Highly sensitive biological and chemical sensors based on reversible fluorescence quenching in a conjugated polymer

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Communicated by Esther M. Conwell, University of Rochester, Rochester, NY, June 11, 1999 (received for review March 12, 1999)

The fluorescence of a polyanionic conjugated polymer can be quenched by extremely low concentrations of cationic electron acceptors in aqueous solutions. We report a greater than million-fold amplification of the sensitivity to fluorescence quenching compared with corresponding ''molecular excited states.'' Using a combination of steady-state and ultrafast spectroscopy, we have established that the dramatic quenching results from weak complex formation [polymer$^{(-)}$/quencher$^{(+)}$], followed by ultrafast electron transfer from excitations on the entire polymer chain to the quencher, with a time constant of 650 fs. Because of the weak complex formation, the quenching can be selectively reversed by using a quencher-recognition diad. We have constructed such a diad and demonstrate that the fluorescence is fully recovered on binding between the recognition site and a specific analyte protein. In both solutions and thin films, this reversible fluorescence quenching provides the basis for a new class of highly sensitive biological and chemical sensors.

With the rising awareness of the public vulnerability to chemical and biological terrorism, there is a heightened need for detection techniques that show both high sensitivity and selectivity. Such techniques also would find wide use in medical diagnostics and biomedical research applications. Methods of identifying biological molecules such as the enzyme-linked immunosorbant assay (ELISA) achieve selectivity by using specific antibody/antigen interactions to anchor the antigen to a substrate, with a subsequent colorimetric change or fluorescence signal on addition of secondary reagents; these techniques can be time-consuming and require multistep procedures. Other approaches have used molecular recognition ligands to link to specific receptor sites on a biological species, usually as a means also of fixing the biomolecule to a substrate or membrane (1–6) It has remained a challenge to incorporate the selectivity offered by ligand/receptor interactions into a sensor that can be extremely sensitive, robust, and versatile.

We have recently explored the photophysical properties of a fluorescent, water-soluble polyanionic conjugated polymer [poly (2-methoxy-5-propyloxy sulfonate phenylene vinylene (MPS-PPV)] (Fig. 1*B*), one of a larger class of related molecules [poly phenylene vinylene (PPV)] (Fig. 1*A* and derivatives) that has been the subject of almost explosive recent interest (7–13). Although much attention has focused on the well known potential for use of PPV derivatives as electronic materials [e.g., electrochemical sensors (14–16) light-emitting diodes (17, 18), and integrated circuits (19, 20)], the highly charged backbone of MPS-PPV (with charge density approximating that of polynucleic acids such as DNA and RNA), also makes it a model polymer for understanding the interactions and self-assembly properties of charged biopolymers. In this paper, we report a striking discovery: the use of this fluorescent anionic polymer leads to a greater than million-fold amplification of the sensitivity to fluorescence quenching, relative to that of corresponding small conjugated molecules with similar structure. The amplification is attributed to a combination of delocalization of the electronic excited state (exciton) and ultrafast exciton mobility along the conjugated polymer chain. We have harnessed this amplification to demonstrate a versatile new class of highly sensitive (and selective) biological and chemical sensors.

MPS-PPV is a water-soluble polymer, with molecular weight estimated from light scattering measurements to be 1–5 $\times$ 10^5 ($\approx$1,000 monomer repeat units). The absorption and fluorescence spectra of MPS-PPV in dilute aqueous solution are similar to those of trans-stilbene and its derivatives, but shifted to longer wavelength because of the extended conjugation in the polymer. It is well established that excited states of trans-stilbene and related molecules are readily quenched by electron-deficient aromatic compounds in both dynamic and static processes (21–24).‖ For example, the fluorescence of trans-stilbene derivatives can be quenched by *N*, *N*′-dimethyl-4,4′-bipyridinium (MV^{2+}) (methyl viologen) (Fig. 1*F*) by formation of relatively weak ground-state ''donor–acceptor'' complexes (21–23). The quenching follows a conventional ''Stern-Volmer'' relationship:

