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Synthesis and Application of Poly(phenylene Ethynylene)s for Bioconjugation: A Conjugated Polymer-Based Fluorogenic Probe for Proteases

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Abstract: A set of carboxylate-functionalized poly(phenylene ethynylene)s (PPEs) has been synthesized in which the carboxylic acid groups are separated from the polymer backbone by oligo(ethylene glycol) spacer units. These polymers are soluble in water and organic solvents and have photophysical properties that are sensitive to solvent conditions, with high salt content and the absence of surfactant promoting the formation of aggregates of relatively low quantum yield and long fluorescence lifetime. Quenching of these materials by the dinitrophenyl (DNP) chromophore ($K_{SV} \sim 10^4$) is also highly solvent-dependent. The presence of carboxylate groups far from the polymer backbone appended to each repeating unit allows for the postpolymerization modification of these PPEs with peptides by methods analogous to those described for carboxylate-functionalized small-molecule dyes. Covalent attachment of the fluorescence-quenching 14-mer Lys(DNP)-GPLGMRGLGGGGK to the PPE results in a nonemissive substrate whose fluorescence is restored upon treatment with trypsin. The rate of fluorescence turn-on in this case is increased 3-fold by the presence of surfactant, though the actual rate of peptide hydrolysis remains the same. A small-molecule mimic of the polymer−peptide system shows a smaller fluorescence enhancement upon treatment with trypsin, illustrating the value of polymer-based amplification in this sensory scheme.

Introduction

Electronically conjugated semiconductive polymers such as poly(phenylene ethynylene)s (PPEs) have been used extensively by our group and others as signal-transducing elements for chemosensors.[1] In particular, electron-deficient analytical targets such as nitroaromatics and methyl viologen (*N*,*N*′-dimethyl-4,4′-bipyridinium) efficiently quench the fluorescence of conjugated polymers by providing a mechanism for the nonradiative recombination of electron−hole pairs (excitons).[2] This phenomenon has been used to create highly sensitive sensors for viologens and vapors of the explosive trinitrotoluene (TNT) based on a PPE fluorescence turn-off mechanism. The extraordinary signal amplification provided by exciton migration along the conjugated polymer backbone has inspired considerable effort by several groups to apply this concept toward the detection of analytes of biological interest. For such applications to be feasible, the inherently hydrophobic nature of conjugated polymer backbones must be overcome by the addition of water-solubilizing groups, usually in the form of ionized side chains. The strong interactions between the resulting conjugated polyelectrolytes[3] and charged molecules can be exploited for analytical purposes. For example, methyl viologen quenches the fluorescence of a sulfonated poly(phenylene vinylene) (PPV) with a Stern−Volmer quenching constant K_{SV} of $\sim 10^7$, and biotinylated viologen derivative was used to create an electrostatically quenched system in which fluorescence is restored upon addition of avidin.[4] Similarly, extremely efficient fluorescence quenching of conjugated polyelectrolyte fluorescence has been observed with oppositely charged Fe^{3+}-containing proteins,[5] and polymeric carbohydrate sensors utilizing viologen−boronic acid quenchers have been prepared wherein the fluorescence quenching ability of the viologen core is reduced upon complexation to the vicinal diol units of monosaccharides.[6] Very recently, PPEs containing pendant mannose units were shown to be effective in the detection of bacteria containing complementary receptors capable of polyvalent binding.[7] Other recent applications have included turn-on sensors for proteases that make use of the release of a nitroanilide group from a

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cationic peptide electrostatically bound to an anionic PPE[8] or of strongly quenching chromophores from a biotinylated peptide.[9] Biotin−avidin interactions have been used to tether nucleic acid probes to PPEs for the detection of specific DNA sequences through fluorescence quenching effects.[10] Conjugated polymer-based biosensors based on fluorescence effects other than nonradiative quenching have also been reported. The groups of Leclerc[11] and Nilsson[12] have made use of changes in the photophysical properties of cationic polythiophenes on complexation to anionic DNA to create highly sensitive DNA sensors, while Bazan and co-workers[13] have used the interaction of cationic polyfluorene derivatives with nucleic acids to control energy transfer between the conjugated polymers and fluorophore-labeled target DNA.

