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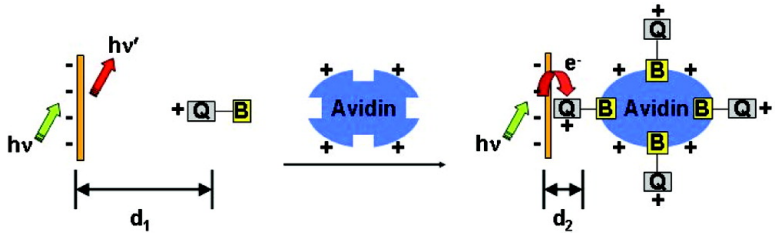

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Perturbation of Fluorescence by Nonspecific Interactions between Anionic Poly(phenylenevinylene)s and Proteins: Implications for Biosensors

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Abstract: The use of anionic water-soluble conjugated polymers (CPs) for sensing the presence of avidin by use of a biotin-modified fluorescence quencher was studied. The molecules involved in the study included poly[2-methoxy-5-(3′-propyloxysulfonate)-1,4-phenylenevinylene] with either lithium (**Li$^+$-MPS-PPV**) or sodium (**Na$^+$-MPS-PPV**) countercations, the well-defined oligomer pentasodium 1,4-bis(4′(2″,4″-bis-(butoxysulfonate)-styryl)-styryl)2-butoxysulfonate-5-methoxybenzene (**5R5$^-$**), the quenchers *N*-methyl-4,4′-pyridyl-pyridinium iodide (**mMV$^+$**) and [*N*-(biotinoyl)-*N*′-(acetyl 4,4′-pyridylpyridinium iodide)] ethylenediamine (**BPP$^+$**), which contains a molecular recognition fragment (biotin) attached to a unit that accepts an electron from a CP excited state, and the proteins avidin, tau, BSA, and pepsin A. Fluorescence quenching experiments were examined in a variety of conditions. Experiments carried out in water and in ammonium carbonate buffer (which ensures avidin/biotin complexation) reveal that nonspecific interactions between the CP and the proteins cause substantial perturbations on the CP fluorescence. The overall findings are not consistent with a simple mechanism whereby avidin complexation of **BPP$^+$** leads to encapsulation of the quencher molecule and recovery of **Li$^+$-MPS-PPV** fluorescence. Instead, we propose that binding of **BPP$^+$** to avidin results in the quenching unit attaching to a positively charged macromolecule. Electrostatic attraction to the negatively charged conjugated polymer results in closer proximity to the quencher. Therefore, more enhanced fluorescence quenching is observed.

Introduction

There are substantial efforts aimed at improving fluorescent biosensors.[1−4] One of the primary qualities of a molecular sensory process is that it must be selective. Diagnostic signals must correspond to the presence of a specific analyte or target, such as a protein structure or a DNA (or RNA) sequence. A clear difference must exist between these signals and those that the sensor produces when nontarget molecular species are interrogated. Biosensors must also be sensitive. The small quantities of diagnostic materials in biological samples, or in the environment, often limit identification speed and accuracy. To address this limitation, signal amplification schemes have appeared which utilize fluorogenic substrate active enzymes,[5−7] modified liposomes,[8] and Au nanoparticles.[9−11] Finally, useful sensors should continue to be selective and sensitive with variations in analyte quantity.

Conjugated polymers (CPs) contain a set of structural attributes that make them useful in optical and electronic detection of chemical and biomolecular targets. Swager pointed out that important properties, such as charge transport, conductivity, emission yield, and exciton migration, are easily perturbed by external agents and lead to substantial changes in measurable signals.[12,13] When the molecular weight of the polymer is large, the backbone holds together a number of segments with an average conjugation length shorter than the average degree of polymerization. Fluorescence resonance energy transfer (FRET) among these segments allows excitations to travel to low energy sites.[14] If the low energy site corresponds to the presence of the target, then the overall collection of optical units has a "light harvesting" effect.

Recent work has revealed new concepts for optimizing the optical amplification of CPs. Theoretical and experimental work demonstrate that the electronic coupling across interchain contacts (either between chains or looped regions in the same

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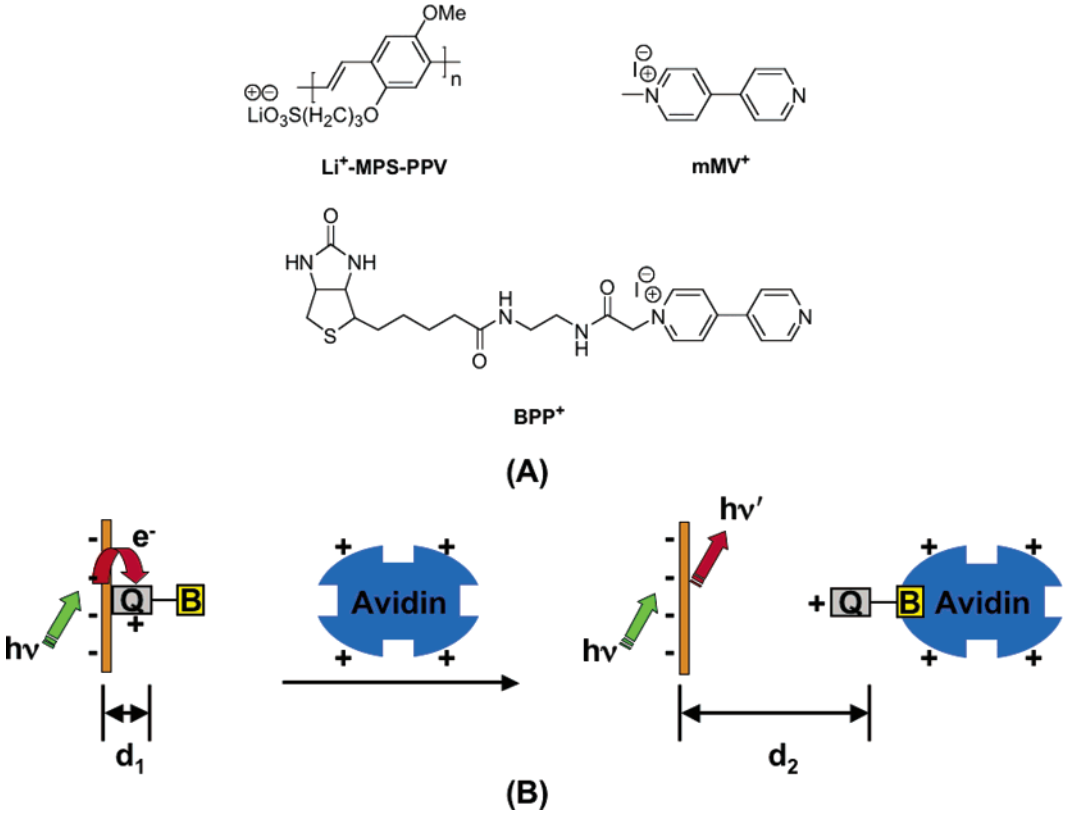
Scheme 1. (A) Chemical Structures of **Li$^+$-MPS-PPV**, **mMV$^+$**, and **BPP$^+$**; (B) Avidin Sensor Description: Orange Line Represents **Li$^+$-MPS-PPV**, and Q and B are the Quencher and Biotin Modules of **BPP$^+$**, Respectively (Avidin Is Positively Charged)



chain) is more efficient than that between conjugated segments adjacent to each other.[15−17] The "dimensionality" of the system (*i.e.*, whether one, two, or three dimensions are available for energy transfer) also plays an important role.[18] The degree to which these effects cooperate to determine the final overall sensitivity remains to be determined and is likely to vary depending on the polymer structure and processing conditions. It should also be noted that exciton migration in a film of small molecules can provide optical amplification similar to these discussed for CPs.[19] These considerations are relevant when the CP is used as a film; however, when sensing is done in solution, perturbations that influence the coil conformation or the aggregation between chains can dominate fluorescence.[20,21]

Water solubility is required for biosensing in solution. This property is most often accomplished in CPs by molecular designs that incorporate charged groups pendant to the electronically delocalized backbone.[22] Because of these charged groups, chain−chain interactions in water-soluble CPs are subject to the same interpolymer forces previously characterized for other polyelectrolytes.[23] Moreover, because of the high content of aromatic subunits, the backbone is considerably hydrophobic, causing extensive aggregation in aqueous media. Even for conjugated oligomers with only four to five repeat units, the aggregation number at low concentrations ($\sim 1 \times 10^{-5}$ M) can be in the order of a thousand.[24,25] Thus, electrostatic and hydrophobic forces, which depend on the chemical structure of a given polymer, work in concert to mediate FRET efficiencies.