$$\phi^o/\phi = 1 + K_{SV}[MV^{2+}] \quad [1]$$

where ϕ^o and ϕ are the quantum efficiencies (or intensities) of fluorescence in the absence and presence of MV^{2+}, respectively, and $[MV^{2+}]$ is the MV^{2+} concentration. The constant K_{sv} thus provides a direct measure of the quenching sensitivity. Although the quenching of trans-stilbene by MV^{2+} in homogeneous solution can only be observed at relatively high concentrations of MV^{2+} (K_{SV} = 15) (Fig. 2*A*), it is much more easily detectable when trans-stilbene or its amphiphilic derivatives are incorporated into anionic assemblies such as micelles or bilayer vesicles (21–24). The amplification in quenching sensitivity from solution to anionic detergent (sodium lauryl sulfate) micelles (K_{SV} = 2 $\times$ 10^3) (Fig. 2*A*) can be readily attributed to a ''concentration enhancement'' effect in which the stilbene and viologen are assembled by a combination of coulombic and entropic interactions in a microphase such that their ''local'' concentrations are greatly enhanced (21–23). Given the net negative charge on MPS-PPV, we anticipated that it might readily bind MV^{2+} in aqueous solution and lead to significant fluorescence quenching

Abbreviations: MPS-PPV, poly (2-methoxy-5-propyloxy sulfonate phenylene vinylene; MV^{2+}, (methyl viologen) *N*, *N*′-dimethyl-4,4′-bipyridinium; PPV, poly phenylene vinylene; B-MV, biotin-methyl viologen; MV^{+}, (mono methyl viologen) *N*-methyl,4,4′-pyridylpyridinium; TA, transient absorption; SE, stimulated emission; PA, photoinduced absorption.

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‖''Dynamic'' refers to interactions that take place because of diffusion of the fluorophore and quencher species within the radiative lifetime whereas ''static'' refers to a bound fluorophore-quencher complex. In stilbene-based chromophores and polymers, the short (nanosecond) radiative lifetime leads to a vanishing contribution from dynamic quenching except at extremely high concentrations. For the purposes of our study, these can be neglected.

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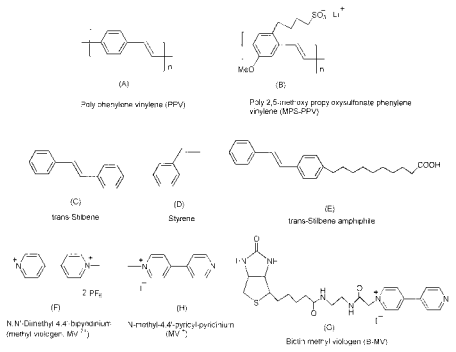

Fig. 1. Chemical structures for the molecules in this study.

at moderate viologen concentrations (Fig. 2*A*). Remarkably, we find that, in dilute solutions of MPS-PPV (10^{-5} M in monomer repeat units), addition of very low concentrations of MV^{2+} leads to noticeable changes in the MPS-PPV absorption spectrum and to a dramatic quenching of its fluorescence (Figs. 3 and 4). The corresponding quenching constant (K_{SV}) is 1.7×10^7, or nearly four orders of magnitude greater than that for stilbene in micelles and six orders of magnitude greater than that for dilute stilbene solutions. As shown by the Stern-Volmer plot in Fig. 4, quenching is nearly (95%) quantitative at 10^{-7} M viologen and is readily detectable at concentrations of 10^{-9} M. Under these conditions, one molecule of MV^{2+} is effectively quenching at a level of ≈1,000 repeat units, or approximately one MV^{2+} molecule per polymer chain! Other quenchers of the "molecular" excited state of trans-stilbene (21–23) are also effective at quenching the fluorescence of MPS-PPV.