A unifying feature of most of the biosensory methods described above is the use of simple, unmodified conjugated polyelectrolytes as sensory signal amplification elements. Because the signal transduction mechanism in these systems is reliant on electrostatic effects—which mediate a wide variety of biomolecular interactions involving charge-bearing proteins and nucleic acids—these sensor designs necessarily suffer from the possibility of nonspecific polymer−analyte interactions that may reduce the sensitivity or fidelity of the system. Previous research by our group has sought to overcome these limitations by use of nonionic water-soluble polymers with dendritic side chains.[14] These polymers can be end-capped with hydrogel-forming, thermally responsive poly(*N*-isopropylacrylamide) blocks, allowing for temperature control of biomolecule−polymer interactions.[15] In this paper, we report an alternative approach to the construction of conjugated polymer biosensors based on water-soluble PPEs containing carboxylic acid-terminated oligo(ethylene glycol) side chains. The presence of multiple carboxylate units tethered to the polymer backbone allows for postpolymerization activation and bioconjugation of the polymer side chains to amine-containing molecules in a manner analogous to that used for small-molecule fluorophores.[16] The photophysical properties and quenching behavior of these polymers toward small-molecule nitroaromatics have been found to be highly dependent on solution pH and surfactant concentration. We have exploited the chemical and physical properties of these materials to generate a polymer-amplified turn-on fluorogenic probe for proteases based on the covalent attachment of a fluorescence-quenching 14-mer peptide to the polymer side chains. The resulting system shows a fluorescence enhancement of 1 order of magnitude upon treatment with the proteolytic enzyme trypsin.

Results and Discussion

Polymer Synthesis and Properties. Among the standard techniques used for the derivatization of biomolecules with small-molecule labels, one of the most common is the formation of amides by use of biomolecular amine groups and activated esters of carboxylate-functionalized small molecules. The broad utility of this approach to biomolecule labeling inspired us to develop parallel methods for the derivatization of bioreactive molecules with activated esters of conjugated polymers. Toward this end, we designed and synthesized a diiodophenylene monomer suitable for use in PPE synthesis in which carboxylic acid moieties are separated from the aromatic core by hydrophilic hexaethylene glycol linkers (Scheme 1). In a variation of a literature procedure,[17] a tetrahydrofuran (THF) solution of pentaethylene glycol was treated with a substoichiometric quantity of sodium metal followed by *tert*-butyl acrylate. Excess unreacted pentaethylene glycol was easily separated from the resultant Michael adduct on the basis of the differential water solubilities of the starting material and product. Conversion of the remaining free alcohol to its *p*-toluenesulfonate ester followed by Williamson etherification with 1,4-diiodohydroquinone provided the diester **6** as a clear oil in good yield. Removal of the *tert*-butyl groups with neat trifluoroacetic acid (TFA) provided the acid monomer **8** as a white powder. A similar monomer **7** based on a tri(ethylene glycol) linker was prepared by an analogous method. The presence of the hydrophilic linker groups in **8** and **7** encourages water solubility and is also expected to reduce nonspecific interactions with biomolecules. Sonogashira−Hagihara copolymerization of **8** with a dialkyne comonomer bearing di(ethylene glycol) side chains provided polymer **9a**, which dissolved in THF, *N*,*N*-dimethylformamide (DMF), and water to give brightly fluorescent solutions. Polymer **9b**, which contains tri(ethylene glycol) comonomer units, was prepared in an analogous manner, and its extremely high water solubility made it useful in photophysical studies where concentrated polymer solutions are required. Successful gel-permeation chromatography (GPC) measurements could not be made for these polymers, perhaps because of nonspecific adsorption to the column packing material, but we estimate their molecular weights to be in excess of 25 000 based on dialysis and gel-filtration behavior.