A recent contribution that details a water-soluble CP-based fluorescence assay has stimulated substantial research.[26] The probe molecule [*N*-(biotinoyl)-*N*′-(acetyl 4,4′-pyridylpyridinium iodide)] ethylenediamine (**BPP$^+$**) consists of a molecular recognition fragment (biotin) attached to a pyridinium unit that can accept an electron from the CP excited state.[27] The structures of the specific CP and **BPP$^+$**, together with the original description of the biosensor process, are illustrated in Scheme 1. When **BPP$^+$** is added to a solution of poly[lithium 2-methoxy-5-(3′-propyloxysulfonate)-1,4-phenylenevinylene] (**Li$^+$-MPS-PPV**),[28] a weak electrostatic complex forms between the pyridinium and sulfonate groups, which brings the quencher

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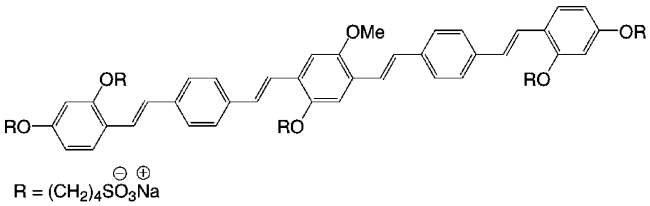
Scheme 2. The Chemical Structure of **5R5**$^-$



5R5$^-$

molecule into close proximity to the optically active backbone. Under these circumstances, the fluorescence quenching of **Li**$^+$-**MPS-PPV** by **BPP**$^+$ is very efficient. The addition of avidin, which selectively binds the biotin substructure, was described to encapsulate **BPP**$^+$ and draw it away from **Li**$^+$-**MPS-PPV**, thereby recovering emission. In other words, the average distance between the polymer and the quenching site is larger upon avidin complexation of the biotin fragment ($d_2 > d_1$). Several control experiments were reported in support of Scheme 1. *N*-Methyl-4,4′-pyridylpyridinium iodide (**mMV**$^+$) quenches the emission of **Li**$^+$-**MPS-PPV** to a similar, but lesser, extent than **BPP**$^+$. Avidin (2.4×10^{-8} M) did not reestablish the emission of **Li**$^+$-**MPS-PPV**/**mMV**$^+$. Finally, addition of avidin (2.0×10^{-7} M) to **Li**$^+$-**MPS-PPV** ([RU] $= 1.0 \times 10^{-5}$ M, where RU = repeat unit of polymer) did not perturb emission.

More recent publications have shown that nonspecific interactions between water-soluble CPs and proteins are more difficult to control than originally anticipated.[29] These unpredictable contacts can dominate the changes in CP emission, even in the presence of target-specific quencher molecules. For example, during studies on how the anionic poly[lithium 2-methoxy-5-(4′-butoxysulfonate)-1,4-phenylenevinylene] (**Li**$^+$-**MBS-PPV**) could be used in "lock-and-key" molecular recognition events, it was found that the anionic protein mouse IgG enhanced the polymer emission by approximately 30%, even at low concentrations ([RU] of **Li**$^+$-**MBS-PPV** $= 9.5 \times 10^{-7}$ M and [IgG] $= 1.5 \times 10^{-7}$ M).[30] The surprising perturbation of emission by a negatively charged protein highlights hydrophobic interactions between the two macromolecules in a buffer solution. Nonspecific interactions can be reduced when **Li**$^+$-**MBS-PPV** is complexed with a cationic polyelectrolyte, however, at the expense of sensitivity.[30]

Surfactants based on small molecules also perturb the optical properties of water-soluble CPs.[31] Addition of dodecyltrimethylammonium bromide (DTA) to **MPS-PPV** increases the fluorescence quantum yield (Φ) by elongating the polymer coil and discouraging interchain aggregation. Surfactants also influence the fluorescence quenching efficiency of external agents. For instance, the quenching efficiency of methyl viologen (**MV**$^{2+}$) is reduced by DTA by more than 2 orders of magnitude.

Surfactant effects are quite complex. In the case of the well-defined oligomer pentasodium 1,4-bis(4′(2″,4″-bis(butoxysulfonate)-styryl)-styryl)2-butoxysulfonate-5-methoxybenzene (**5R5**$^-$) (see Scheme 2), which has a structure that mimics a well-defined repeat segment of **Na**$^+$-**MBS-PPV**, the addition of DTA also increases Φ.[24] However, a comparison of **Na**$^+$-**MBS-PPV** and **5R5**$^-$ with approximately similar [RU] (2.0×10^{-5} M) and [DTA]/[RU] ≈ 10, shows that the oligomer is more effectively quenched by **MV**$^{2+}$. A relevant observation here is that **5R5**$^-$ forms large aggregates ($\sim$1000 molecules), even under these low concentrations. Incorporation of surfactants within these aggregates appears to increase the electronic communication between optical subunits and may also increase the surface-to-volume ratio by forming smaller aggregates. Further evidence that the nonspecific interactions of surfactants influence the FRET between oppositely charged oligomers in solution has also appeared.[32]

Proteins themselves may also behave as electron acceptors.[33] The electron-transfer protein cytochrome *c* (cyt *c*) quenches the **Li**$^+$-**MBS-PPV** fluorescence very efficiently. A plausible mechanism involves quenching by the Fe(III) site in cyt *c*. This photoinduced electron-transfer step yields the ferrous state of cyt *c*. Fluorescence quenching was observed, even when the pH of the solution rendered the cyt *c* slightly negative. Furthermore, the protein lysozyme, which does not contain an electroactive center, was able to quench up to 50% of the **Li**$^+$-**MBS-PPV** emission.

The collected work on nonspecific interactions described above led us to reconsider the original **Li**$^+$-**MPS-PPV**/**BPP**$^+$/avidin sensor. In pure water, which would maximize the **Li**$^+$-**MPS-PPV**/**BPP**$^+$ electrostatic attraction,[34] the biotin/avidin complexation is not optimized.[35] In buffer solutions, wherein the biotin/avidin binding is enhanced, the electrostatic binding between **Li**$^+$-**MPS-PPV** and **BPP**$^+$ would be severely attenuated, rendering the biosensor scheme sensitive to nonspecific hydrophobic interactions.[30] To address these concerns, we report in this contribution a systematic investigation of the fluorescence quenching of anionic CPs and oligomers with **BPP**$^+$, **mMV**$^+$ in the presence and absence of avidin and nonspecific proteins. The resulting conclusions contradict the original mechanism provided in Scheme 1.

Results and Discussion

Synthesis and General Consideration of Polymer Structures. We propose the following system for abbreviating the nomenclature of water-soluble PPVs: **M**$^+$-**MPS-PPV** for the methoxypropyloxy sulfonate-substituted PPV, for example, **Na**$^+$-**MPS-PPV** or **Li**$^+$-**MPS-PPV**, or **M**$^+$-**MBS-PPV** for the methoxybutoxy sulfonate counterparts. This system provides a complete description of the pendant groups and the countercations of the polymer.

There are no synthetic details or characterization of **BPP**$^+$ provided in the literature. Our procedure and assignment are as follows. An excess of 4,4′-bipyridine (4 equiv) was added to a solution of commercially available *N*-(biotinoyl)-*N*′-(iodoacetyl)-ethylenediamine in DMSO-d_6 (eq 1). The disappearance of the singlet due to the iodoacetyl protons (3.61 ppm) was monitored by ^{1}H NMR spectroscopy. Once the reaction was complete (room temperature), the solvent was removed and the product was purified by extraction of unreacted 4,4′-bipyridine with

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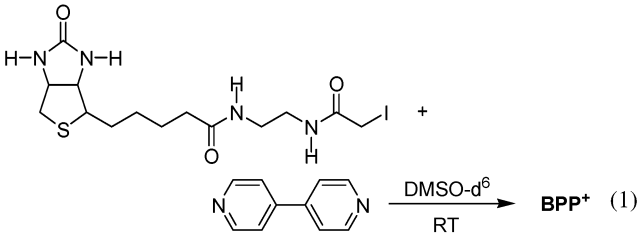
Table 1. K_{sv} Values (in M^{-1}) for Chromophores ([RU] = 1.7×10^{-5} M) in Water[40]

	Li^+-MPS-PPV	Na^+-MPS-PPV	5R5$^-$
mMV$^+$	6.6×10^6	4.0×10^6	1.9×10^4
BPP$^+$	3.9×10^6	2.3×10^6	4.2×10^4



(1)

dichloromethane. The structure was confirmed by electrospray mass spectrometry.