The remarkably low levels of viologen and other reagents that are effective in quenching the fluorescence for MPS-PPV may be attributed to several phenomena not generally encountered for molecular excited states or even excitonic states of aggregates. To gain an understanding of the mechanism for this dramatic quenching, the system was studied by using femtosecond transient absorption (TA). The experimental setup used for the TA measurements has been described in detail elsewhere (25). The samples were photoexcited at 3.1 eV, within the π-π^* absorption band of the MPS-PPV polymer. Cross-correlation measurements between the pump and the probe using two-photon absorption in a sapphire plate showed a system resolution time of 150 fs over the entire spectral range studied (25). As a measure of transmission changes, we use the differential transmission (DT), defined as $DT = (T - T_0)/T_0 = DT/T_0$, where T_0 and T are the transmission of the probe beam in the presence and absence of the pump, respectively. In our data, we plot the pump-induced absorption change ($\Delta\alpha$), which is related to DT by the expression $\Delta\alpha = -1/d \ln(1 + DT)$, where d is the sample thickness.

It is well known that the relatively large energy difference between absorption and emission leads to efficient population inversion and lasing in PPV derivatives (26–28); the corresponding stimulated emission (SE) leads to a positive DT signal, and this signal provides a dynamic measure of the exciton population

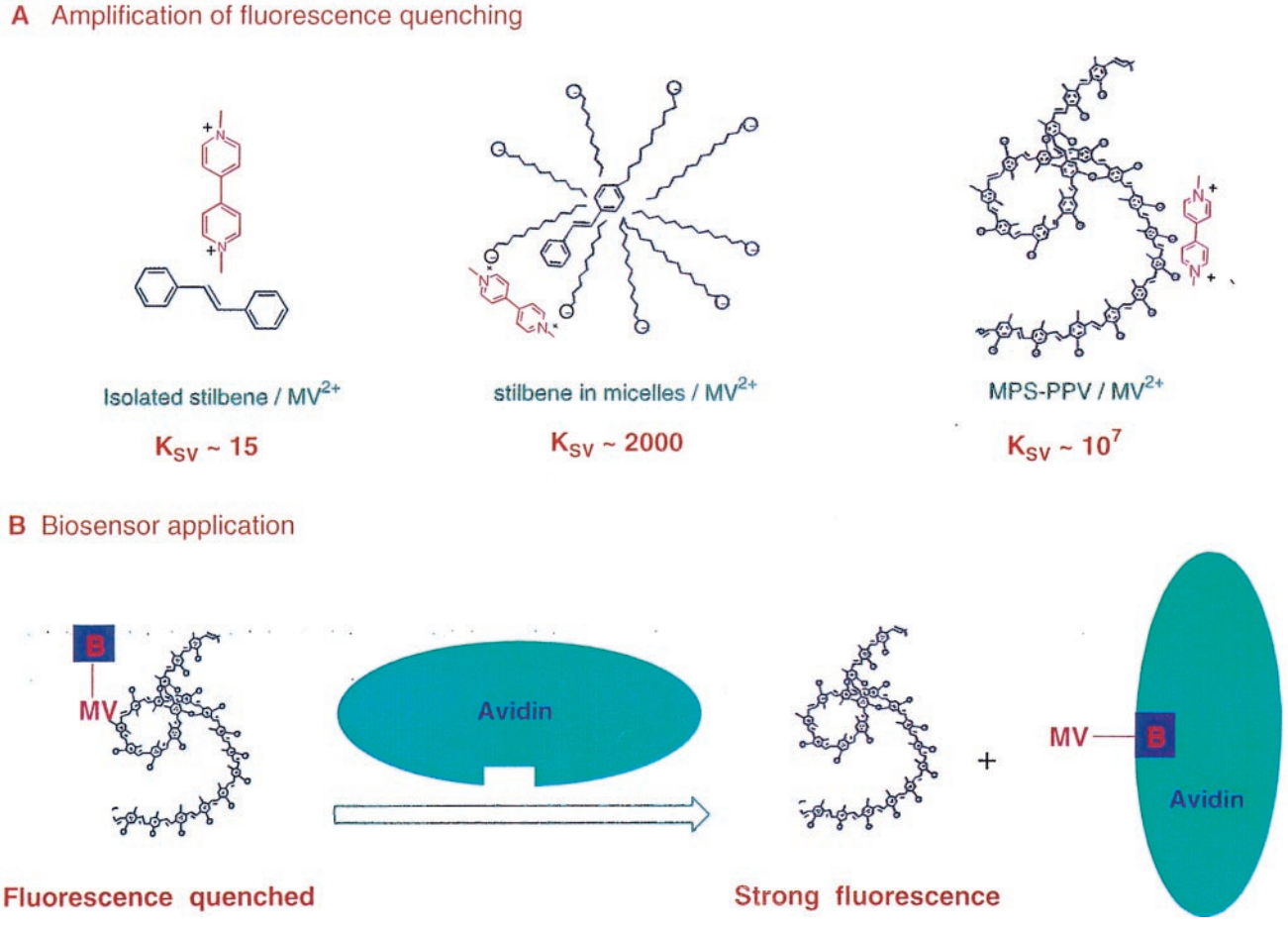

Fig. 2. (*A*) Amplification of fluorescence quenching sensitivity. (*B*) Biosensor application.

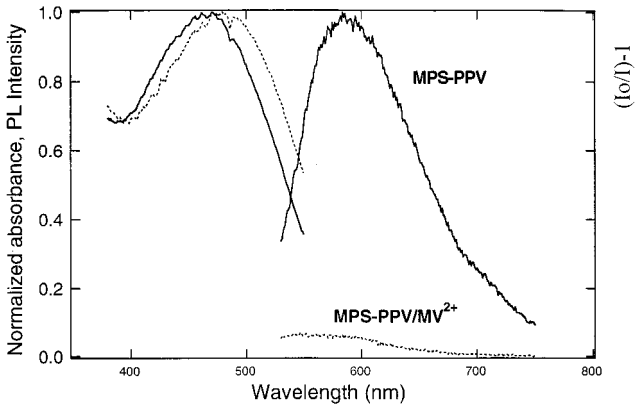