The spectral properties of **9a** and **9b** in DMF solution (λ_{abs} 430 nm, λ_{em} 470 nm) are similar to those of conventional PPEs in organic solvents. However, we noted that the shapes of the absorbance and fluorescence spectra in aqueous solution are highly pH-dependent, displaying features characteristic of polymer−polymer aggregation in unbuffered solutions that disappear when the solution is rendered alkaline (pH 11) with dilute NaOH (data not shown). These observations would seem to suggest that this polymer undergoes a pH-sensitive aggregation process in which alkaline environments increase the negative charge on the carboxylate-functionalized PPE, augmenting the water solubility of individual polymer chains and discouraging interchain interactions. A similar mechanism has been proposed to explain the pH- and solvent-dependent spectroscopic features of phosphonate- and sulfonate-substituted

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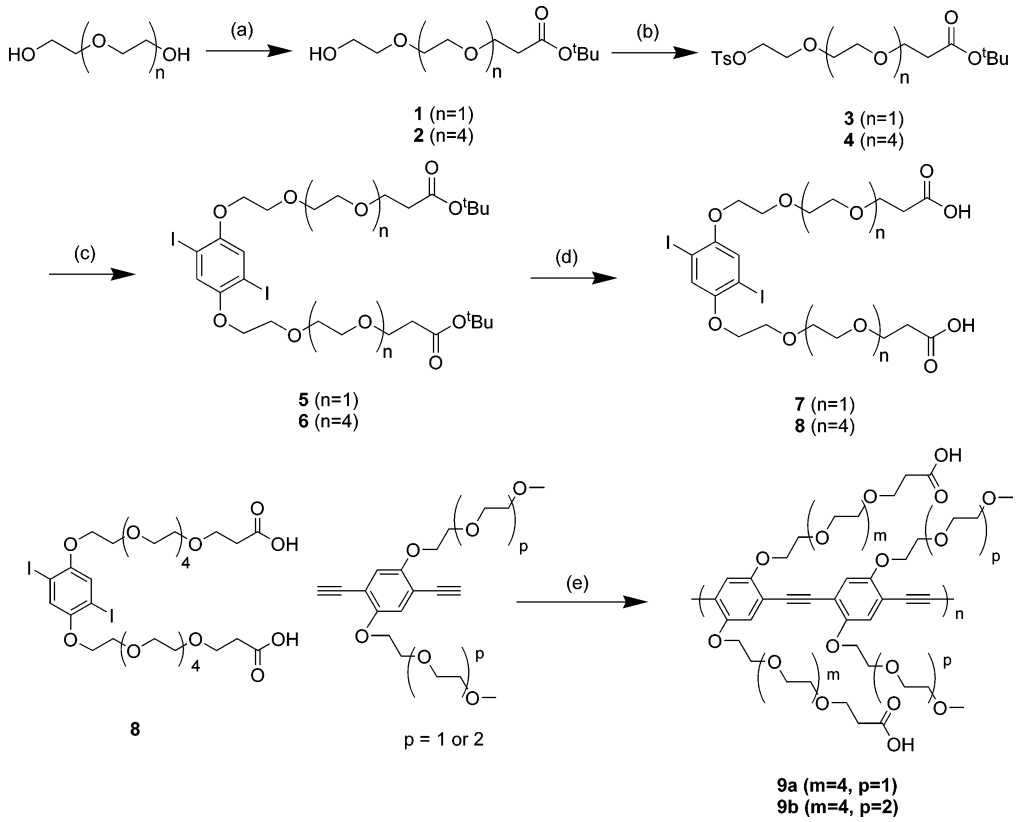
Scheme 1. Synthesis of Polymers **9a** and **9b**[a]



[a] Synthetic conditions: (a) (i) Na, THF, (ii) *tert*-butyl acrylate, 16 h; (b) *p*-toluenesulfonyl chloride, Et_3N, CH_2Cl_2, 16 h; (c) 2,5-diiodohydroquinone, K_2CO_3, KI, 2-butanone, reflux; (d) CF_3COOH, neat; (e) $Pd(PPh_3)_4$ (cat.), CuI (cat.), morpholine, 60 °C.

