
Polyelectrolyte-Based Fluorescent Sensors

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2.1 General Introduction

The organic semiconductors that are discussed in this chapter are polyelectrolytes that contain a fluorescent chromophore that is present either as (1) a single large conjugated system, or as (2) an array of strongly aggregated chromophores that exhibit distinct fluorescence from the corresponding monomer by virtue of excitonic delocalization. The latter “class” of organic semiconductors consists of two different systems. In one the chromophores are covalently linked to a polymer backbone and the ensemble is thus a “permanent” molecule. In the other example to be discussed, the aggregated chromophore is associated with an oppositely charged support via adsorption or a templating polyelectrolyte scaffold via “host–guest” interactions. These different systems we will discuss are remarkable for their structural differences as well as for the similarity of their photophysical behavior and potential utility in sensing applications. Two characteristics stand out for these macromolecules and macromolecular assemblies: they are water soluble, but can be easily adsorbed onto oppositely charged supports, and they are also relatively hydrophobic and thus can associate with organic and inorganic counterions and biopolymers by nonspecific association. As we shall discuss, all three systems show amplified fluorescence quenching (super-quenching) upon association with molecules capable of acting as energy transfer or electron transfer quenchers. Structures of the three systems to be discussed are shown in Fig. 2.1.

2.1.1 Amplified Fluorescence Quenching

Amplified fluorescence quenching was first observed for the conjugated polyelectrolyte **1** by the electron acceptor methyl viologen (**5**) [1]. The Stern–Volmer quenching constant for **1** with **5** as a quencher was found to be $\sim 10^7 \text{ M}^{-1}$ in aqueous solution [1]. The quenching is “static” in nature and attributed to a ground-state “charge transfer” complex between the viologen

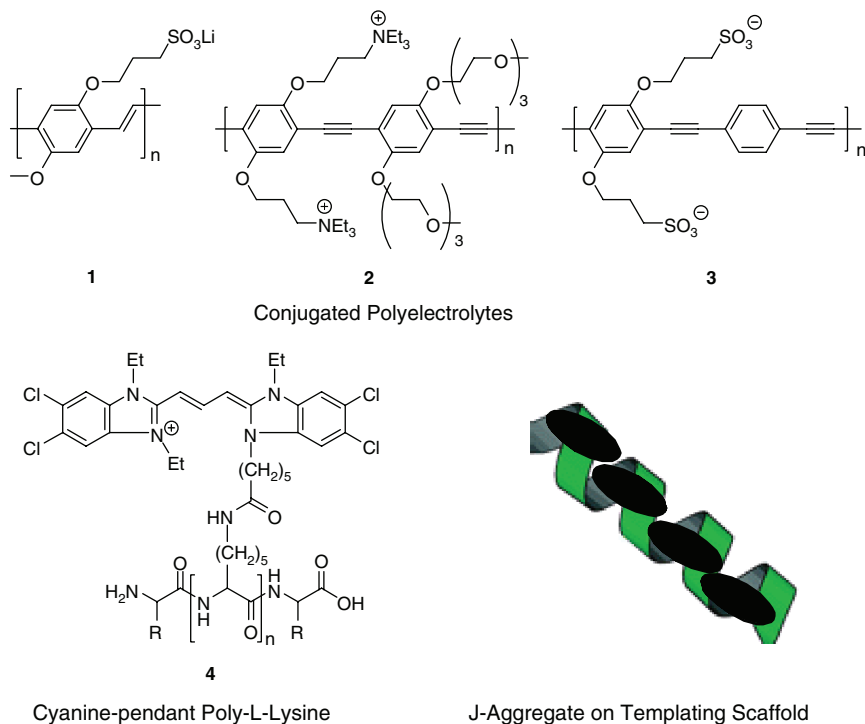


Fig. 2.1. Examples of polyelectrolyte-based fluorescent sensors

and one (or more) of the polymer repeat units. A similar ground-state charge transfer complex has been found with viologen and aromatic hydrocarbons, including the “monomer” chromophore for **1**, *trans*-stilbene; the equilibrium constant for formation of the complex in acetonitrile is $\sim 15 \text{ M}^{-1}$ and this is also the Stern–Volmer constant for fluorescence quenching of *trans*-stilbene by viologen [2]. Thus the quenching constant for the polymer with **5** is enhanced by six orders of magnitude compared with the quenching of *trans*-stilbene or other aromatic hydrocarbons. Similar enhanced fluorescence quenching can be obtained with cationic organic dyes such as cyanines and in this case sensitized fluorescence of the dye may or may not be observed [3].

A series of cyanine-pendant poly-L-lysine polyelectrolytes (**4**) also exhibit similarly enhanced fluorescence quenching by anionic electron transfer or energy transfer quenchers (Fig. 2.2) [4]. For the cyanine-pendant poly-L-lysines it was possible to vary the number of polymer repeat units (PRU) systematically and gain an understanding of what factors influence the magnitude of the amplified quenching [5]. For the cyanine monomer (a monocation) quenching by the electron acceptor anthraquinone disulfonate (**6**) (AQS, a dianion) shows a $K_{\text{SV}} = 630 \text{ M}^{-1}$ (larger than the above-mentioned value for quenching of neutral *trans*-stilbene with the dicationic viologen), and quenching constants

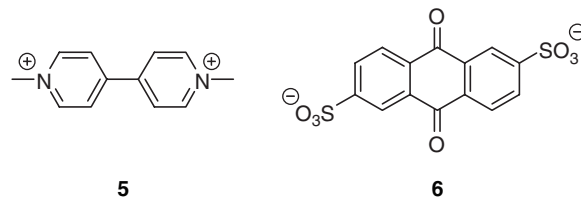


Fig. 2.2. Structures of quencher ions

for oligomers and polymers show a monotonic increase with the number of PRUs [5]. The highest value obtained is $K_{SV} = 1.2 \times 10^9 \text{ M}^{-1}$ for a polymer having ~ 900 PRU [5]. The initial increase as PRU goes from 1 to about 33 can be mainly attributed to increased Coulombic attraction between the multicationic oligomer and the AQS; however, as the number of PRU increases beyond 33 the cyanine can be observed to form largely J-aggregates and the number of quenchers per polymer at 50% quenching $(Q/P)_{50}$ undergoes a further decrease; a minimum value of 2.6 for $(Q/P)_{50}$ is reached for the polymer with 250 PRU [5, 6]. Subsequent increases of polymer molecular weight lead to an increase in $(Q/P)_{50}$. More significantly the value of PRU/Q at 50% quenching levels off at ~ 100 suggesting that excitonic delocalization over patches of ~ 100 chromophores can be intercepted by a single quencher [5, 6]. Thus the amplified quenching (super-quenching) can be clearly shown to be a combination of enhanced Coulombic (and hydrophobic) attraction between oppositely charged polyelectrolyte and quencher, and delocalization of the excitation over an extended array of chromophores associated either by direct conjugation and/or aggregation.