Fig. 3. Absorption and fluorescence spectra (excited at 500 nm) of MPS-PPV (1.7×10^{-5} M in monomer repeat units) in water in the presence (dotted line) and absence (solid line) of MV^{2+} (1×10^{-7} M).

(29–30). It is also known that aggregation of polymer chains in solution and films leads to quenching of excitons by formation of nonemissive interchain excited states (excimers or interchain excitons) (31). The consequences of these processes on the TA dynamics in neat MPS-PPV solutions (1.5×10^{-3} M) are illustrated in Fig. 5*A*. The inset to Fig. 5*A* shows the TA spectrum from 450–750 nm, showing both SE (positive $\Delta T/T_0$) and photoinduced absorption (PA) (negative $\Delta T/T_0$) bands, attributable to the photoinduced transition of the exciton back to the ground-state, or to a higher excited-state, respectively. In the first 2 ps, the spectrum decays, with an apparent blue shift attributable to the formation of secondary interchain excited-states, with a competing PA (see the difference spectrum in the inset to Fig. 5*A*). The temporal evolution of excitons to interchain excited-states (excimers) can be directly monitored by comparing the dynamics near the peak of the SE (500 nm) and near the zero crossing of the TA spectrum (600 nm, where the exciton cross-section is nearly zero, but the interchain state has a finite PA) as shown in Fig. 5*A*; the initial decay of the SE (exciton) has a time constant of 1.5 ps, and there is a complementary growth of the excimer population with the same time constant. Hence, aggregation of MPS-PPV at these relatively

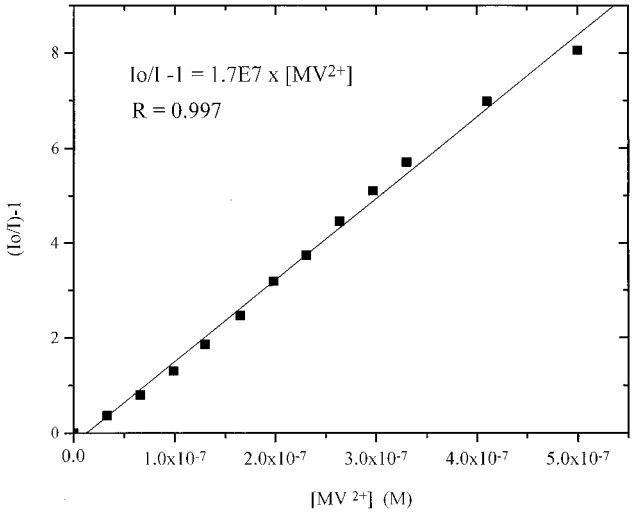

Fig. 4. Stern-Volmer plot for quenching of the fluorescence of 1.2×10^{-5} M (repeat units) MPS-PPV by MV^{2+} in aqueous solution.