PPEs.[18] Although the minimization of polymer aggregation effects would seem to be necessary to achieve the greatest possible sensitivity in assay schemes that use PPE labels, the need for stable, near-neutral buffered solutions precludes the use of a highly alkaline environment to control aggregation in these applications. Instead, we screened a number of commercially available nonionic surfactants for their ability to increase the fluorescence quantum yield (QY) of these PPEs in neutral and buffered solutions. Triton X-100, an oligo(ethylene glycol) with a single hydrophobic headgroup, was found to be remarkably effective in this regard: the addition of about 0.5 wt % of this surfactant to a solution of **9b** in unbuffered water increased the QY of the polymer from 0.11 to 0.20. The same effect was observed in a solution of **9b** in a pH 7.5 Tris-buffered saline (TBS) solution, although the magnitude of the enhancement was not as large (Figure 1). This difference can be attributed to the larger inherent hydrophobic forces in solutions of high ionic strength.

The data in Figure 1 clearly show that 30−50% increases in QY are possible with the addition of small concentrations of Triton X-100. Concomitant with the fluorescence intensity increases is a gradual change in the shape of the fluorescence spectra, in which the 480 nm red-shifted emission maximum suggestive of an aggregate[19] gives way to a single fluorescence feature at 460 nm. In this case, the addition of surfactant results in a fluorescence profile for **9b** that resembles the spectral features of PPEs typically observed in good solvents. The results

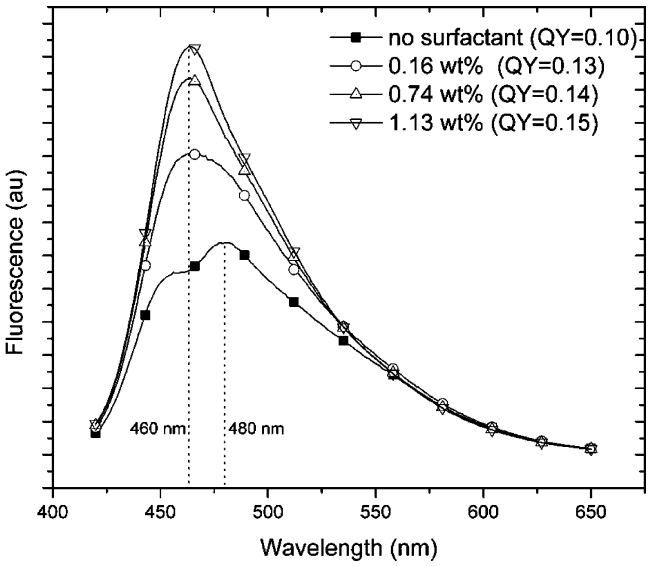


Figure 1. Fluorescence enhancement of polymer **9b** (0.7 μM in repeat units) in Tris-buffered saline (TBS; 50 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM $CaCl_2$) upon addition of Triton X-100, with approximate quantum yields. The concentration of polymer is identical in each of the scans.

of the pH and surfactant experiments suggest that in aqueous solution the polymer exists in equilibrium between an aggregated, π-stacked form of relatively low QY and a highly solubilized form of higher QY in which interchain interactions are minimized. To determine if these two forms can be identified photophysically, the fluorescence lifetime of **9b** was measured in DMF and three different buffers, both with and without added surfactant (Table 1).

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Table 1. Fluorescence Lifetimes of **9b** in Aqueous Buffer Solutions and in DMF[a]

surfactant/buffer	pH 5.0 (acetate)	pH 7.3 (PBS)	pH 8.5 (borate)	DMF
no surfactant	0.64 (0.82) 3.81 (0.18)	0.64 (0.82) 4.05 (0.18)	0.67 (0.93) 3.97 (0.07)	0.77
0.1 wt % Triton X-100	0.77 (0.97) 3.90 (0.03)	0.70 (0.87) 3.24 (0.13)	0.75 (0.98) 3.71 (0.02)	
0.2 wt % Triton X-100		0.73 (0.92) 4.24 (0.08)		

[a] Fluorescence lifetimes are given in nanoseconds. In cases where the fluorescence follows a biexponential decay pattern, the two component lifetimes are given along with their relative contributions in parentheses (to a total of 1.0). Polymer solutions were excited at 405 nm and fluorescence was recorded at 470 nm by use of RF phase modulation (see Supporting Information).