When equimolar (in PRU) aqueous solutions are made from cationic-conjugated polyelectrolyte **1** and the cyanine-pendant poly-L-lysine **4** (~ 250 PRU), there is clear evidence of energy transfer from the higher energy absorbing **1** to the lower energy emitting **4** [3]. Although the absorption spectrum of the mixture resembles an “addition” spectrum of the two polymers, the emission spectrum is dominated by emission from the J-aggregate of **4**. The emission is also independent of the exciting wavelength [3]. This suggests that (not surprisingly) there is strong association between the oppositely charged polyelectrolytes. Interestingly, the roughly neutral (overall) ensemble can undergo super-quenching by addition of either cationic methyl viologen or anionic AQS [3]. This indicates that the mixture must consist of regions of each individual polymer that possess sufficient residual charge to strongly bind counterions and permit quenching by either quencher. Although the two polymers, **1** and **4**, associate strongly when solutions of the polymers are mixed, there is little evidence for interaction when one of the polymers is first adsorbed onto an oppositely charged support. For example, polymer **4** (cationic) can be coated onto Laponite clay nanoparticles at submonolayer coverage such that the nanoparticles still retain a net overall negative

charge. The Laponite-supported **4** exhibits J-aggregate fluorescence and can be quenched by both anionic and cationic electron acceptors (*vide infra*) [7]. Interestingly, mixing aqueous solutions of **1** with a suspension of Laponite-coated **4** results in a superposition of absorption/excitation and fluorescence from **1** and **4** suggesting that there is independent photophysical behavior for the two polymers and hence little interaction or association between them under these conditions [3]. Since both Laponite supported **4** and solution-phase **1** are anionic, it is reasonable to assume that at low concentrations there should be little interaction between them.

When polymer **4** is treated with suspensions of Laponite clay until no further uptake of polymer occurs, there is still less than a monolayer of polymer and since the clay has a higher charge density than the polymer, the overall charge on the nanoparticles is negative. Under these conditions the polymer J-aggregate fluorescence is quenched by both **5** and AQS. Interestingly, the quenching constant, K_{SV} , for AQS is 50% higher for the Laponite-supported **4** than for aqueous solutions [3]. For coating of the polymers onto other supports, a “quench reversal” may be obtained. For example, both **1** and **4** can be coated by adsorption onto commercially available cationic or anionic polystyrene microspheres. Under these conditions, the fluorescence of polymer **1** (on cationic polystyrene) is quenched by AQS, but not by **5**. Similarly, anionic polystyrene-supported **4** is quenched by **5** but not by AQS.

Not surprisingly amplified quenching of polymers adsorbed to, or otherwise bound onto, supports can be observed and also “tuned” depending on the properties (size, structure, charge density) of the support [3]. An interesting example involves the series of cyanine-pendant poly-L-lysines whose solution-phase quenching is described earlier. When this series (ranging from monomeric to small oligomers to relatively large polymers) of polymers **4** was studied in solution, there was a large increase in quenching constant with polymer molecular weight. This series of polymer **4**, including the monomer, can be coated onto silica microspheres, which at neutral pH have a negative charge, but with a charge density much lower than the Laponite clay. Under these conditions, the adsorbed monomer and range of MW polymers are all quenched by AQS. However, the magnitude of the quenching for the series of cyanines is quite different from that observed in solutions [6]. Most notable is the difference between quenching of the monomer in solution and when adsorbed on the silica. The monomer exhibits a quenching constant with AQS, $K_{SV} = 630 \text{ M}^{-1}$, which is reasonable for a small cationic monomer quenched by a dication. The quenching constant for the monomer adsorbed on silica is enhanced by almost 20,000 and approaches those of the larger polymers in solution. In fact, the quenching constants for the adsorbed series of different molecular weight cyanine polymers **4** exhibit only a small increase in quenching constant with increase in molecular weight [6]. Similar super-quenching through adsorption-mediated aggregation has been observed for a series of cyanines adsorbed on Laponite and thus defines the third system indicated in the first paragraph of this chapter [8]. Although self-assembly of

small molecules (monomers–oligomers) onto supports can result in amplified quenching as a general process, its utility may be limited if the “building blocks” can reversibly dissociate from the support and thus it has not been widely used in sensing applications to date.

2.1.2 General Sensor Schemes: Bioassays Based on Quench/Unquench

The high sensitivity of polymers **1** and **4** to fluorescence quenching by electron acceptors and energy transfer acceptors suggested that the quenching might be the basis for sensitive chemical or biosensing [1,4]. Initial studies with **1** confirmed that both types of sensing approaches are possible. Several studies have indicated that one of the simplest approaches, linking a small recognition molecule (or ligand) to a quencher, can lead to a molecule (bioconjugate) that quenches the fluorescence of the polymer and wherein the quenching is reversed when a larger biomacromolecule associates with the ligand portion of the bioconjugate [1,3,4,9–11]. This quenching followed by quench reversal can be observed for solution phase polymers such as **1** and similar conjugated polyelectrolytes. However, in several cases, it has been shown that although quenching by the bioconjugate occurs, quenching reversal does not readily occur. An example is the finding for the higher molecular weight cyanine polymer **4** in combination with a bioconjugate containing an AQS quencher and a biotin ligand in aqueous solution [4]. Although strong quenching is observed, there is no quench reversal when a protein (avidin) recognizing and binding very strongly to biotin is added. Interestingly, a clear quench/quench reversal is observed for the same components when the polymer **4** is supported on nanoparticle Laponite [4]. One of the problems with trying to develop fluorescence-based sensors for polymers such as **1** and **4** in solution is the occurrence of nonspecific interactions with proteins, nucleic acids, or other biopolyelectrolytes [12]. Not surprisingly in view of the results described earlier for mixtures of **1** and **4**, there is strong association between oppositely charged polyelectrolytes, which can modify fluorescence in a variety of ways. A general solution to this problem is to anchor the sensing polymer to a support such as described above, either by adsorption or by covalent attachment. In the supported format, the polymer has lower mobility but still may exhibit strong fluorescence and susceptibility to highly amplified quenching. Additionally, the choice of polymer is important in maximizing effectiveness as a sensor. Polymers **1** and **4** have very low fluorescence yields and thus relatively lower effectiveness as fluorescent sensors in quench/unquench applications. In contrast the poly(phenylene ethynylene) polyelectrolytes such as **2** or **3** (Fig. 2.1) have more “rigid rod”-like structural units and exhibit much higher fluorescence efficiencies and lower sensitivity to fluorescence modulation via nonspecific interactions [12].

Several different sensing schemes have been developed on the basis of the quench/unquench of a highly fluorescent polymer. Previously reported

examples include fluorescence quenching by association of a small molecule bioconjugate with either a solution-phase or particle-supported polymer and subsequent dissociation and unquenching when a biomacromolecule binds to the ligand portion of the bioconjugate. Other formats include microsphere-bound polymer that is colocated with a biological receptor (or “capture strand”); typically these bioassays involve a competition whereby a quencher-bioconjugate competes with an unlabeled analyte for sites on the surface of the microparticle. Assays of this type have been developed for both nucleic acids and proteins [12–14].