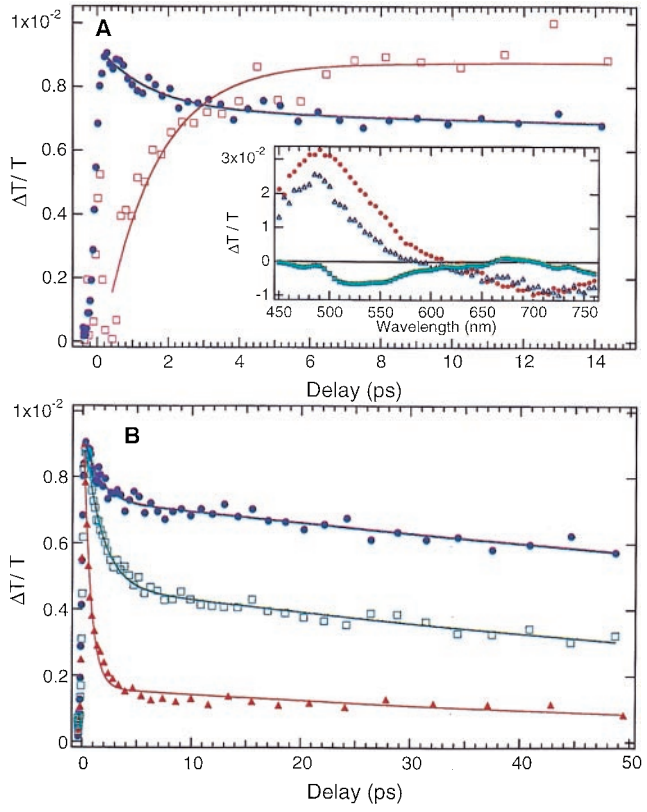

Fig. 5. (*A Inset*) TA spectra in 1.5×10^{-3} M MPS-PPV solution at zero time delay (circles) and 2-ps delay (triangles), showing SE peak (500 nm) and PA peak (720 nm), together with difference spectra showing secondary PA peak caused by excimer formation in the aggregated polymer. (*A*) SE decay (circles) and excimer PA growth (squares) showing complementary dynamics and 1.5-ps decay/growth time. (*B*)SE decay in 5×10^{-4} M MPS-PPV solution before (circles) and after addition of 1×10^{-5} M MV^{2+}, with equilibration of solution (squares) and after agitation (triangles).

high concentrations provides a direct quenching mechanism in neat MPS-PPV solutions. Fig. 5*B* compares the decay of the SE (500 nm probe) in a 5×10^{-4} M MPS-PPV solution, with that for the same solution with addition of 10^{-5}M MV^{2+}. When the MPS-PPV/MV solution is allowed to equilibrate for several minutes, the dynamics show an increase of the 1.5-ps decay component, with no change in the initial 1.5-ps lifetime. This indicates that the dicationic MV^{2+} is promoting additional aggregation of the relatively concentrated MPS-PPV solutions. Interestingly, agitation of the solution (either by gentle shaking or sonication) leads to a dramatic increase in both the magnitude and the rate of SE quenching, with a time constant of 650 fs. The evolution between these two types of dynamics is fully reversible. This dramatic change in the ultrafast exciton decay points to two competing quenching mechanisms: aggregation quenching caused by formation of interchain states and electron-transfer quenching caused by the MPS-PPV/MV^{2+} complex. The addition of divalent cations to anionic polyelectrolytes is known to lead to aggregation (32), and, hence, the MV^{2+} intrinsically plays a dual role. The fact that other non-electron-deficient divalent cations such as Ca^{2+} and Mg^{2+} (which do not quench stilbene) also quench the MPS-PPV emission, but not as efficiently as MV^{2+} (K_{SV} for Ca^{2+} is 10^4), supports this picture of competition between aggregation and electron-transfer quenching. It is also significant that addition of monovalent cations (K^+ and Na^+) (which do not promote aggregation) had a negligible quenching effect. It is important to note that, at MPS-PPV/MV concentrations used in Fig. 3 and elsewhere in this study, no changes in