M$^+$**-MPS-PPV** was synthesized by the method of Shi and Wudl.[28] The polymer countercation is controlled by the metal-alkoxide base, NaOMe or LiOMe, used in the polymerization reaction and conversion of precursor polymer to the conjugated form. The polymers are subjected to dialysis with 12 000−14 000 molecular weight cut-off cellulose membranes for 48 h to remove any low molecular weight products. Due to the nature of the polymerization reaction, batch-to-batch variations of molecular weight, molecular weight distributions, and conjugation defects are often encountered.[36] These factors, together with aggregation size and polymer conformation, can influence the fluorescence of different polymer samples.[37]

Fluorescence Quenching Experiments and Nonspecific Interactions in Water. Fluorescence quenching efficiencies can be quantified by use of the Stern−Volmer equation:[14]

$$F_0/F = 1 + K_{sv}[Q]$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, and [Q] is the quencher concentration. The Stern−Volmer constant, K_{sv}, provides a direct measure of the quenching efficiency and is determined from the linear portion of a plot of F_0/F versus [Q]. The values obtained for anionic conjugated polymers and cationic electron acceptors are too high for diffusion-controlled quenching, indicating the formation of nonfluorescent ground-state complexes.[38,39] Furthermore, linear plots are observed only under low quencher concentrations (for [RU] = 1.7×10^{-5} M, [**mMV**$^+$] ≤ 3.4×10^{-7} M, and [**BPP**$^+$] ≤ 4.6×10^{-7} M). Deviations from linearity in the Stern−Volmer plots at higher concentrations have been attributed to a "sphere of action" mechanism.[14,39]

Table 1 provides the K_{sv} values from fluorescence quenching experiments in water using **Na**$^+$-, **Li**$^+$**-MPS-PPV**, and **5R5**$^-$ as donors and **mMV**$^+$ and **BPP**$^+$ as acceptors. The quenching data are reported from the linear region of the Stern−Volmer plots. The two polymers are quenched approximately to the same extent and are over 2 orders of magnitude more sensitive than the oligomer. Differences in quenching efficiency between **mMV**$^+$ and **BPP**$^+$ can be attributed to steric, electrostatic, and hydrophobic interactions inherent to the differences in molecular structures that are difficult to deconvolute. For both **M**$^+$**-MPS-PPV** structures, **mMV**$^+$ is nearly twice as effective as **BPP**$^+$. The opposite effect is true for the oligomer **5R5**$^-$. The smaller **mMV**$^+$ molecules perhaps more easily associate with the larger polymer structure, whereas the larger **BPP**$^+$ would allow more of the smaller oligomers to surround a single quencher. Additionally, the large hydrophobic component that is added with the attachment of biotin and the linking moiety to **mMV**$^+$ to make **BPP**$^+$ makes hydrophobic interactions with the donor more significant. Previous light scattering experiments[24] have demonstrated that the hydrophobic content of **5R5**$^-$ leads to extensive aggregation in aqueous media, and therefore we propose that it is more sensitive to the hydrophobic **BPP**$^+$. To further support this claim, we have observed that in high ionic strength media where electrostatic interactions between **M**$^+$**-MPS-PPV** and the cationic quenchers are substantially reduced, the more hydrophobic **BPP**$^+$ becomes the more effective quencher.

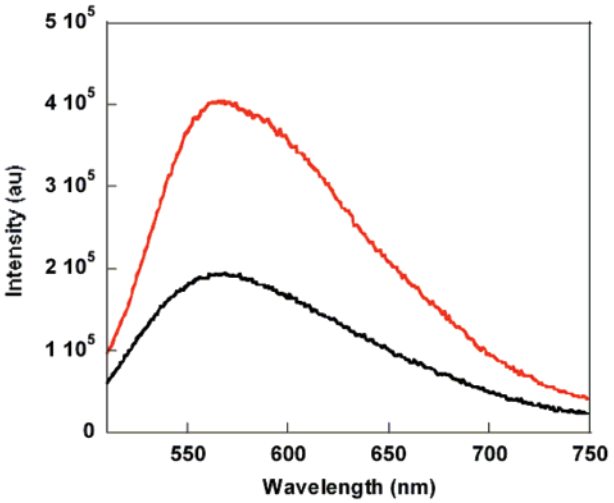


Figure 1. Fluorescence spectra (λ_{ex} = 500 nm) in water: **Li**$^+$**-MPS-PPV** ([RU] = 1.7×10^{-5} M) (black line) and **Li**$^+$**-MPS-PPV** and [avidin] = 3.0×10^{-7} M (red line).

The sensitivity of the **Li**$^+$**-MPS-PPV** fluorescence is illustrated in an experiment where avidin was added to an aqueous solution of the polymer, resulting in an increase of emission (λ_{ex} = 500 nm, Figure 1). The increase in polymer fluorescence by addition of avidin is consistent with previously reported nonspecific interactions between the charged conjugated polymers and other proteins.[30,32] Avidin has an isoelectric point (pI) of 10 and will be positively charged in a dilute aqueous solution.[41,42] Under these conditions, interactions of the protein with the anionic polymer arise from a combination of attractive electrostatic and hydrophobic forces. Once [avidin] ≈ 3.0×10^{-7} M, further additions, up to [avidin] = 5.0×10^{-7} M, do not give rise to detectable changes, and we refer to this [avidin] as the "saturation" point (see Experimental Section). Use of

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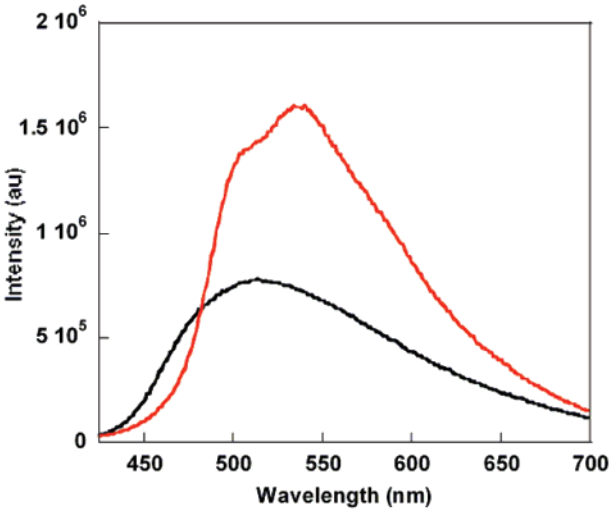

Figure 2. Fluorescence spectra (λ_{ex} = 400 nm) in water: **5R5**$^-$ ([RU] = 1.7 × 10^{-5} M) in water (black line) and **5R5**$^-$ and [avidin] = 3.0 × 10^{-7} M (red line).

Na$^+$**-MPS-PPV** ([RU] = 1.7 × 10^{-5} M) results in similar observations (Supporting Information).

An experiment similar to that in Figure 1 was performed using **5R5**$^-$ ([RU] = 1.7 × 10^{-5} M), and the results are shown in Figure 2. A red shift in fluorescence and a pronounced increase in intensity are observed. The emission saturation point occurs when [avidin] ≈ 3.0 × 10^{-7} M.