Among the most sensitive and effective assays that have been developed are those measuring enzyme activity [10, 15, 16]. A typical case involves protease assays. Both solution phase and microsphere-based assays have been developed. In these assays, a quencher is tied to a bioconjugate peptide, which contains a recognition site and a cleavage site. Before protease catalyzed cleavage of the bioconjugate peptide, the bioconjugate associates with the fluorescent polymer and quenches its fluorescence. Subsequent to the cleavage, the quencher is released and no longer associates with the polymer [15]. Thus, these assays function as fluorescence “turn on” assays. The same principle has been used to develop assays for kinase and phosphatase enzymes [16]. In this case, differential binding of a peptide, protein, or other moiety, before or after phosphorylation, to a microsphere on which a phosphate-binding site and fluorescent polymer are colocated, can be used as the basis for fluorescence “turn-on” and “turn-off” assays. Since these assays and their development have been described in detail and reviewed elsewhere, [12, 15, 16] this chapter will focus on assay technologies that have been more recently developed. The next section of the chapter will discuss enzyme activity assays that have been developed with the fluorescent dyes and polyelectrolytes. Although some of these involve quench/unquench of fluorescent polymers, in several cases new formats and/or new approaches to assay technologies will be described.

2.2 Enzyme Activity Assays

2.2.1 Assay Formats and Types

The assays that have been described in the previous section can be carried out by measurements of fluorescence of the polymer with a conventional fluorometer or a microwell plate reader. In the latter format, they are particularly convenient to use in high throughput screening (HTS) applications and have been carried out for small samples in up to a 3,456 well plate format. More recently, we have studied amplified quenching for microsphere-supported polymers such as **2**, by flow cytometry and in microfluidic channels [17]. In addition to providing new ways of adapting the super-quenching technology, they have provided evidence that in several cases the quenchers associate very strongly with the microsphere-adsorbed polymer and are only removed by “washing”

for long periods. Interestingly, the same Stern–Volmer quenching constant is obtained for **2** with AQS by direct measurement of fluorescence and by flow cytometry.

In addition to the measurements of enzyme activity through the quench-unquench procedures described earlier, we have also found a new strategy for using super-quenching unquench/quench by what we refer to as “frustrated super-quenching.” A microsphere-supported polymer such as **2** (and other cationic-conjugated polymers) can be coated by a lipid bilayer to yield a layered system in which the conjugated polymer layer is separated by the bilayer from reagents present in the aqueous medium. The lipid bilayer can “protect” the polymer from quenching by electron transfer quenchers such as AQS, and for good bilayers such as dimyristoyl phosphatidyl glycerol (DMPG) the bilayer protects the polymer, such that lower than usual ($\sim 20\%$, reduced from 95%) quenching occurs by AQS under the same conditions [17]. The small amount of quenching that does occur may be attributed to defects in the bilayer but in general the bilayer provides strong attenuation of quenching. In the case of DMPG bilayers over the polymer, disruption of the bilayer in the presence of AQS by addition of Triton-X 100, a nonionic surfactant, results in efficient quenching of the polymer fluorescence. This has been demonstrated for microsphere supported **2** and “overlayers” of DMPG through flow cytometry and in microfluidic channels. Extensions of this principle of assay development will be discussed in more detail subsequently.

A final type of assay involves incorporating biopolymer-mediated controlled self-assembly as a means of a fluorescent switch [8, 18, 19]. In the examples to be discussed, advantage is taken of the assembly of nonfluorescent cyanine dye monomers to form highly fluorescent J-aggregates on a templating biopolymer or inorganic scaffold. For the biopolymers such self-assembly provides a fluorescence “turn-on” assay for the biopolymer as well as an enzyme activity assay for enzymes that can degrade the biopolymer to monomer or small oligomers. In the latter case, the fluorescence is quenched as the enzyme digests the biopolymer scaffold required for fluorescent J-aggregate formation.

2.2.2 Proteolytic Enzyme Assays Using Conjugated Polyelectrolytes

Proteolytic enzymes like proteases and kinases/phosphatases play a crucial role in many physiological and pathological processes, rendering the necessity for inventing high throughput screening assays for their real time analysis of crucial importance. Most of the assays developed so far are low sensitivity assays, requiring high concentrations of the enzyme or the substrate. Enzyme assays using fluorescent-conjugated polymers that are in solution as well as supported on solid surfaces like borosilicate or polystyrene microspheres accommodate real time analysis of enzyme kinetics, and have very low detection limits (nanomolar). In this section, we describe in some detail a protease sensor system that operates via a “turn on” mechanism.

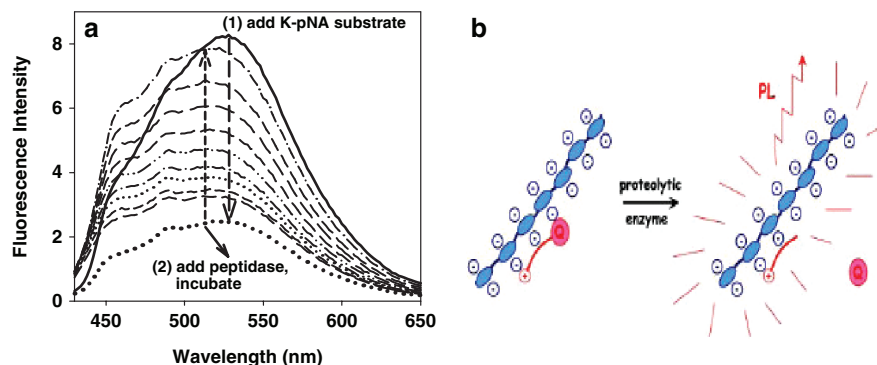


Fig. 2.3. (a) Fluorescence spectroscopic changes observed in a proteolytic enzyme assay using PPE-SO₃ (**3**) and K-pNA. Initial addition of KpNA quenches fluorescence, and then addition of peptidase gives rise to fluorescence recovery. *Solid line* Initial fluorescence, [PPE-SO₃] = 1.0 μ M, phosphate buffer solution, pH 7.1; *Dotted line* fluorescence after addition of 167 nM K-pNA. Fluorescence intensity as a function of time (5–200 min) after addition of porcine intestinal peptidase (3.3 μ g mL⁻¹). (b) Mechanism of the “turn-on” CPE-based sensor

Protease activity has been monitored quantitatively and in real-time using the anionic fluorescent-conjugated polyelectrolyte **3** (Fig. 2.1) [11]. The sensing mechanism is based on the electrostatic interaction between anionic **3** and a peptide substrate with a positive charge. For example, the cationic monopeptide L-lysine-*p*-nitroanilide (K-pNA) was used as a substrate-quencher for development of a prototype assay. Polyelectrolyte **3** is strongly fluorescent in aqueous buffer solution, but the fluorescence is very efficiently quenched by K-pNA ($K_{SV} \approx 10^7$ M⁻¹). The efficient quenching is believed to arise via a charge transfer mechanism (the nitroanilide moiety is a good electron acceptor) and because the dicationic substrate-quencher ion pairs strongly to the polyelectrolyte chains. Upon addition of a peptidase enzyme (a nonspecific aminopeptidase), [20,21] the fluorescence of **3** recovers (Fig. 2.3). The fluorescence recovery arises because the enzyme cleaves the peptide bond linking the L-lysine residue to the pNA quencher moiety. As a result, the pNA is no longer ion-paired to the polyelectrolyte, so its ability to quench falls dramatically. The mechanism by which this sensor operates is illustrated schematically in Fig. 2.3.