The fluorescence lifetime of **9b** in DMF solution was 0.77 ns, a value typical for PPEs in good solvents. In the case of aqueous solutions, we found that the fluorescence decay was biexponential, indicating the presence of two distinct fluorescent species in solution or two radiative decay pathways.[20] The components of the fluorescence decay correspond to a dominant short-lived species of $\tau = 0.6-0.7$ ns—similar to the lifetime of the polymer in DMF—together with minor contributions (2−18% of the observed fluorescence decay) from a longer-lived 3−4 ns component. As Table 1 indicates, the relative populations of the two species are strongly affected by the addition of surfactant, which was found to greatly increase the contribution of the short-lifetime component at the expense of the longer-lived species. Alkaline buffer environments also generally favored increased contributions from the short-lifetime component. In general, the data indicate that the same conditions that promote large quantum yields and sharp emission spectra (organic solvents, alkaline pH values, or surfactant in aqueous solutions) also result in an increase in the relative contribution of the short-lived species. These observations are consistent with a model in which the polymer chains are aggregated in neutral to acidic solutions and well-solvated in organic solvents, when the solution is rendered alkaline, or when surfactant is added. In this case the short-lived component of the fluorescence lifetime corresponds to the nonaggregated polymer and makes a larger contribution to the overall fluorescence lifetime when the relative proportion of polymer chains in this state is increased. Similarly, the long-lived fluorescence decay can be assigned to polymer aggregates that are broken up at conditions of high pH and surfactant concentration and are completely absent in DMF solutions. These aggregates are expected to form through strong hydrophobic and π-stacking interactions between polymer chains, and their long fluorescence lifetime is the signature of a weakly allowed transition from the lowest excited state of the aggregate to the ground state.[18] In phosphate-buffered saline (PBS) solutions of **9b**, the high ionic strength of the medium encourages greater aggregation through hydrophobic interactions, requiring higher concentrations of surfactant to break up aggregates relative to buffer solutions lacking added salts.

Polymer-Based Protease Substrate Design and Quenching Efficiency. In conventional fluorogenic protease substrates, a fluorescent donor and nonfluorescent acceptor dye are separated by a peptide sequence known to be cleaved by the enzyme of interest.[21] Internal energy transfer maintains the system in a nonfluorescent state until the peptide is cleaved. Modifications of this general design have given rise to substrates in which the peptide and fluorophore are tethered to a dendrimer[22] or a soluble, nonconjugated polymer support.[23] As an initial demonstration of the utility of the carboxylate-containing polymers described above, we chose to construct a fluorogenic protease substrate in which a water-soluble PPE acts as both soluble support and fluorophore. The electron-accepting 2,4-dinitrophenylamino group (DNP) stood out as an excellent potential fluorescence quencher for this system because of the known sensitivity of PPE fluorescence to the presence of nitroaromatics[2] and the commercial availability of Fmoc-protected DNP-substituted lysine (Fmoc-LysDNP) to facilitate incorporation of the quenching unit during peptide synthesis.

To verify the quenching ability of the DNP group toward carboxylate-functionalized water-soluble PPEs, Stern−Volmer quenching constants K_{SV} were measured for LysDNP under a variety of conditions. In unbuffered water solutions, polymer **9b** was quenched by free LysDNP with a K_{SV} of 5.8×10^4 M^{-1}. The presence of Triton X-100 at low concentration reduced this value by a factor of 4 to 1.4×10^4 M^{-1}, similar to the K_{SV} determined in Tris-buffered saline (1.5×10^4 M^{-1}). Addition of surfactant to the TBS solution reduced K_{SV} still further to 1.0×10^4 M^{-1}. Comparable behavior was observed for polymer **9a**. Given the nanosecond-scale fluorescence lifetimes described above, the large magnitudes of K_{SV} in all these cases strongly implies that static binding of LysDNP is responsible for the observed quenching, and in this context the differences in K_{SV} observed under various solvent and surfactant conditions can be understood in terms of variations in the effective hydrophobically driven association constant of LysDNP and the PPE. Here the addition of surfactant and the use of a controlled near-neutral pH reduces hydrophobic interactions between LysDNP and the PPE, suggesting that the pH, ionic strength, and surfactant concentration of the assay medium will be important in determining residual background fluorescence and maximum fluorescence enhancement in our proposed protease substrate.