Fluorescence Recovery by Avidin in Water. A set of experiments using **Li**$^+$**-MPS-PPV** ([RU] = 1.0 × 10^{-5} M), [**BPP**$^+$] = 3.2 × 10^{-7} M, and avidin (aqueous environment) were previously described in the literature.[26] In those studies, **BPP**$^+$ quenched approximately 50% of the initial polymer emission. When avidin (3.0 × 10^{-8} M) was added, approximately 95% of the initial intensity was recovered. It is important to note that there are four biotin binding sites in avidin.[42] Assuming quantitative binding upon mixing, a solution with [**BPP**$^+$] = 3.2 × 10^{-7} M and [avidin] = 3.0 × 10^{-8} M will yield an unbound [**BPP**$^+$] ≈ 2 × 10^{-7} M, and one would expect, based on Scheme 1, significant residual fluorescence quenching.

Similar experiments were repeated with **Li**$^+$**-MPS-PPV** in deionized water purified by nanofiltration (pH = 5.6), and the results are given in Figure 3. We observe more enhanced quenching by **BPP**$^+$, relative to that previously reported. However, only 47% emission recovery is observed upon addition of [avidin] = 3.0 × 10^{-8} M. At a stoichiometric avidin-binding site/biotin ratio ([avidin] = 8.0 × 10^{-8} M), the system appears to behave with specificity (Figure 3d), and 86% of the polymer emission is recovered. However, polymer emission continues to grow upon further avidin addition, up to [avidin] = 2.3 × 10^{-7} M. At this point, the polymer emission intensity is approximately twice that observed in the absence of **BPP**$^+$ and avidin (Figure 3e). Further avidin addition (up to 3.5 × 10^{-7} M) does not cause the fluorescence to change.[43]

Previous studies[26] show that with **mMV**$^+$ (3.2 × 10^{-7} M), the addition of avidin (2.4 × 10^{-8} M) did not result in **Li**$^+$**-MPS-PPV** fluorescence recovery. Using our materials, when

(43) Fluorescence measurements show negligible signs of photobleaching, even after 15 or more consecutive scans using the same polymer solution (see Supporting Information).

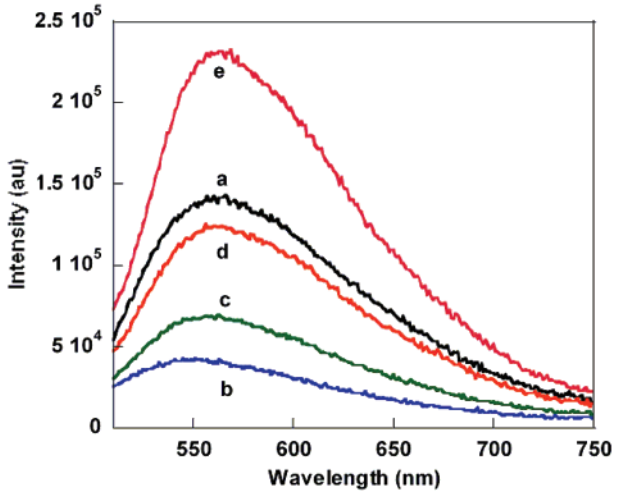

Figure 3. Fluorescence spectra (λ_{ex} = 500 nm) in water: (a) **Li**$^+$**-MPS-PPV** ([RU] = 1.0 × 10^{-5} M) (black line); (b) **Li**$^+$**-MPS-PPV** and [**BPP**$^+$] = 3.2 × 10^{-7} M (blue line); (c) **Li**$^+$**-MPS-PPV**, **BPP**$^+$, and [avidin] = 3.0 × 10^{-8} M (green line); (d) **Li**$^+$**-MPS-PPV**, **BPP**$^+$, and [avidin] = 8.0 × 10^{-8} M (red line); (e) **Li**$^+$**-MPS-PPV**, **BPP**$^+$, and [avidin] = 2.3 × 10^{-7} M (pink line).

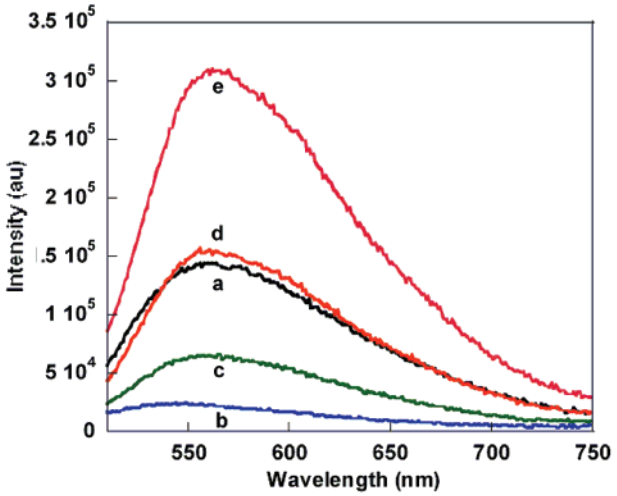

Figure 4. Fluorescence spectra (λ_{ex} = 500 nm) in water: (a) **Li**$^+$**-MPS-PPV** ([RU] = 1.0 × 10^{-5} M) (black line); (b) **Li**$^+$**-MPS-PPV** and [**mMV**$^+$] = 3.2 × 10^{-7} M (blue line); (c) **Li**$^+$**-MPS-PPV**, **mMV**$^+$, and [avidin] = 3.0 × 10^{-8} M (green line); (d) **Li**$^+$**-MPS-PPV**, **BPP**$^+$, and [avidin] = 8.0 × 10^{-8} M (red line); (e) **Li**$^+$**-MPS-PPV**, **mMV**$^+$, and [avidin] = 2.3 × 10^{-7} M (pink line).

mMV$^+$ is used as the quencher (Figure 4), the addition of 3.0 × 10^{-8} M avidin results in a 44% increase of fluorescence. When [avidin] = $^1/_4$[**mMV**$^+$], the emission intensity is nearly identical to that of the unperturbed polymer. The data behave as if there was binding and encapsulation of **mMV**$^+$ by avidin. Polymer emission continues to grow upon further avidin addition, up to [avidin] = 2.3 × 10^{-7} M. At this point, the emission intensity is approximately twice that observed in the absence of **mMV**$^+$ and avidin. Further avidin addition (up to 3.5 × 10^{-7} M) does not result in additional changes in emission intensity. Similar results were obtained with **Na**$^+$**-MPS-PPV** (Supporting Information).

Because there is no specific avidin/**mMV**$^+$ binding, the spectral changes resulting from avidin addition to solutions of **Li**$^+$**-MPS-PPV** and **mMV**$^+$ in Figure 4 must arise from nonspecific interactions. Comparison of Figures 3 and 4 shows that the saturating [avidin] is similar for either quencher. The

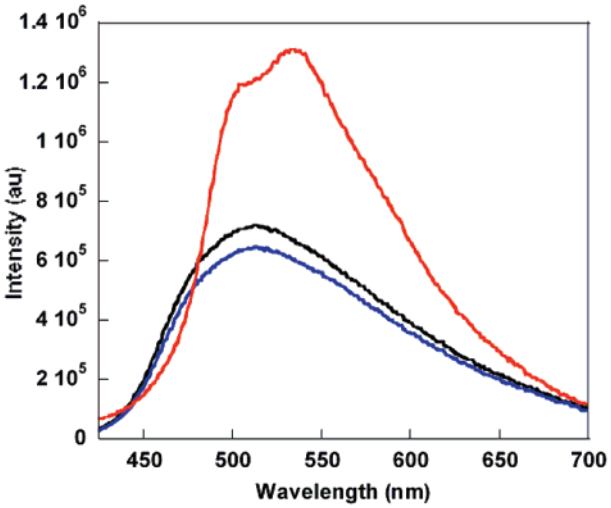

Figure 5. Fluorescence spectra ($\lambda_{ex} = 400$ nm) in water: **5R5**$^-$ ([RU] = 1.7×10^{-5} M) (black line); **5R5**$^-$ and [**mMV**$^+$] = 2.0×10^{-6} M (blue line); and **5R5**$^-$, **mMV**$^+$, and [avidin] = 1.0×10^{-6} M (red line).