2.2.3 Phospholipase Assays Using Conjugated Polyelectrolytes

Water-soluble conjugated polyelectrolytes interact with oppositely charged surfactants forming polymer-surfactant complexes. The polymer-surfactant interaction induces changes in the conformation [22] and optical properties [22–24] of the polymer including narrowed absorption and enhanced fluorescence quantum efficiency. In addition, the fluorescence quenching efficiency of

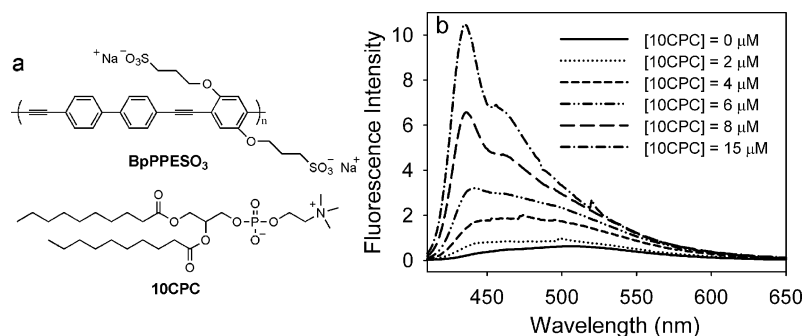


Fig. 2.4. (a) Structures of polymer and substrate. (b) Fluorescence spectroscopic changes observed upon addition of 10CPC. [BpPPESO₃] = 1 μM in water at ambient temperature. Reproduced from ref. [60] with permission. Copyright 2007, American Chemical Society

a conjugated polyelectrolyte by ionic and neutral quenchers can be modified significantly in the presence of surfactants [7–9]. Phospholipids are naturally occurring surfactants that feature an ionic headgroup (either cationic, anionic, or zwitterionic) and two hydrophobic tails (Fig. 2.4a). In a recent series of investigations, we have explored the effect of phospholipids on the optical properties of conjugated polyelectrolytes. As shown in Fig. 2.4b, the fluorescence intensity of an aqueous solution of the anionic polymer BpPPESO₃ increases dramatically and blue-shifts with increasing concentration of the added phospholipid 10CPC. Addition of 15 μM 10CPC to a BpPPESO₃ solution leads to a greater than 50-fold increase in the polymer's fluorescence intensity at 435 nm. The significant change of the polymer fluorescence suggests that the phospholipid interacts with the polymer via a combination of the electrostatic attraction between the anionic units on the polyelectrolyte and the cationic head-group of the phospholipid, as well as a hydrophobic interaction between the hydrocarbon tails of the lipid and the polymer backbone. The phospholipid molecules likely bind to the polymer chains in a manner that maximizes the hydrophobic interactions, thereby inducing the polymer to take on a more extended conformation as well as disrupting the polymer–polymer interactions (aggregation).

We have taken advantage of this lipid induced change in fluorescence of the conjugated polyelectrolyte to develop a real-time fluorescence turn-off assay for the lipase enzyme phospholipase C (PLC). PLC plays a critical role in cell function and signal transduction cascades in mammalian systems [25, 26]. It catalyzes hydrolysis of the phosphate ester in a phospholipid at the glycerol side, [27] yielding as products diacylglycerol (DAG) and a phosphoryl base. Neither of these lipid hydrolysis products has a significant effect on the fluorescence of BpPPESO₃ when they are present at relatively low concentrations. Figure 2.5a illustrates the mechanism of the PLC turn-off assay. The polymer aggregates in aqueous solution, but upon addition of the phospholipid

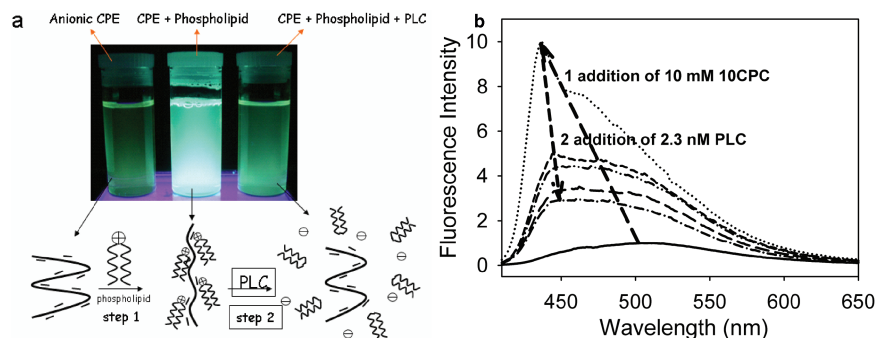


Fig. 2.5. (a) Mechanism and demonstration of PLC turn-off assay. (b) Fluorescence spectroscopic changes observed in the PLC turn-off assay in water. *Solid line* Initial fluorescence, $[\text{BpPPESO}_3] = 1.0 \mu\text{M}$; *Dotted line* fluorescence after step 1: addition of $10 \mu\text{M}$ 10CPC. Fluorescence intensity as a function of time after step 2: addition of 2.3 nM PLC: 1 (---), 5 (- · - · -), 20 (— —), 45 (—●—) min. Reproduced from ref. [60] with permission. Copyright 2007, American Chemical Society

a polymer–lipid complex forms, which disrupts the polymer aggregates that are present in solution (step 1). As a result, the fluorescence of the polymer is enhanced. After addition of the lipid, the solution containing the polymer–lipid complex is then incubated with the PLC enzyme (step 2). PLC hydrolyses the lipid, thereby decreasing its amphiphilic character, and disrupting the polymer–lipid complex. This effect causes the polymer to reaggregate, causing a decrease in the fluorescence intensity. As shown in Fig. 2.5, the decrease in fluorescence intensity continues as long as lipid substrate remains in the solution and the PLC-catalyzed hydrolysis reaction continues.

After optimizing the assay conditions, including ionic strength, pH, temperature, activator (Ca^{2+}) concentration, and polymer concentration, a calibration curve was developed, which allows the lipid substrate concentration to be determined from the fluorescence intensity. The calibration curve allows the enzyme catalysis kinetics parameters (e.g., K_m and V_{max}) to be measured. This PLC turn-off assay is effectively inhibited by known inhibitors (F^- and EDTA), which demonstrates that the sensor relies on the specific catalysis reaction by PLC. It has been demonstrated to be a sensitive (detection limit $\sim 0.5 \text{ nM}$ enzyme concentration), fast ($< 5 \text{ min}$), and selective (good specificity over phospholipase A and D, and other nonspecific proteins) PLC assay, which can be carried out at very low initial substrate concentration (in the range of micromolar to nanomolar).