To link the fluorescence-quenching DNP group to the PPE, we chose to use a peptide sequence (GPLGMRGL) that has previously been identified as a potent substrate for a variety of matrix metalloproteases, most notably the cancer-associated human collagenase III (MMP-13).[24] We also noted that the single arginine residue in the middle of the peptide sequence renders it susceptible to cleavage by trypsin, an inexpensive and easily handled digestive enzyme that hydrolyzes peptides on the carboxyl side of unmodified arginine and lysine residues. The peptide sequence AcHN-Lys(DNP)-GPLGMRGLGGGG-Lys-OH was designed to incorporate both the fluorescence-quenching DNP group as part of the amino acid sequence and

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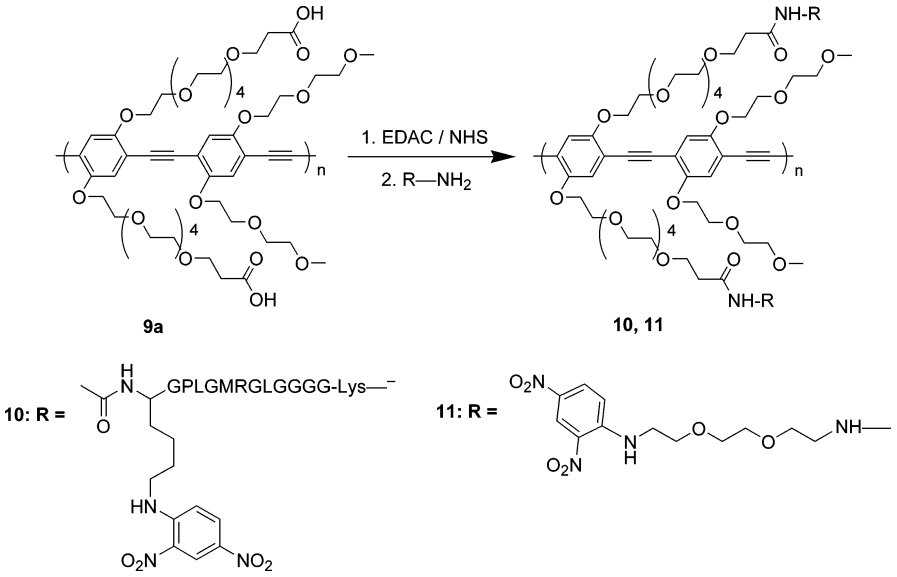
Scheme 2. Synthesis of Quenched Peptidic (**10**) and Nonpeptidic (**11**) Fluorogenic Substrates[a]



[a] Substrates are shown fully substituted for simplicity (see text).

a C-terminal lysine residue through which coupling to the PPE can take place via an amide linkage. The point of attachment is separated from the cleavage sequence by four glycine spacer units. The peptide was synthesized by solid-phase techniques and end-capped with an acetyl group on the resin. The peptide was then coupled to **9a** after activation of the polymer with excess EDAC and *N*-hydroxysuccinimide, providing **10** after dialysis.[16] Polymer **11**, an analogue of **10** containing a nonpeptidic oligo(ethylene glycol) spacer, was prepared by similar means (Scheme 2). The peptide loading in **10** was estimated by quantifying the contributions of the DNP and PPE chromophores to the absorption spectrum of dialyzed **10**, giving values between 0.25 and 1.7 peptide chains/phenylene ethynylene repeat unit, depending on the stoichiometry and activation time of the conjugation reaction. Solutions of **10** were visibly nonfluorescent, and related control experiments revealed that prior activation of **9a** with carbodiimide was necessary for the generation of internally quenched substrates.

Protease Detection by Use of a Polymer-Based Fluorogenic Substrate. To test the efficacy of **10** as a protease sensor, a sample of **10** (1.1 μM in repeat units) with high peptide loading (1.7 peptides/repeat unit) was subjected to hydrolysis by trypsin in dilute solution (3 μg/mL). As shown in Figure 2, the system exhibits an increase in fluorescence of approximately 1 order of magnitude after hydrolysis, while the absorbance spectrum remains largely unchanged. Control experiments (Figure 2b) showed that turn-on of fluorescence in the presence of trypsin is rapid but is slowed considerably by the trypsin inhibitor benzamidine hydrochloride. No fluorescence increase occurs with the nonpeptidic substrate **11** or in the presence of the inhibitor alone.