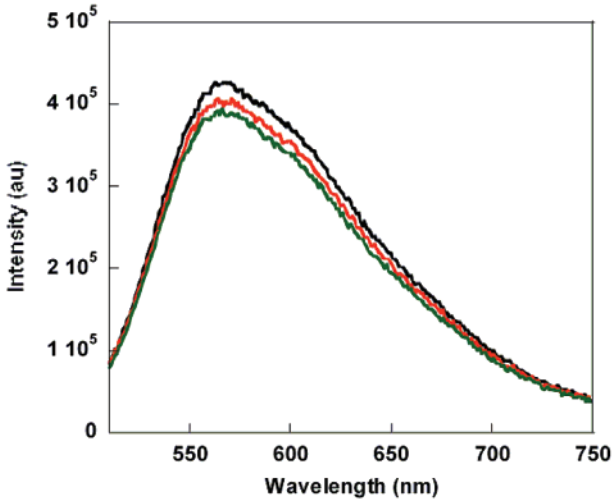

Figure 6. Fluorescence spectra ($\lambda_{ex} = 500$ nm) in 0.1 M $(NH_4)_2CO_3$ at pH 8.9 of **Li**$^+$**-MPS-PPV** ([RU] = 1.7×10^{-5} M) (black line); **Li**$^+$**-MPS-PPV** and [avidin] = 3.0×10^{-7} M avidin (red line); **Li**$^+$**-MPS-PPV** and [avidin] = 5.0×10^{-7} M avidin (green line).

interaction between **mMV**$^+$ and the polymer/avidin complex is negligible, and the solution behaves like the avidin and polymer pair. Ultimately, it is possible to obtain experimental conditions where the emission of the **Li**$^+$**-MPS-PPV**/avidin/**mMV**$^+$ solution behaves as if the avidin is removing **mMV**$^+$ from the vicinity of the polymer; however, this cannot be due to a specific interaction. Significant deviations in the behavior of **Li**$^+$**-MPS-PPV** versus **Na**$^+$**-MPS-PPV** were not observed (see Supporting Information for the **Na**$^+$**-MPS-PPV** spectra).

Experiments in water using **5R5**$^-$ ([RU] = 1.7×10^{-5} M, $\lambda_{ex} = 400$ nm) and **mMV**$^+$ (2.0×10^{-6} M) are shown in Figure 5. Similar to **Li**$^+$ or **Na**$^+$**-MPS-PPV**, the oligomer fluorescence over-recovers when avidin is added. The saturation point occurs at [avidin] = 1.0×10^{-6} M. Large variations due to protein−fluorophore contacts are therefore expected even when the statistical uncertainties due to the polymer structure are removed.

Fluorescence Recovery by Avidin in Buffer. The secondary structure, and thus the affinity between a protein and its corresponding ligand, is influenced by the environment; that is, temperature, solvent, ionic strength, and pH.[44] With this perspective, we conducted avidin detection experiments using an optimized buffer system that ensures binding. We chose 0.1 M ammonium carbonate at pH 8.9, because previous work demonstrated high biotin/avidin affinity under these conditions.[35] Similar conditions are commonly used in assays that determine the biotin binding capacity of an avidin-containing sample.[45] The emission intensity of the **M**$^+$**-MPS-PPV** in this buffer solution was within 25% of the intensity in water.

Stern−Volmer analysis of **Li**$^+$**-MPS-PPV** quenching ([RU] = 1.7×10^{-5} M) in buffer provided K_{sv} values approximately 2−3 orders of magnitude lower than those in water (**BPP**$^+$ = 6.3×10^4 M^{-1}, **mMV**$^+$ = 1.5×10^4 M^{-1}). According to the Debye−Hückel theory, the relatively high buffer ion concentration will reduce the Coulombic attraction between the negatively charged polymer and the positively charged quencher, thereby lowering the concentration of nonemissive ground-state complexes and diminishing the quenching efficiency. For this reason, the quenching of **5R5**$^-$ is reduced to a point where it is negligible as compared to the magnitudes observed with the polymer structures and will not be discussed. It is interesting to note that, in buffer, **BPP**$^+$ is a more efficient quencher than **mMV**$^+$, whereas **mMV**$^+$ is the more efficient quencher in water. A possible explanation is that, in a high ionic strength solution, the more hydrophobic **BPP**$^+$ provides for a stronger interaction with the noncharged polymer backbone, when compared to **mMV**$^+$.[46]

Avidin addition to **Li**$^+$**-MPS-PPV** ([RU] = 1.7×10^{-5} M) in buffer has only a minor fluorescence quenching effect (Figure 6). Recalling that addition of similar amounts of avidin to a solution of the polymer in water doubled the PL intensity (Figure 1) indicates that the protein is less effective at perturbing the polymer conformation when in a buffer solution. Small-angle neutron scattering experiments have shown that under high salt conditions **Li**$^+$**-MBS-PPV** assumes a rigid rod conformation.[47,48] The polymer structure can be thought of as being "locked" into this conformation.[49] It is reasonable that avidin cannot perturb this "locked" backbone substantially, and the corresponding change in fluorescence is negligible.

Avidin sensing and corresponding control experiments were conducted in the $(NH_4)_2CO_3$ buffer. Addition of [avidin] = 3.0×10^{-7} M to a solution of **Li**$^+$**-MPS-PPV** ([RU] = 1.7×10^{-5}) and [**mMV**$^+$] = 2.0×10^{-6} M does not change the emission (Figure 7). In contrast, one observes further fluorescence quenching upon addition of avidin to a solution of **Li**$^+$**-MPS-PPV** quenched with [**BPP**$^+$] = 2.0×10^{-6} M. For example, 1.0×10^{-7} and 3.0×10^{-7} M avidin quenches an additional 17% and 34% of the emission intensity, respectively (Figure 8). Subsequent additions of avidin (up to 5.0×10^{-7} M) do not produce further changes.

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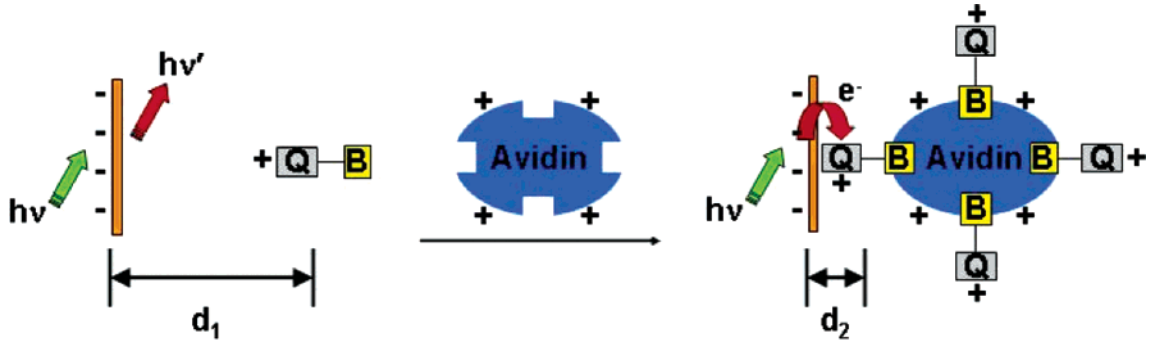
Scheme 3. Proposed Mechanism for Enhanced Fluorescence Quenching by Addition of Avidin under Buffer Conditions



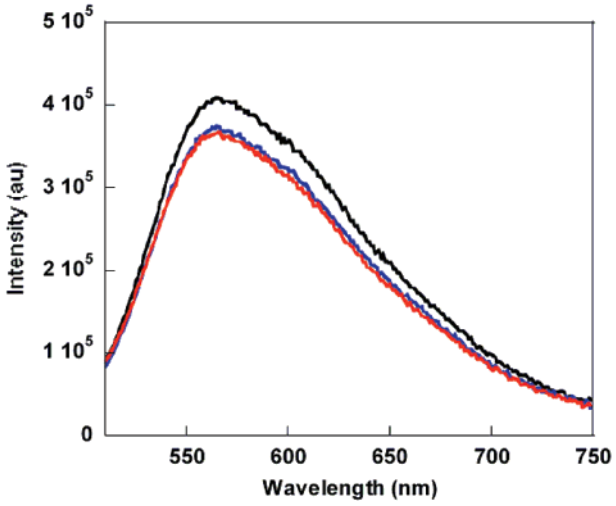


Figure 7. Fluorescence spectra (λ_{ex} = 500 nm) in 0.1 M $(NH_4)_2CO_3$ at pH 8.9: **Li$^+$-MPS-PPV** ([RU] = 1.7×10^{-5} M) (black line); **Li$^+$-MPS-PPV** and [**mMV$^+$**] = 2.0×10^{-6} M (blue line); **Li$^+$-MPS-PPV**, **mMV$^+$**, and [avidin] = 3.0×10^{-7} (red line).