This method is general and can be applied to other enzyme systems utilizing different types of substrates. For example, a similar assay to sense phosphatidylinositol phospholipase C (PI-PLC) can be constructed by utilizing the anionic phospholipid phosphatidylinositol biphosphate (PIP_2) as substrate along with a cationic-conjugated polyelectrolyte as the fluorescent signaling element. By taking the advantage of the rapid and strong response of

the polymer to phospholipids, it is possible to image the interactions between polymer and liposome by means of confocal or epifluorescence microscopy. Finally, some natural or synthesized cationic lipids have been applied as DNA transfection agents, [28, 29] so this assay might open another way to detect the cationic lipids used in gene transfer.

2.2.4 Assays Based on “Frustrated Super-Quenching”

Phospholipase A₂ (PLA₂) also belongs to the family of lipase enzymes. It catalyzes the hydrolysis of the *sn*-2 fatty acid ester bond of the phospholipid substrate liberating a fatty acid and lysophospholipids. When the liberated fatty acid is arachidonic acid, a cascade of reactions promoting the formation of proinflammatory moieties is triggered. PLA₂ is also associated with circulatory low density lipoprotein and is now being exploited as a novel biomarker for coronary artery diseases. A simple fluorescence turn off assay for PLA₂ was developed by quantifying enzymatic activity of PLA₂ using frustrated super-quenching, which is achieved through the ionic interaction between a solid supported fluorescent-conjugated polyelectrolyte and its specific quencher. A cationic-conjugated polyelectrolyte (**2**) is physically adsorbed onto a solid support such as nonporous borosilicate microspheres. As illustrated in Fig. 2.6a, fluorescence of microsphere supported polymer is quenched in the presence of AQS. Using the layer by layer method of assembly, microsphere supported cationic polymer is coated with DMPG, an anionic lipid bilayer that serves the dual purpose of acting as a substrate for PLA₂ enzymatic activity and shielding the fluorescent polymer from quenching by AQS. When these lipobeads are

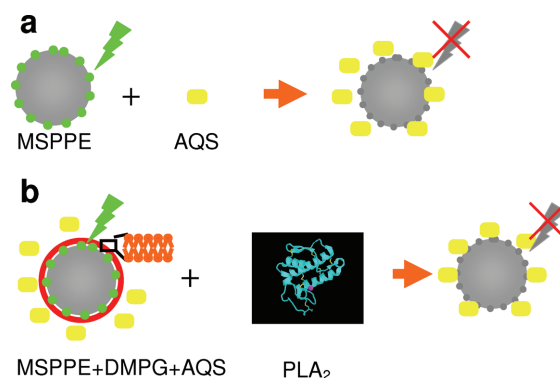


Fig. 2.6. (a) Microsphere supported PPE (MSPPE) with desired concentration of AQS. This results in the turning off of the fluorescence of PPE. (b) MSPPE is coated with a layer of DMPG lipid bilayer and incubated with AQS. The DMPG layer protects the fluorescence of PPE from being quenched by AQS. When PLA₂ is added, it disrupts the lipid bilayer by cleaving the *sn*-2 ester bond of the lipid bilayer, resulting in the exposure of the fluorescence of PPE, which is then quenched by AQS

incubated with a mixture of PLA₂ and AQS, the cleaving of the *sn*-2 acyl ester bond of the DMPG is catalyzed by PLA₂, exposing the fluorescent polymer to AQS to be quenched (Fig. 2.6b). This reaction occurring on the surface of the bead is detected by a flow cytometer. Compared with previously developed radiometric, [30] calorimetric, [31] fluorogenic, [32, 33] or spectrophotometric [34] assays reported in the literature, this assay demonstrates the use of a simple homogeneous platform to detect PLA₂ enzymatic activity directly by using a natural substrate of PLA₂ without any modification.

The assay for PLA₂ activity described above, using frustrated super-quenching, is a fluorescence turn off assay. This assay can also be adapted as a fluorescence turn on assay for screening of potential inhibitors of PLA₂. A fluorescence turn on assay using the principle of FRET has also been developed using solid supported polymer and rhodamine (Rh)-labeled DMPG. As described previously, the solid supported polymer is coated with DMPG. In this particular assay, however, the head group of the DMPG beads is labeled with rhodamine. Polymer and rhodamine are brought in close contact with each other when the solid supported conjugated polyelectrolyte is coated with Rh-DMPG lipid bilayer, enabling FRET to occur. When these lipobeads are incubated with PLA₂, the Rh-DMPG bilayer is disrupted, resulting in an increase in the intermolecular distance between the polymer and Rh. An increase in the fluorescence of conjugated polyelectrolyte can thus be observed.

The principle of frustrated super-quenching as a detection technique for quantifying the catalytic activity of hydrolytic enzymes can also be extended by using solid supported polymer and enveloping it with anionic biopolymers like carboxymethyl amylose (CMA) or carboxymethyl cellulose (CMC) to shield its fluorescence from electron transfer or energy transfer quenchers.

2.2.5 Supramolecular Self-Assembly and Scaffold Disruption/Destruction Assays

As described in Sect. 2.1, cyanine dyes can form J-aggregates that are characterized by sharp red-shifted absorbance and fluorescence compared with the monomer. As discussed in the introduction, J-aggregates can form on inorganic nanoparticles or microspheres as well as within polymeric ensembles. Other studies have indicated that cyanines can self-assemble on biopolymeric scaffolds to form aggregates with properties that depend on the structure of the host.

2.2.6 Cyanines and Supra-Molecular Self-Assembly

At the core of the scaffold formation and disruption/destruction assay is the electronic exciton concept that is relevant to molecular aggregation [35–41]. Cyanine dyes and other aromatic or heteroaromatic organic compounds can form molecular aggregates with varying structural and physical properties,

which are often very sensitive to medium, potential host structure and chromophore substituents. Two common forms of dye aggregates are the so-called J-type and H-type. H-type dimers or aggregates are arrays of molecules where the principal transition moments are largely parallel and individual chromophores are arranged either face-to-face or edge-to-face. H-type aggregates are usually characterized by absorption spectra that show a strong maximum at higher energy than the principal transition of the monomer and a much weaker, red-shifted absorption [35]. H-aggregates are nonfluorescent or weakly fluorescent. In contrast, J-type dimers or aggregates have transition moments of monomers either end-on-end or in an otherwise extended structure, wherein the aggregate principal absorption is sharp and red-shifted compared with the monomer transition. J-type aggregate fluorescence is typically sharp and only modestly red-shifted compared with the J-type aggregate absorption [39–41]. Self-assembling cyanine dyes aggregate on chem-bio-helices (helicophilic cyanines) or assemble onto linear polymers, transforming the latter to adopt helical or supra-helical structures (helicogenic cyanines). During this process, nonluminescent molecules become highly fluorescent [35–39]. Helices of proteins, peptides, carbohydrates, lipids, and nucleic acids and nonhelices such as phospholipids, membranes, or liposomes may mediate J-type aggregate fluorescence [42–44]. Finally, J-type aggregate transformations are observed with several types of solid supports and nanoparticles [4, 6, 7, 45]. Thus, the spectral properties of J-type aggregates make them attractive candidates for developing a variety of chem-bio-sensing applications.