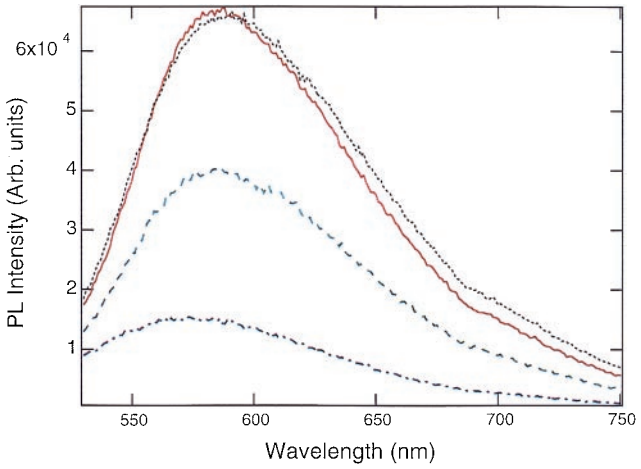

Fig. 6. Fluorescence spectra from aqueous solution of MPS-PPV (1.7×10^{-5} M in monomer repeat units) excited at 500 nm in water alone (solid line), after addition of 2×10^{-6} M B-MV (dash-dot line), after addition of 1×10^{-7} M avidin (dash line), and after addition of 2×10^{-7} M avidin (dot line).

the quantitative quenching were observed with time, and agitation was not necessary to achieve efficient quenching. Hence, the aggregation quenching likely plays a minor role for MV^{2+} at low concentration.

Because the ground state binding of trans-stilbene and even negatively charged assemblies containing trans-stilbene derivatives by MV^{2+} is relatively weak (21–23), it was interesting to determine whether the highly effective fluorescence quenching observed in the presence of viologen and other cationic reagents could be reversed. An attractive possibility involves the synthesis and use of a molecule in which a viologen-type quencher and a second recognition unit were combined, separated by a relatively short "tether." Accordingly, we prepared biotin-methyl viologen (B-MV) (Fig. 1*G*), which combines a viologen unit linked to a biotin molecule by a short but flexible tether. Biotin is an excellent receptor for proteins such as avidin and streptavidin, but it was not expected to react with MPS-PPV (33–35). The complex between biotin and avidin is extremely strong ($K \approx 10^{15}$ M) (36), and, consequently, the binding is expected to be rapid and effectively irreversible. The avidin-biotin complexation has been very well studied (and used in several biomedical diagnostic assays) (37); avidin contains four biotin binding sites, and the protein has a molecular weight of ≈64,000 (38). Consequently, it was anticipated that, in the absence of receptor protein (avidin), the small biotin group in B-MV would not hinder association of the viologen portion of B-MV with MPS-PPV and that its addition to solutions of MPS-PPV would result in strong fluorescence quenching. Because the protein is a much larger molecule than either biotin or MPS-PPV, and because protein-biotin complexation should be much stronger than that for the polymer-viologen combination (33–35, 38), we anticipated that addition of protein to these "quenched" solutions might reverse the quenching (Fig. 2*B*).