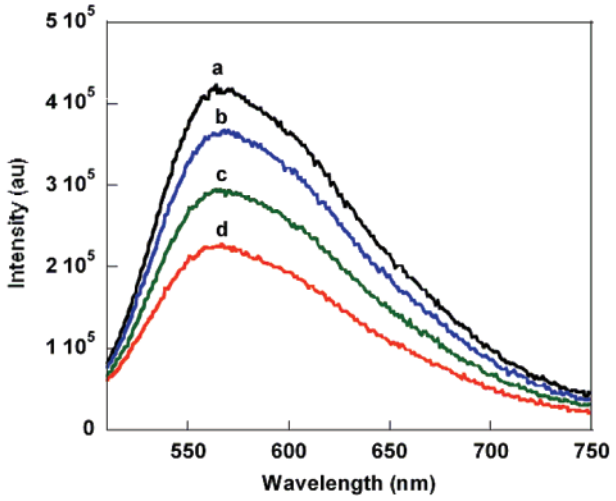
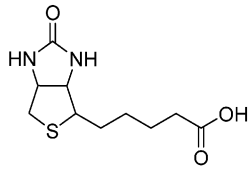


Figure 8. Fluorescence spectra (λ_{ex} = 500 nm) in 0.1 M $(NH_4)_2CO_3$ at pH 8.9: (a) **Li$^+$-MPS-PPV** ([RU] = 1.7×10^{-5} M) (black line); (b) **Li$^+$-MPS-PPV** and [**BPP$^+$**] = 2.0×10^{-6} M (blue line); (c) **Li$^+$-MPS-PPV**, **BPP$^+$**, and [avidin] = 1.0×10^{-7} M avidin (green line); (d) **Li$^+$-MPS-PPV**, **BPP$^+$**, and [avidin] = 3.0×10^{-7} M avidin (red line).

One possible explanation for the enhanced fluorescence quenching of avidin when using **BPP$^+$** in buffer is shown in Scheme 3. These conditions make the biotin−avidin interaction favorable, leading to binding of the biotinylated quencher. Avidin, which possesses a pI of 10, will have an overall net positive charge at pH 8.9 and complexes with the anionic polymer by electrostatic interactions.[50] Encapsulation of the entire **BPP$^+$** molecule by the protein is not complete, with the biotin group (B in Scheme 1) inside the protein[51,52] and the cationic electron acceptor portion (Q in Scheme 1) extending toward the protein surface. It appears that Q is either outside the protein or sufficiently close to satisfy the distance requirement for quenching the conjugated polymer. This complex is more able to compensate for the electrostatic screening of the buffer ions than **BPP$^+$** alone. Under these conditions, Q is more strongly bound to the CP ($d_1 > d_2$), and more effective fluorescence quenching takes place.

Scheme 4. The Structure of D-Biotin



We note that there is precedence for the self-quenching of fluorophores attached to biotin via a linker of similar dimensions to that in **BPP$^+$** upon binding to avidin.[3,53] These studies indicate that there is substantial optical coupling between dyes, despite the dimensions of the biotin/avidin complex.[51] It is therefore reasonable to expect photoinduced charge-transfer fluorescence quenching between an anionic CP and the **BPP$^+$** on the surface of the **BPP$^+$**/avidin complex.

A subsequent set of tests probed the mechanism in Scheme 3. The experiments involve blocking biotin binding sites on avidin by pretreating the protein with its natural ligand, D-biotin (Scheme 4). Under these conditions, **BPP$^+$** cannot complex to avidin, and, according to Scheme 3, one would not expect to see additional fluorescence quenching when the blocked protein is added to **Li$^+$-MPS-PPV**/**BPP$^+$**.

The D-biotin stoichiometry can be varied to create solutions of avidin with zero to four vacant binding sites. For example, pretreating a 1.0×10^{-5} M avidin solution with 2.0×10^{-5} M D-biotin leaves, on average, two biotin binding sites per avidin. The emission from solutions of **Li$^+$-MPS-PPV** ([RU] = 1.7×10^{-5} M in 0.1 M $(NH_4)_2CO_3$ at pH 8.9) was partially quenched

(50) Strong electrostatic interactions between avidin and negatively charged quantum dots have been reported by others. See: Goldman, E. R.; Balighian, E. D.; Mattoussi, H.; Kuno, M. K.; Mauro, J. M.; Tran, P. T.; Anderson, G. P. *J. Am. Chem. Soc.* **2002**, *124*, 6378−6382.
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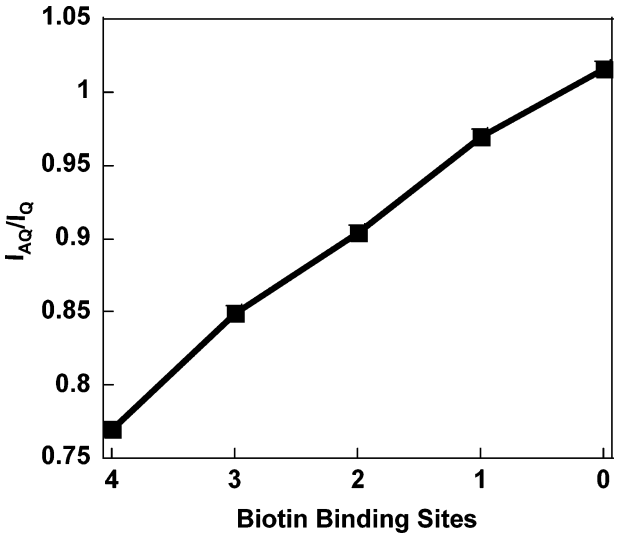

Figure 9. Emission intensity ratio (I_{AQ}/I_Q) for solutions of **Li$^+$-MPS-PPV** ([RU] = 1.7×10^{-5} M) and **BPP$^+$** (2.0×10^{-6} M) without avidin (I_Q) and with avidin (I_{AQ}) as a function of the average number of biotin binding sites on avidin ([avidin] = 2.0×10^{-7} M, 0.1 M $(NH_4)_2CO_3$ at pH 8.9, λ_{ex} = 500 nm).

by **BPP$^+$** (2.0×10^{-6} M) prior to addition of pretreated avidin solutions. The emission intensities of these solutions (I_Q) were then compared to the intensities observed after addition of the pretreated avidin (I_{AQ}, [avidin] = 2.0×10^{-7} M) as a function of the number of available binding sites (Figure 9, emission spectra can be found in the Supporting Information).

When all of the binding sites on avidin are empty (four biotin binding sites), the fluorescence quenching is similar to that observed in Figure 8 ($I_{AQ}/I_Q = 0.77$). Conversely, when all sites are blocked, no **BPP$^+$** can bind to the avidin, and the emission intensity of the **Li$^+$-MPS-PPV**/**BPP$^+$** solution remains unperturbed ($I_{AQ}/I_Q \approx 1$). It is important to recall that solutions that contain no specific biotin−avidin (or **BPP$^+$**-avidin) recognition (Figures 6 and 7) also show no perturbation of the emission upon avidin addition. Intermediate levels of avidin blocking result in intermediate degrees of additional quenching. For example, at 50% blocking, one observes roughly one-half of the quenching enhancement observed when unblocked avidin is used. The data in Figure 9 indicate that specific interactions of **BPP$^+$** and avidin lead to the formation of ground-state complexes that are more effective at quenching and are consistent with the mechanism in Scheme 3.

Effect of Nonspecific Proteins on the Fluorescence Quenching of M$^+$-MPS-PPV by BPP$^+$. In a realistic analysis, a given sample may contain nontarget species that should not perturb the diagnostic signals of the sensory mechanism. Given that avidin was able to nonspecifically perturb the fluorescence quenching by **BPP$^+$** and **mMV$^+$**, it was of interest to examine proteins without biotin binding sites. For the purpose of the study, we chose BSA,[54,55] pepsin A,[56,57] and the microtubule associated protein tau.[58,59] Properties of immediate interest for this study are summarized in Table 2. In both water and 0.1 M ammonium carbonate buffer (pH 8.9), BSA and pepsin-A are negatively charged, while tau is positively charged.