2.2.7 Cyanine Chemistry

The structures of representative examples of cyanines with the potential to form helicogenic and/or helicophilic J-type aggregates are shown in Fig. 2.7. The syntheses of some of these cyanines were described previously [45]. These

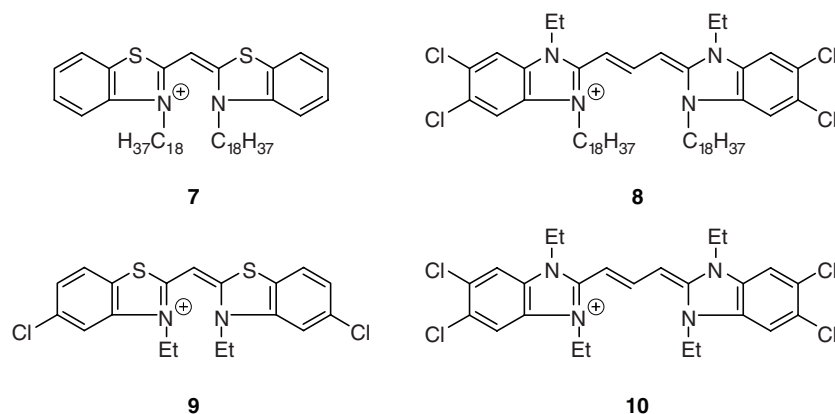


Fig. 2.7. Chemical structures of cyanine dyes that may form J-type aggregates

water soluble and amphiphilic cyanines are anionic or cationic and span a range of absorption from 375–600 nm. The cyanines, depending on their structures, can form either J- or H-type aggregates. However, since normally only J-type aggregates exhibit strong fluorescence, the detection is dependent on the use of a cyanine that forms a fluorescent J-type aggregate. Cyanines **9** and **10** are cationic and they both form J-type aggregates when associated with anionic supports such as the biopolymer CMA or CMC, as well as nanoparticles of clay, anionic microspheres, or silica microparticles [4, 6–8, 18, 45]. Anionic structures having the same chromophores as **9** and **10** have also been prepared and may be anticipated to form aggregates when exposed to chemical and biological helices that are cationic. We have already observed that clay nanoparticles (anionic) “overcoated” with cyanines **9** and **10** associate with some of the anionic cyanines [4, 6, 7]. Both **9** and **10** are slightly water soluble and we have shown that they “exchange” with anionic sites on different nanoparticles or polymeric supports. We have also studied amphiphilic cyanines **7** and **8** with the same chromophores as **9** and **10**. These are nearly water insoluble and our investigations indicate that once bound to an anionic binding site (clay, silica, or biopolymer) they do not dissociate. These amphiphilic structures offer the possibility of preparing a precoated sensor that will be useful in continuous (one-step, real time, kinetic) assays. Sensors prepared thus may be highly sensitive and are subject to very few nonspecific effects, which might be triggered by other components in the complex milieu that offer binding sites for the cyanine monomer and/or the J-type aggregate. Several physico-chemical properties of cyanines might be involved in forming fluorescent J-type aggregates including side-chain length, side-chain substituents, and dye chirality [46]. The wide range of absorption for the cyanines (375–600 nm) offers the possibility of multiplexing the assays. Some cyanines form J-type aggregates with low fluorescence efficiency; yet when there is no background from the fluorescence of the monomer or the H-type aggregate, the fluorescence may still be sharp and strong enough to provide a useful assay. Furthermore, even where the cyanine fluorescence efficiency is low, if it has virtually zero background emission from monomers, then it will yield high signal/background; a valuable assay metric.

2.2.8 Glycosidases and Scaffold Disruption/Destruction Assay

We described a new optical sensing system for the high-throughput screening (HTS) of a broad range of chemical and biological molecules, based on the principle of scaffold formation or disruption/destruction [8, 19]. The technology offers the flexibility of configuring the assays in *label free* or *labeled* formats and operating in continuous or discontinuous (multistep, endpoint) modes with complex sample formulations, under operationally relevant testing conditions [8]. The new assay platform was first demonstrated using carbohydrates and carbohydrate metabolizing glycosidase enzymes. Cellulose is the most abundant plant carbohydrate. It is a linear polysaccharide composed of

β -D-glucose linkage – (1 \rightarrow 4). Cellulose is of interest to the bioenergy/biofuel industries. Amylose is a major component of starch and the second major plant carbohydrate. It is a linear polymer of α -D-glucose linkage – (1 \rightarrow 4). Amylase converts glycogen and starch into sugars. We used the cyanine **9** that undergoes a cooperative self-assembly with CMA and CMC. The water soluble **9** exists as a nonfluorescent monomer in the absence of CMA or CMC, but is converted to a highly fluorescent, red-shifted J-aggregate when it self-assembles on the anionic polymers; the induced circular dichroism (CD) spectrum for the achiral cyanine, as well as other evidence, indicates that the linear, chiral CMA polymers convert to supramolecular helical assemblies as the cyanine and polymer self-assemble [8, 18]. We utilized this property to develop an assay for amylase, based on the concept of “scaffold destruction,” wherein the destruction of CMA by amylase enzyme is accompanied by attenuation of light emission from the J-aggregate [8]. The extent of light attenuation was an index of amylase activity (Fig. 2.8). A similar assay format can be applied to monitor the enzymatic digestion of cellulose by cellulases.

Hyaluronidase is involved in bacterial and fungal infections because of virulence factors evoked by tissue degradation and mediates host–pathogen interactions [47]. Since hyaluronic acid (HA) is a major component of the extracellular matrix involved in joint lubrication, a sensitive hyaluronidase assay is important. Current hyaluronidase assays rely on turbidimetric techniques that require high levels of the enzyme and are relatively inaccurate [47]. HA was previously shown to bind cyanines [48, 49]. The detection scheme designed for CMA, CMC, and amylase enzyme described earlier was also applicable to HA and hyaluronidase activity [19]. “Scaffold Destruction”