Indeed, as shown in Fig. 6, addition of B-MV to solutions of MPS-PPV results in quenching of its fluorescence. B-MV is somewhat less effective as a quencher than MV^{2+}, which is reasonable attributable to its lower positive charge; its quenching is quite comparable to that of the "mono" viologen cation (mono methyl viologen) *N*-methyl,4,4′-pyridylpyridinium (MV^{+}) (Fig. 1*H*). Addition of very small amounts of avidin reverses this quenching, as anticipated in Fig. 2*B*. As shown in Fig. 6, the amount of avidin necessary to produce significant fluorescence recovery is remarkably low. Partial quenching and reversal may be demonstrated by using even lower concentrations of quencher

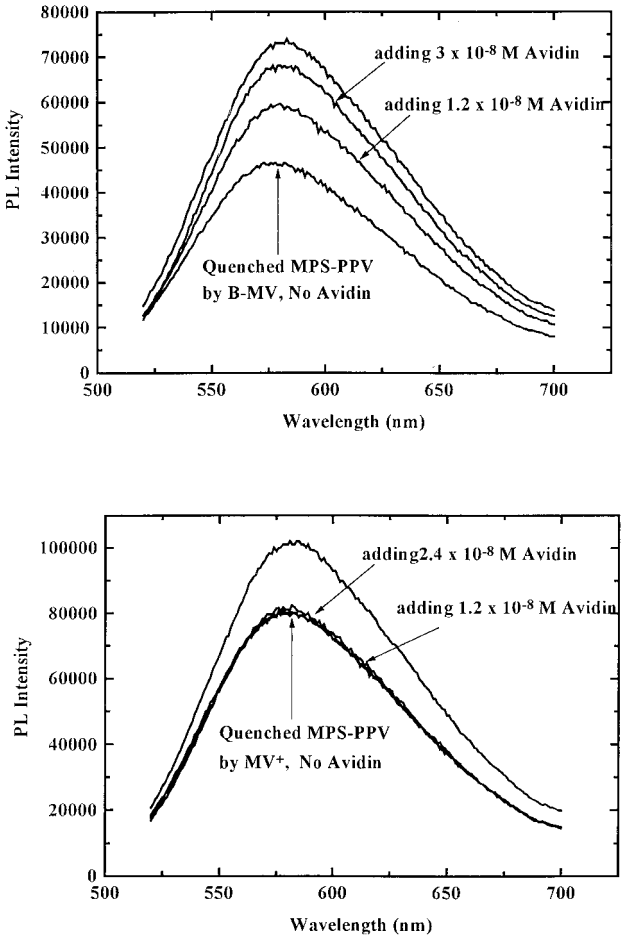

Fig. 7. Partial quenching of fluorescence of MPS-PPM (10^{-5} M in "repeat units") by B-MV (*Upper*) and MV^{+} (*Lower*) (quencher concentration 3.2×10^{-7} M in each case). In the upper plot, the fluorescence quenching is reversed by addition of 1.2×10^{-8} M and 3×10^{-8} M avidin whereas addition of similar amounts to the MV^{+}-quenched sample (*Lower*) shows no increase.

and protein. Fig. 7 shows that addition of either MV^{+} or B-MV at $2–3 \times 10^{-7}$ M produces significant quenching of the fluorescence of MPS-PPV; the fluorescence quenching is reversed in the case in which avidin (1.2×10^{-8} M) is added to the B-MV-quenched sample. However, no change occurs when the same amount of avidin is added to the MV^{+}-quenched sample, verifying that the reversal of fluorescence quenching is caused by the avidin-biotin complex formation. The levels of avidin "sensed" by the recovery of polymer fluorescence are in reasonable accord with an expectation of nearly complete complexation of avidin with a four-fold equivalent of B-MV. Based on these experiments, it is evident that nanomolar amounts of avidin (or lower) may be sensed by this process. Addition of dilute solutions of avidin (2×10^{-7} M) does not produce any detectable change in the fluorescence of "unquenched" MPS-PPV. Furthermore, addition of aqueous solutions of cholera-toxin protein (which lacks a biotin binding site) to B-MV-quenched MPS-PPV produces no increase in the MPS-PPV fluorescence. These results provide strong evidence that the recovery of fluorescence shown in Figs. 6 and 7 occurs as a consequence of the specific biotin-avidin interaction. Taken together, these results for this nonoptimized case provide demonstration of an attractive and versatile biosensor based on fluorescence recovery from the conjugated polymer.