Ionic Strength and Surfactant Effects in Fluorescence Turn-on. To further study the mechanism of fluorescence turn-on in this system, we examined the overall response and relative kinetic behavior of **10** lightly loaded with fluorescence-quenching peptide (0.25 peptide chain/polymer repeat unit) with trypsin in solutions containing varying concentrations of buffer (Tris, pH 7.5), salts (NaCl, $CaCl_2$), and surfactant (Triton X-100). The data in Figure 3 show that larger trypsin-induced

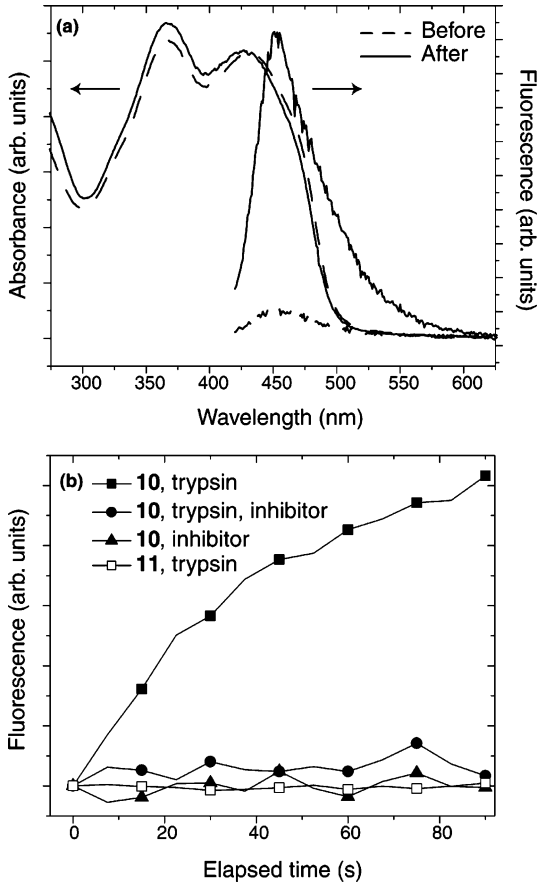
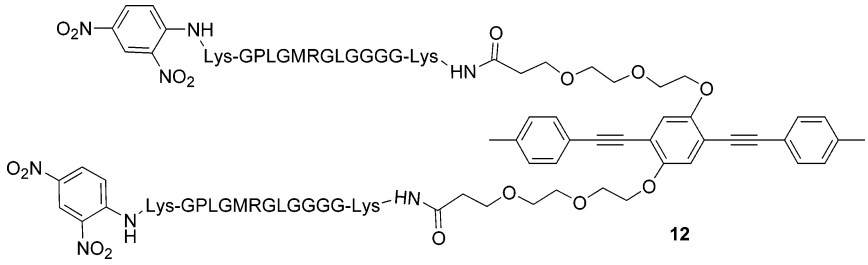


Figure 2. (a) Absorbance (left) and fluorescence (right) spectra of substrate **10** (1.1 μM in repeat units) before and after treatment with trypsin (3 μg/mL). (b) Generation of fluorescence as a function of time on treatment of **10** and control substrate **11** with trypsin in the absence and presence of an inhibitor (benzamidine hydrochloride).

fluorescence enhancements are generally favored by conditions of lower ionic strength and the absence of surfactant. In the latter case, the presence of surfactant reduces the overall degree of fluorescence enhancement by augmenting the fluorescence of both the initial (internally quenched) and, to a smaller extent,