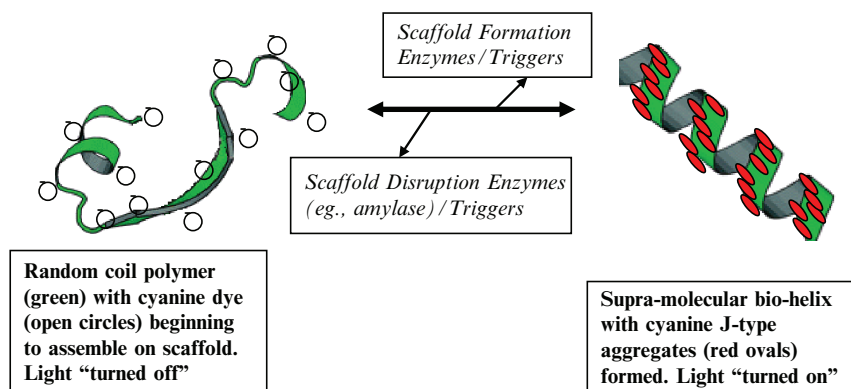


Fig. 2.8. Scaffold formation/disruption assays based on supramolecular self-assemblies. In this scheme, a cyanine is helicogenic. Molecular self-assembly of cyanine upon linear chiral polymer such as CMA, CMC, or HA results in a conformation transition of the polymer to adopt a super-helix structure. Scaffold disrupting glycosidases (hyaluronidase, amylase, cellulase) trigger fluorescence attenuation by disruption of the helical scaffold

hyaluronidase assay has applications in pathogen/clinical diagnostics [19]. Other polymers of carbohydrates, proteins, nucleic acids, and chemical polymers might provide similar scaffolding for helicogenic cyanines upon which molecular aggregation occurs. This is in addition to – and different from – the previously demonstrated helicophilic property of cyanines [8, 18, 19, 50, 51]. By controlling and regulating the interactions of cyanines with various helices and nonhelices in a solvent-directed fashion, a variety of chem-bio-sensing may be accomplished, in either optical “turn on” or “turn off” modes (Fig. 2.8).

We described earlier fundamental investigations for developing optical sensors with broad, interdisciplinary applications to the fields of biology, chemistry, and physics. Novel, *label-free* or *labeled*, rapid, HTS assays were described that are suitable for screening drugs in optical “turn on” or “turn off” modes for a variety of chem-bio helices. Since J-aggregation is accompanied by spectral shift toward longer (red) wavelengths compared with monomeric cyanine, these assays could avoid the effects of interfering substances that absorb in the blue region [52, 53]. The above assay formats offer the additional advantages of orthogonality, redundancy, and degrees of freedom to maximize the specificity of detection for biosensor applications, besides exploiting nature’s own selectivity [54]. These assay metrics are achieved through ratiometric analysis of light absorption and fluorescence emission intensities as well as spectral shifts of the monomer dye relative to cooperatively self-assembled aggregates, as a consequence of scaffold formation or scaffold destruction. By appropriately tuning the interactions between the scaffold and the cyanine, a variety of chem-bio-sensing applications are possible. Thus, a broad range of solid-phase, solution-phase, and interfacial sensing applications are feasible using this novel fluorescence platform technology.

2.3 Conjugated Polyelectrolyte Surface-Grafted Colloids

Conjugated polyelectrolytes can also be synthesized as covalently attached coatings on nano- or micro-spheres such as silica [55] or latex [56] particles. In a general approach, grafting points are introduced to the particles as an aryl halide group. Polymers can be surface grafted via metal-catalyzed step growth polymerization. These conjugated polymer grafted colloids show promise as a new fluorescence sensor platform, with several advantages over other forms of coatings.

In our recently developed approach, introduction of aryl iodide groups on the surface of silica particles was accomplished by reacting the surface with a trialkoxysilane bearing an aryl iodide group (Fig. 2.9). One advantage of this method is that the surface density of the grafting points can be controlled by varying the amount of silane, thus allowing the number of polymer brushes on the surface to be adjusted accordingly. A PPE-type polymer was grown from these aryl iodide groups via Pd(0) catalyzed A-B type polymerization under Sonogashira conditions. Although a large amount of free polymer

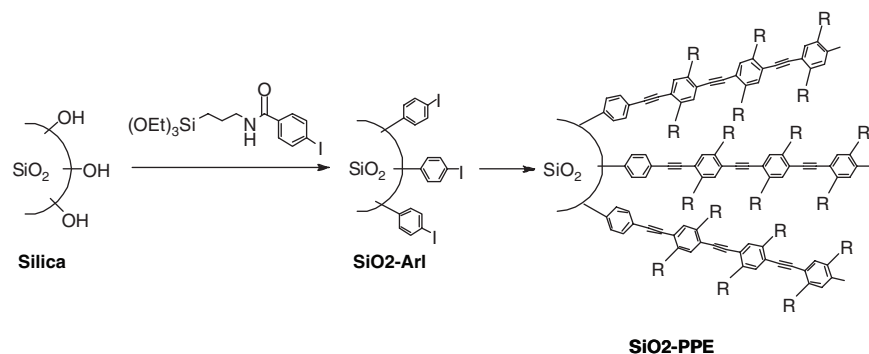


Fig. 2.9. Synthesis of polymer and surface-grafted silica particles. Reproduced from ref. [55] with permission. Copyright 2007, American Chemical Society

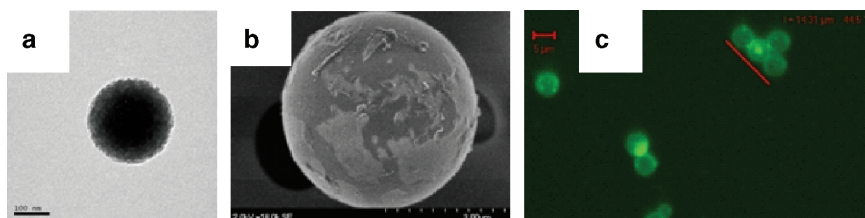


Fig. 2.10. Electron microscope images of silica particles: (a) 300 nm CP-grafted particle (TEM); (b) 5 μm CP-grafted particle (SEM); (c) Confocal fluorescence microscope image of 5 μm CP-grafted particles. Reproduced from ref. [55] with permission. Copyright 2007, American Chemical Society

was obtained as a byproduct of the reaction, isolated silica particles ($\text{SiO}_2\text{-PPE}$) exhibit yellow coloring with bright green fluorescence upon irradiation with a long wavelength UV lamp, which are characteristics of PPE. In a control experiment, the identical polymerization reaction was performed with unmodified silica particles in place of aryl iodide modified silica particles. Free polymer and unmodified silica particles without coloring were obtained after a standard work-up procedure. This suggests the importance of aryl iodide functionality on the surface for covalent attachment of the polymer. The surface modification process can be monitored by use of infrared (IR) spectroscopy to confirm the presence of functional groups on the surface [55]. Although the signals are suppressed by a strong peak from Si-O-Si stretch, the presence of the polymer can be confirmed by comparing IR spectra of polymer-grafted particles and free polymer.