The system described above (and the many possible variations of it) are remarkable from a number of different perspectives. The key component is the ionic conjugated polymer, which leads to two critical effects. First is amplification of the quenching sensitivity, which we attribute to the large number (>1,000) of monomer units per chain, and the high mobility of the exciton along the chain to find the quenching site. Second, once the quenching reagent has been stripped away by the analyte protein, the relatively large sizes of both the MPS-PPV polymer and the protein prevent further association with the quencher, so that the strong fluorescence can be completely recovered. The strategy of using a relatively small amount of a quencher-recognition molecule such as B-MV, and MPS-PPV or a similar conjugated polymer as the optical transduction element, results in a sensing device that may be effectively in the "off" position (near zero fluorescence background) in the absence of the reagent to be sensed. The very short lifetime of the excited states of quenched polymer (<1 ps) should result in relatively little "photobleaching" in the absence of the molecule to be sensed and thus to a potentially robust sensor. The sensitivity and generality of the fluorescence quenching of MPS-PPV (and related polymers) by a wide family of acceptors, and its ready reversal by what is probably best described as a steric effect when the second recognition element binds to the protein, suggests that the approach outlined here may be applicable to a wide variety of specific sensing applications for proteins and other biological macromolecules.

The potential sensing applications of MPS-PPV and related polymers (refs. 39–43, ref. 44 and references therein, and refs. 45–49) are not confined to ionic species or solutions. Neutral, electron-deficient aromatics such as 9,10-dicyanoanthracene and nitroaromatics quench in aqueous solutions at higher concentrations than for MV^{2+} but still at levels where no "dynamic" quenching could occur given the short (≈1 ns) lifetime of the fluorescent state of MPS-PPV. Even more remarkable, quenching is observed for these compounds in solid films of MPS-PPV. As a demonstration, single monolayer films of MPS-PPV were prepared on glass substrates by using polyelectrolyte self-assembly, as described in more detail elsewhere (50). These films show similar fluorescence and absorption to the solutions of MPS-PPV (Fig. 8); interestingly, exposure of these films to the vapor of nitroaromatics such as nitrobenzene or dinitrotoluene leads to substantial quenching of the fluorescence from the films. Fig. 8 shows the rapid quenching that can be observed from dinitrotoluene vapor at room temperature. From the vapor pressure of dinitrotoluene, it can be determined that the film senses (by fluorescence quenching) the nitroaromatic at a level of $<8 \times 10^{-9}$ M. Because the films of MPS-PPV may be readily overcoated with other films of varying thickness and composition, it should be possible to develop a variety of vapor-based "chemical" sensors of high sensitivity and selectivity.

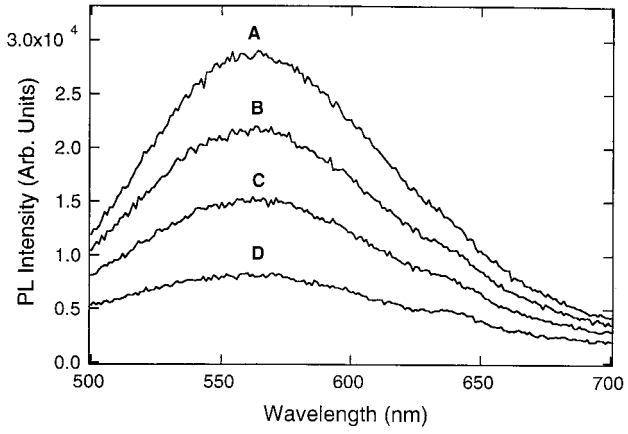

Fig. 8. Fluorescence of a solid film of MPS-PPV excited at 400 nm on exposure of dinitrotoluene vapor as a function of time before exposure (A) and at 10 s (B), 30 s (C), and 60 s (D).

We thank B. I. Swanson, V. Klimov, X. Yang, A. Parikh, and A. Shreve for helpful comments. L. Chen acknowledges financial support from the Los Alamos Center for Material Science. Funding for this research was provided by the Los Alamos Directed Research and Development Program, under the auspices of the U.S. Department of Energy.

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