Table 2. Molecular Weight and pI of Proteins Used in the Nonspecific Recovery of Fluorescence Quenching by **BPP$^+$**

protein	MW (kD)	pI
avidin	66	10−10.5
BSA	66	4.7
pepsin A	35	3.3
tau	42	10

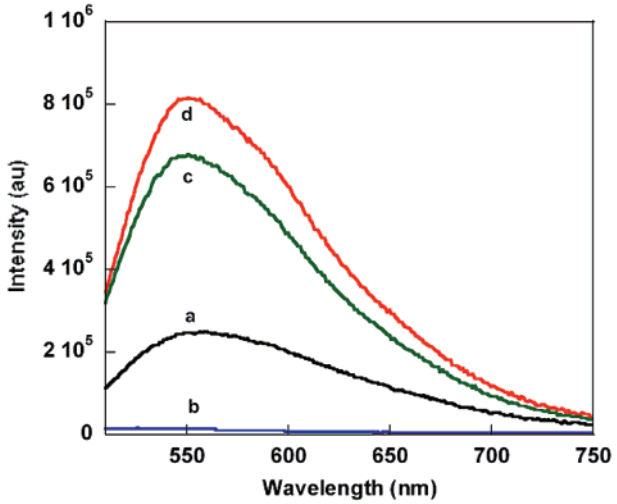

Figure 10. Fluorescence spectra (λ_{ex} = 500 nm) in water: (a) **Li$^+$-MPS-PPV** ([RU] = 1.7×10^{-5} M) (black line); (b) **Li$^+$-MPS-PPV** and [**BPP$^+$**] = 2.0×10^{-6} M (blue line); (c) **Li$^+$-MPS-PPV**, **BPP$^+$**, and [BSA] = 3.0×10^{-7} M (green line); (d) **Li$^+$-MPS-PPV** and [BSA] = 3.0×10^{-7} M (red line).

The addition of all three nonspecific proteins leads to substantial increases in the emission intensity of **Li$^+$-MPS-PPV** solutions in water ([RU] = 1.7×10^{-5} M). A ∼2-fold enhancement was observed for pepsin-A and tau (3.0×10^{-7} and 1.0×10^{-7} M, respectively), while a ∼3-fold enhancement was seen for BSA (3.0×10^{-7} M) (Figure 10, a and d). These proteins can also nonspecifically re-establish (or over-establish) the polymer emission in the presence of **BPP$^+$** or **mMV$^+$** (2.0×10^{-6} M). For example, in solutions of **Li$^+$-MPS-PPV** ([RU] = 1.7×10^{-5} M) and **BPP$^+$**, both BSA (Figure 10) and tau (Supporting Information) resulted in more than a 2-fold over-recovery in signal relative to the original polymer emission intensity. The addition of pepsin-A to a quenched polymer solution also displayed nonspecific recovery of the emission but to a lesser extent than tau or BSA (∼25%, Supporting Information). These results are in accord with the observations made using avidin. Furthermore, none of these proteins have the capacity to specifically bind the biotinylated quencher, and thus the recovery seen from the polymer/**BPP$^+$** solutions can only be explained by nonspecific interactions between the charged macromolecules.

Perturbations in buffer (0.1 M ammonium carbonate, pH 8.9) by the two anionic, nontarget proteins are severely attenuated. The addition of pepsin-A to **Li$^+$-MPS-PPV** (same concentrations as above) lowers the original emission intensity by only

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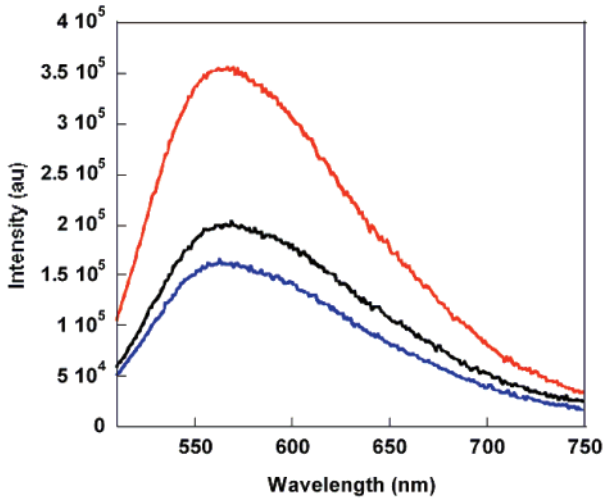


Figure 11. Fluorescence spectra (λ_{ex} = 500 nm) in 0.1 M $(NH_4)_2CO_3$ at pH 8.9: **Li$^+$-MPS-PPV** ([RU] = 1.7 × 10^{-5} M) (black line); **Li$^+$-MPS-PPV** and [**BPP$^+$**] = 2.0 × 10^{-6} M (blue line); **Li$^+$-MPS-PPV**, **BPP$^+$**, and [tau] = 1.0 × 10^{-7} M (red line).

~5%, while addition to quenched polymer solutions (**BPP$^+$** or **mMV$^+$**) results in no notable change in fluorescence intensity. Similarly, addition of BSA to the polymer gives rise to an increase of emission intensity of only ~7%, while only ~12% of the quenched emission was recovered in solutions containing **BPP$^+$** or **mMV$^+$** (data shown in Supporting Information).

Addition of the cationic tau in buffer (0.1 M ammonium carbonate, pH 8.9) provides a response different from those observed with the anionic BSA and pepsin-A. Tau and avidin have comparable charges and molecular weights. The addition of tau (1.0 × 10^{-7} M) in buffer yielded results similar to those in water for solutions of **Li$^+$-MPS-PPV** ([RU] = 1.7 × 10^{-5} M), where ~2-fold increases in the polymer emission intensity were observed (Supporting Information). Tau also brings the emission intensity of a polymer/**BPP$^+$** solution to a level comparable to that of a solution containing only tau and **Li$^+$-MPS-PPV**, again without any specific recognition of the biotin fragment (Figure 11). Similar behavior is seen when **mMV$^+$** is the quencher. A decrease in the emission of the polymer in the presence of **BPP$^+$** by addition of protein is therefore only observed with avidin (Figure 8 and Scheme 3).

Conclusion and Summary Discussion

A subtle interplay between steric bulk, overall molecular size, hydrophobic content, and charged groups within the structure of the quencher molecules promotes differences in fluorescence quenching abilities. In water, **mMV$^+$** quenches **Li$^+$-** and **Na$^+$-MPS-PPV** more efficiently than **BPP$^+$**; for **5R5$^-$**, **BPP$^+$** is the better quencher. The exact reason for this trend is difficult to explain, mainly because the dimensions and overall geometries in which the polymers and oligomers associate and/or aggregate in water are not available. Our current thinking is that the smaller size of **mMV$^+$** allows for more intimate contact with the polymer structures and makes it a more effective quencher. The surfactant-like structure of **BPP$^+$** may break up the large **5R5$^-$** aggregates and allows better donor/acceptor complexation.

Mixing of avidin and the emitting conjugated structures in water results in an unambiguous increase of fluorescence intensity. This result is consistent with substantial previous work that explored basic protein/CP interactions. The large and positively charged avidin structure elongates and separates polymer chains. In the case of **5R5$^-$**, the protein breaks up aggregates and increases the average interchromophore distance. For both types of molecules, the net result is a decrease in self-quenching. The appearance of vibronic structure in the fluorescence spectra of **5R5$^-$**, characteristic of "monomer-like" emission,[60] further supports this claim (Figure 2).

In water, the electrostatic binding of the singly charged quenchers to the anionic polymers is overcome by the interactions with avidin. When using **BPP$^+$**, addition of a stoichiometric amount of avidin ([biotin] = [biotin binding-sites] = $^1/_4$[avidin]) "recovers" the emission to an intensity similar to that of the unperturbed sample. At first sight, the system appears to behave with specificity. Further addition of avidin "over-recovers" emission to an intensity roughly 150% that of the unquenched sample. The analogous negative control experiments with **mMV$^+$** show similar behavior. Therefore, the resemblance of the emission intensity from a CP-stoichiometric biotin/avidin solution to the emission intensity of the unperturbed CP solution is coincidental.

