between avidin and BLY reaches 0.25. We believe this

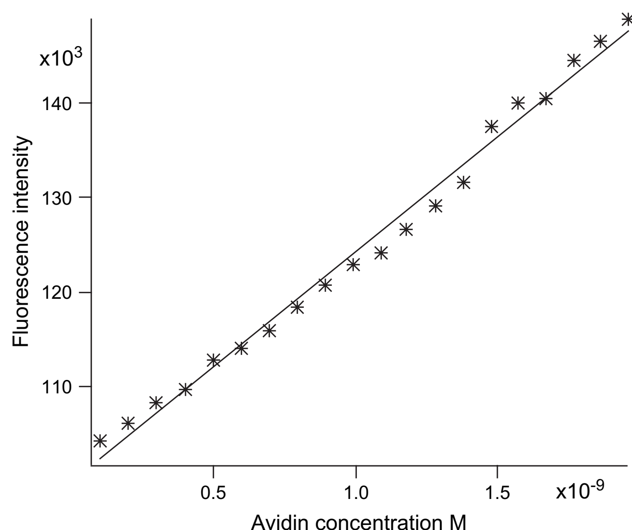


Fig. 4. Photoluminescence recovery by incremental addition of 100 pM avidin to a BLY solution fully quenched by PAH.

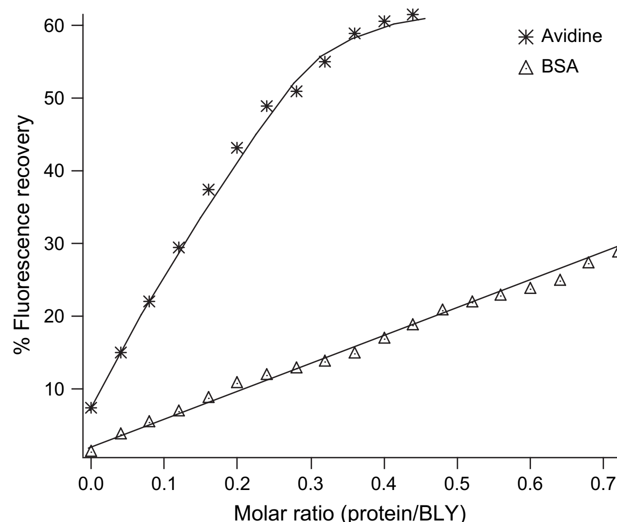


Fig. 5. Fluorescence recovery as a function of protein/BLY molar ratio.

is because the PAH-bound BLY molecules are pulled away by avidin through specific interaction. After this point, the fluorescence recovery tends to level. The competing interaction between protein and polyelectrolyte for DLC is similar to that of a specific interaction competing with an electrostatic interaction. The avidin/biotin interaction has a binding constant of 10^{15}, which is one of the strongest amongst all specific interactions. Typical binding constants of 10^9-10^{12} between other potential bioanalytes and their receptors are also expected to be significantly stronger than the weak electrostatic interaction between the DLC and polyelectrolyte. As a reference point for DLC interaction with polyelectrolyte, equilibrium constants for dye association with polyelectrolyte is a useful comparison. The association constant for dye/polyelectrolyte complex is in the order of 10^5-10^6 [35,36], which is in general much weaker than the specific interaction between the analyte and the receptor. Therefore, the so-called ‘‘weaker’’ specific (ligand/protein) interactions will remain strong enough to overturn the original DLC/polyelectrolyte complex formation and regain fluorescence. Thus, the DLC approach represented here is expected to be a general protein sensing platform with specific interactions.

In contrast to recovery by avidin, we find a linear increase of fluorescence with an increase in molar ratio between BSA and BLY up to 0.72. In addition, the recovery per nanomolar amount of BSA is approximately 1/5 that of the recovery for avidin, demonstrating the specificity of the ligand for the target. This limited recovery by BSA is probably due to nonspecific interactions between the charged residues on BSA with the ionic binding sites on the quencher and dye [37–39]. More specifically, BSA has an isoelectric point of 4.7, i.e., BSA is highly negatively charged at the neutral reaction condition, $pH = 6.9$. The highly negatively charged BSA can generate release of DLC to the aqueous solution and/or disrupt the DLC aggregate from a quenched state to a non-quenched state through competitive interaction with polyelectrolyte, which hence reorients the quenched DLC pairs. For proteins with an isoelectric point higher than BSA, such interferences are expected to be minimized relative to the BSA interaction, due to subsequent reduction of the protein/DLC interaction.

The specificity of this polyelectrolyte sensing was also investigated by allowing avidin to compete with BSA. In this experiment, 2.35 nM BSA was added to a quenched DLC/polyelectrolyte solution and resulted in a 26% increase in the fluorescence. To the same solution 0.69 nM of avidin was then added, resulting in a further doubling of the fluorescence intensity. In this competitive assay experiment, the recovery per nanomolar amount of BSA is approximately 1/15 that of the recovery for avidin. Aiming at further improving the specificity, we will focus on using $\pi-\pi$ interactions to enhance the stability of DLC/polyelectrolyte complex, which in turn will minimize the fluorescence recovery due to nonspecific interactions resulting from protein/polyelectrolyte interaction.

Finally, we recognize the importance of having this system to be effective in an aqueous buffer environment. Our preliminary study shows that 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (a dye molecule with three negative sulfonate groups) quenched by PAH shows very little (<5%) fluorescence recovery as up to 0.1 M of NaCl solution was added. This stability in aqueous solutions of high ionic strength can be rationalized by the enhanced electrostatic interaction between polyelectrolyte and dye molecule.

4. Conclusions

We have studied the quenching behavior of several fluorophores using fluorescence and UV–vis spectroscopies. Several unconjugated, non-fluorescent polyelectrolytes were used to quench these fluorophores. Quenching was due to the formation of aggregates through electrostatic interactions between dyes and an oppositely charged polyelectrolyte. Electrostatic interaction is well manifested by the recovery of fluorescence upon addition of salt. Quenching efficiency was affected by the amount of salt present, structure and charge of the fluorophore and/or polymer, and length of the polymer chain. The quenched biotinylated probes were used in the detection of avidin with subnanomolar sensitivity. Our highly sensitive system also demonstrated specificity for avidin over another protein, BSA. We believe that this method will serve as a novel platform for the development of highly sensitive biosensors.

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