The presence of polymer brushes on the surface of silica particles can also be confirmed by electron microscopy and confocal fluorescence microscopy. As seen in Fig. 2.10a, transmission electron microscope (TEM) images of $\text{SiO}_2\text{-PPE}$ particles show rough surface texture. It should be noted that no significant change in particle size was observed after grafting the polymer. The

thickness of the polymer layer was estimated using thermal gravimetric analysis data to be approximately 12 nm [55]. Although the particle size was not affected, the TEM data clearly show a uniformly covered surface with a thin layer-grafted polymer. Scanning electron microscope (SEM) images also show the presence of polymer on the surface of the particles. As shown in Fig. 2.10b, the polymer-grafted particles exhibit an “orange peel”-like appearance, which is associated with the polymer. In addition to a thin layer of polymer, some large aggregates are also observed on the surface. The origin of the material is unclear; however, one possible explanation is that the material was initially produced in solution during the polymerization and then became either chemically or physically adsorbed onto the surface. The confocal images (Fig. 2.10c) of polymer-grafted particles clearly show the green fluorescence from the polymer on the surface of the particles. Although the emission seems to be from the entire surface of the particles, some clustering of the fluorescent material can also be observed. Such observation correlates with the SEM images of the surface of the particles, which suggests that the polymer graft layer is not completely uniform.

Photophysical characterization of the polymer-grafted particles was performed to investigate their potential for fluorescent sensor material. Suspensions of SiO₂-PPE particles in water and methanol exhibit strong fluorescence with $\lambda_{\text{max}} = 535$ nm. Attempts were made to measure UV-vis absorption property of the particles; however, the colloidal nature of the suspension caused strong interference in the near UV region because of light scattering. This also precluded fluorescence quantum efficiency measurement of the particles. Although direct measurement of the absorption spectrum was not possible, the absorption profile can be approximated by the fluorescence excitation spectrum of colloidal suspensions of particles. It was found that the excitation spectrum closely resembles the absorption spectrum of the free polymer [55].

A series of fluorescence quenching studies of SiO₂-PPE particles were conducted using a variety of quenchers including methyl viologen (**5**), Cu²⁺, diethyldicarbocyanine (DEDCC), diethylcyanine (DEC), and diethylthiadibocyanine (DETDC). Among these quenchers, **5** and Cu²⁺ quench via a charge-transfer mechanism, while cyanine dyes quench via a Förster energy-transfer mechanism. As illustrated in Fig. 2.11a, charge-transfer type quenchers only quench $\sim 70\%$ of the fluorescence emission from the particle. Energy-transfer type quenchers, on the other hand, exhibit complete quenching, with Stern–Volmer constants (K_{SV}) ranging from 10^5 to 10^7 M⁻¹ as seen in Fig. 2.11b. For charge-transfer type quenchers, K_{SV} values were calculated using a modified Stern–Volmer expression [57–59] to give values in the 10^6 M⁻¹ range. Such large K_{SV} constants indicate that the grafted polymers retain the amplified quenching property, which is desirable for sensor materials. Although the K_{SV} values are in a similar range, there exists a distinct difference in quenching behavior between these different types of quenchers. The exciton and quencher must be in proximity (<1 nm) for charge-transfer

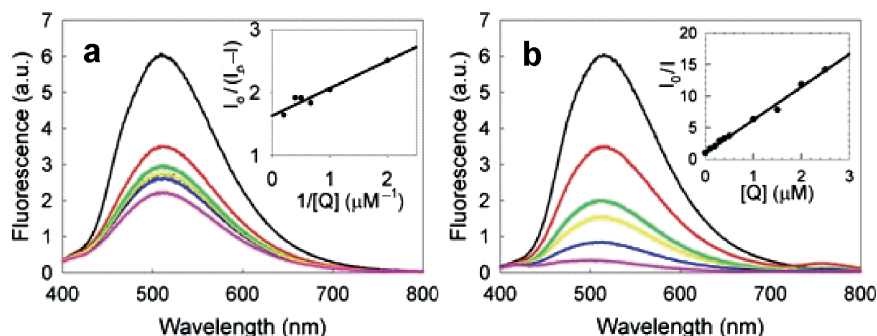


Fig. 2.11. Stern–Volmer quenching of CP-grafted particles suspended in water: (a) Electron transfer quenching by MV^{2+} (0–5 μM) and (b) energy transfer quenching by DEDCC (0–2.5 μM). Reproduced from ref. [55] with permission. Copyright 2007, American Chemical Society

type quenching to be efficient, whereas energy-transfer type quenching can be efficient even when the exciton and quencher are separated by distances greater than 5 nm. Considering the difference in quenching mechanism, the fact that charge-transfer type quenchers only quench a portion of the fluorescence from the particle suggests that the grafted polymers on the colloid surface exist in a strongly aggregated state and that some fraction of excitons are trapped within the aggregates. Such aggregates prevent quenchers from penetrating into the polymer matrix. Therefore, energy-transfer quenchers with longer effective distances can quench excitons that are trapped deeply inside the aggregates, while charge-transfer quenchers only quench excitons on the exterior of the grafted layer. In addition to such quenching effects, a very efficient sensitized fluorescence emission from DETDCC was observed at 700 nm [55].

As illustrated above, conjugated polymers can be covalently attached to silica particles to yield colloidal particles with bright fluorescence emission. The surface-grafted polymer retains its amplified quenching property, in which the fluorescence emission can be quenched efficiently with K_{SV} constants in 10^5 – 10^7 M^{-1} range. Such characteristics suggest that conjugated polymer-grafted colloids may be useful for applications in fluorescence sensors for biological targets.

2.4 Summary and Conclusions

This chapter describes a broad range of optical biosensor technologies, based on interchromophore interactions and the super-quenching phenomenon. Several specific biosensor schemes are described that are able to sense the activity of kinase, protease, lipase, and cellulase enzymes. Although the systems are disparate, the underlying sensor mechanisms are related, in that they all

rely on the unique optical and photophysical properties of excitons that are present in these polymer-based or self-assembled chromophore aggregates. The excitons are strongly delocalized and highly mobile within the polychromophore assemblies; in this way these solution chromophore-based assemblies are analogous to solid-state semiconductors, which display optical and electronic properties that are quite sensitive to the presence of traps at low concentration.

We believe that there is great promise for the use of polyelectrolyte-based fluorescence sensors in optical biosensor applications. The systems are flexible in their design and format, they are highly sensitive and can be quite specific, and they are easily adapted to high-throughput screening. Although we have described a number of possible applications in the present chapter, many other applications have been described elsewhere, and there are clearly many more potential target analytes and biosensor systems that undoubtedly will be developed as a result of ongoing and future investigations.

Acknowledgments

The work at UNM was partially funded by the National Science Foundation (NSF) under Award CTS-0332315 awarded to Dr. Gabriel P. Lopez. The work at UF was partially funded by an award (W911NF-07-1-0079) from the Defense Threat Reduction Agency. Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company for the United States Department of Energy's National Nuclear Security Administration under contract DE-AC04-94AL85000. This work was partially funded by the Defense Threat Reduction Agency (DTRA) under Contract MIPRG089XR076 awarded to Komandoor Achyuthan. Thanks are due to Dr. Stephen Casalnuovo for facilities and support.

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Organic Semiconductors in Sensor Applications

Bernards, D.A.; Owens, R.M.; Malliaras, G.G. (Eds.)

2008, XVI, 290 p. 183 illus., Hardcover

ISBN: 978-3-540-76313-0