Buffer conditions that improve avidin/biotin complexation lead to electrostatic screening. Reduced Coulombic attraction results in K_{sv} values 2−3 orders of magnitude lower than those obtained in water. Perturbations by avidin on the CP emission are also severely attenuated under buffer conditions. Avidin does not influence quenching by **mMV$^+$**. However, when the polymer is quenched by **BPP$^+$**, addition of avidin causes additional fluorescence quenching. These data form the basis for Scheme 3, where avidin−biotin binding yields a complex with a larger net positive charge and therefore a larger association constant with **M$^+$-MPS-PPV**. Additionally, we suspect that hydrophobic interactions between the polymer backbone and the protein may also enhance association. Within the avidin/**BPP$^+$** complex, encapsulation is not complete, and the acceptor unit of **BPP$^+$** is exposed and capable of quenching polymer emission.

Proteins with no biotin binding sites provide supporting details. In water, BSA, pepsin-A, and tau increase substantially the polymer emission and recover the quenching by **BPP$^+$** or **mMV$^+$**. The fluorescence recovery cannot be accounted for by specific binding of quenchers and is best accommodated under a scenario where the quencher/chromophore ground-state complex is broken up by the proteins. The end result is displacement of the quencher from the proximity of the conjugated framework. None of the nontarget proteins in a buffer medium was able to increase the quenching by **BPP$^+$** in a manner similar to that displayed by avidin in Figure 8.

Overall, the work described in this paper is inconsistent with the mechanism in Scheme 1 and is better described by that shown in Scheme 3, where complexation of **BPP$^+$** by avidin ultimately results in more effective fluorescence quenching.

Experimental Section

General Comments. The concentration at which protein addition induces no further fluorescence change is determined as follows. As an example, Figure 1 shows that saturation occurs at an avidin concentration of 3.0 × 10^{-7} M. Avidin is added in 1.0 × 10^{-7} M

(60) (a) Bazan, G. C.; Oldham, W. J.; Lachicotte, R. J.; Tretiak, S.; Chernyak, V.; Mukamel, S. *J. Am. Chem. Soc.* **1998**, *120*, 9188−9204. (b) Renak, M. L.; Bartholomew, G. P.; Wang, S.; Ricatto, P. J.; Lachicotte, R. J.; Bazan, G. C. *J. Am. Chem. Soc.* **1999**, *121*, 7787−7799. (c) Wang, S.; Bazan, G. C.; Tretiak, S.; Mukamel, S. *J. Am. Chem. Soc.* **2000**, *122*, 1289−1297.

increments, and an emission spectrum is taken after each addition. In the case of Figure 1, each addition caused an increase in emission intensity. Avidin additions exceeding 3.0×10^{-7} M do not induce any significant changes in fluorescence, and the saturation point is reached.

Polymer and **5R5**$^-$ concentrations were calculated on the basis of repeat units, that is, moles of repeat units/liter. For **M**$^+$**-MPS-PPV**, one repeat unit is as shown in Scheme 1. For **5R5**$^-$, the formal concentration of the oligomer solution is multiplied by a factor of 5, corresponding to 5 phenyl ring repeat units. This normalization of chromophore concentration allows for a comparison of chromophore numbers on a "ring-by-ring" or charge basis.

When determining Stern−Volmer constants, the fluorescence of a chromophore in the presence of a quencher at a given concentration is compared to the fluorescence of the chromophore in the absence of the quencher. For this comparison to be meaningful, it is assumed that the number of photons absorbed in the presence or absence of quencher is the same. When **mMV**$^+$ or **BPP**$^+$ is added, small changes (1−4%) in the absorbance spectra are observed at the excitation wavelengths.[61] For the above assumption to be valid, a correction for the increase or decrease in absorption is made. A normalization of fluorescence intensity can be made by manipulating the expression

$$A = \log(P_o/P)$$

where A is equal to absorbance of a sample, P_o is equal to the radiant power entering the sample, and P equals the radiant power emerging from the sample. An expression for the radiant power absorbed by the sample is given by

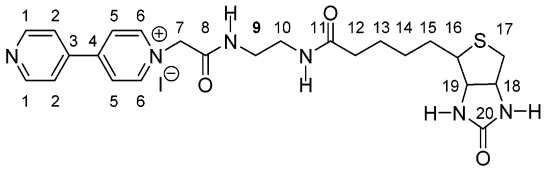
$$P_o(1 - 10^{-A}) = P_o - P$$

A ratio of the $1-10^{-A}$ terms in the absence (A_0) and presence (A) of quencher gives a correction factor to adjust F (fluorescence intensity at a given quencher concentration). The expression describing the correction of F is given by

$$F_{corrected} = F(1 - 10^{-A_o}/1 - 10^{-A})$$

Corrected K_{sv} values are obtained from the slopes of the linear regions of plots $F_o/F_{corrected}$ versus [quencher]. K_{SV} values obtained from raw or corrected data did not deviate significantly (<15%). Fluorescence measurements were performed in quartz cuvettes with [RU] = 1.7×10^{-5} M. To 3 mL of chromophore diluted in water (or in buffer) were added small aliquots of concentrated quencher solutions followed by mixing. The absorbance at the chromophore excitation wavelength was recorded after each addition of quencher followed by a fluorescence measurement. Fluorescence and absorbance measurements were performed on a PTI Quantum Master fluorometer and a Shimatzu spectrophotometer, respectively. [*N*-(Biotinoyl)-*N*′-(iodoacetyl)] ethylenediamine and avidin were purchased from Molecular Probes, Inc.

[*N*-(Biotinoyl)-*N*′-(acetyl 4,4′-pyridylpyridinium iodide)] Ethylenediamine (BPP$^+$). [*N*-(Biotinoyl)-*N*′-(iodoacetyl)] ethylenediamine (13.0 mg, 0.0286 mmol) and an excess of 4,4′-bipyridine were dissolved in DMSO-d_6 in an NMR tube. The 400 MHz ^{1}H NMR spectrum was monitored until the singlet arising from the iodoacetyl protons (3.61 ppm) disappeared (overnight). The reaction mixture was poured into a glass centrifuge tube, and the solvent was removed under vacuum. The crude product was dissolved in a minimal amount of DMF and was added dropwise into a glass centrifuge tube containing CH_2Cl_2 under vigorous stirring. The mixture was allowed to stir for 15 min, and the solids were obtained by action of a centrifuge. The precipitation procedure was repeated until the 4,4′-bipyridine was no longer visible on a reverse phase TLC plate (4,4′-bipyridine rf = ∼0.4, **BPP**$^+$ rf = ∼0.2 in 55% acetonitrile:45% water). Removal of the bipyridine was confirmed by ^{1}H NMR spectroscopy. The purified product was dried under vacuum to give 6.3 mg of a dark yellow solid (49%). ^{1}H NMR 400 MHz (DMSO-d_6, RT, ppm): 9.1 (d, 3H), 8.7 (d, 2H), 8.5 (d, overlapping peaks, 3H), 7.8 (m, 1H), 6.43 (s, 1H), 6.39 (s, 1H), 5.4 (s, 2H), 4.3 (m, 1H), 4.1 (m, 1H), 3.1 and 3.05 (2 multiplets, overlapping peaks, 6H), 2.75 (dd, 1H), 2.0 (t, 2H), 1.5 and 1.6 (2 multiplets, overlapping peaks, 4H), 1.3 (m, 2H). ^{13}C NMR 125 MHz (DMSO-d_6, RT, ppm) (see structure below for carbon assignments): 172.4, 164.4, 162.7 (C_8, C_{11}, and C_{20}), 152.9 (C_1), 151.0 (C_2), 146.8 (C_3), 140.8 (C_4), 124.8 (C_5), 122.0 (C_6), 61.2, 61.0 (C_7 and C_{19}), 59.2 (C_{18}), 55.4 (C_{16}), 41.2, 41.1 (C_9 and C_{10}, peaks observed only in MeOD-d_4), 37.9 (C_{12}), 35.3 (C_{17}), 28.2, 28.1 (C_{14} and C_{13}), 25.2 (C_{15}). MS (ESI-positive): (M − I$^-$), 483.3. MS (ESI-negative): (I$^-$), 126.9.



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Supporting Information Available: Additional fluorescence spectra obtained with the conjugated polymers, quenchers, and different proteins under different conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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