Chart 1. Structure of **12**, a Small-Molecule Mimic of **10**



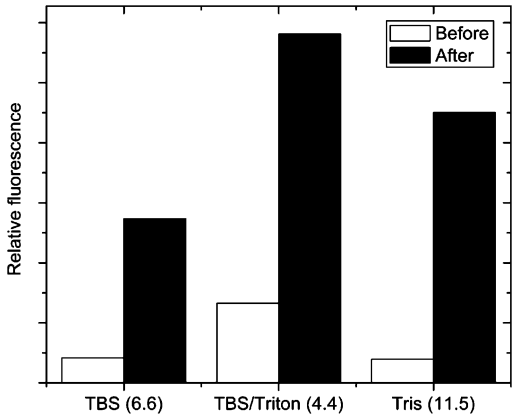


Figure 3. Overall fluorescence enhancement in substrate **10** (0.25 peptide/repeat unit) before and after hydrolysis by trypsin. Numbers in parentheses indicate the overall fluorescence enhancement. TBS: 50 mM Tris, pH 7.5, 150 mM NaCl, and 5 mM $CaCl_2$. TBS/Triton: as for TBS, with 0.08% Triton X-100. Tris: 50 mM Tris, pH 7.5.

Table 2. Relative Initial Reaction Rates and Initial Rates of Fluorescence Increase for the Hydrolysis of **10** under Conditions of Varying Ionic Strength and Surfactant Concentration

	relative rates	
conditions[a]	peptide cleavage	fluorescence generation
TBS	1	1
TBS/Triton	1.1 ± 0.2	2.9 ± 0.2
Tris	4.1 ± 0.6	5.3 ± 0.2

[a] See caption to Figure 3.

the final (cleaved) forms of **10**.[25] The fact that this lightly loaded polymeric substrate shows an overall fluorescence enhancement of a magnitude similar to that seen in our initial experiment (1.7 peptide chains per polymer repeat unit) attests to the ability of relatively few DNP groups to efficiently quench the fluorescence of an entire PPE chain.

The kinetic behavior of the lightly loaded protease substrate described above was investigated by measuring the relative initial rates of peptide hydrolysis (measured by HPLC) and fluorescence turn-on under the same set of ionic strength and surfactant conditions. As shown in Table 2, the initial reaction rate is greatly increased under conditions of lower ionic strength. This follows general trends previously reported for the trypsin hydrolysis of *N*-α-benzoylarginine ethyl ester (BAEE).[26] Interestingly, we found that while the addition of Triton X-100 to the reaction medium did not significantly change the rate of peptide cleavage, it did increase the rate of fluorescence generation by a factor of 3. The kinetic and overall fluorescence enhancement data suggest that a strong hydrophobic effect mediates the interaction between the PPE backbone and the fluorescence-quenching peptide fragment both before and after hydrolysis of the peptide linker. By solubilizing hydrophobic portions of the oligopeptide, the surfactant reduces quencher−polymer interactions both before and after peptide cleavage and leads to a faster rate of fluorescence increase.

To quantify the sensory advantage provided by use of a conjugated polymer as the fluorophore in **10**, we synthesized the substrate **12**, a small-molecule mimic of **10** (Chart 1). Relative to **10**, substrate **12** has a shorter side-chain linker between the fluorophore and the peptide chain in addition to a larger effective peptide loading (2 peptide units/molecule). Although both of these factors would ordinarily be expected to improve the efficiency of **12** as a fluorogenic probe, its fluorescence intensity increases only about 2.9-fold upon hydrolysis by trypsin, an overall increase several times smaller than that seen for **10**. In this case, the favorable contributions of hydrophobic effects and exciton migration in **10** negate the effects of the larger fluorophore−quencher distance and lower quencher loading relative to **12**.

In conclusion, we have prepared water-soluble poly(phenylene ethynylene)s containing carboxylate-functionalized side chains. These materials show solvent- and surfactant-dependent photophysical properties and are efficiently quenched by the dinitrophenyl (DNP) chromophore. Derivatization of these polymers with a fluorescence-quenching peptide susceptible to proteolytic cleavage by trypsin provides a fluorogenic substrate that shows large fluorescence increases upon hydrolysis by this enzyme, even in cases of extremely low peptide loading.

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Supporting Information Available: Synthetic and experimental procedures (PDF). This information is available free of charge via the Internet at http://pubs.acs.org.

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(25) We have found that the addition of Triton X-100 increases the fluorescence quantum yield of neutral, PEGylated PPEs in water approximately 14-fold. Surfactants have previously been found to significantly increase the quantum efficiency of poly(phenylene vinylene)s: Chen, L. H.; Xu, S.; McBranch, D.; Whitten, D. *J. Am. Chem. Soc.* **2000**, *122*, 9302.

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