Anal. Chem. **2008,** *80,* 150−158

Conjugated Polyelectrolyte Based Real-Time Fluorescence Assay for Phospholipase C

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A fluorescence turnoff assay for phospholipase C (PLC) from *Clostridium perfringens* is developed based on the reversible interaction between the natural substrate, phosphatidylcholine, and a fluorescent, water-soluble conjugated polyelectrolyte (CPE). The fluorescence intensity of the CPE in water is increased substantially by the addition of the phospholipid due to the formation of a CPE−lipid complex. Incubation of the CPE−lipid complex with the enzyme PLC causes the fluorescence intensity to decrease (turnoff sensor); the response arises due to PLC-catalyzed hydrolysis of the phosphatidylcholine, which effectively disrupts the CPE−lipid complex. The PLC assay operates with phospholipid substrate concentrations in the micromolar range, and the analytical detection limit for PLC is <1 nM. The optimized assay provides a convenient, rapid, and real-time sensor for PLC activity. The real-time fluorescence intensity from the CPE can be converted to substrate concentration by using an *ex situ* calibration curve, allowing PLC-catalyzed reaction rates and kinetic parameters to be determined. PLC activation by Ca^{2+} and inhibition by EDTA and fluoride ion are demonstrated using the optimized sensor.

Several recent studies have shown that water-soluble, fluorescent π-conjugated polyelectrolytes (CPEs) provide a useful platform for the development of highly sensitive fluorescence-based sensors for biomolecules[1−4] such as proteins,[1,5−7] DNA,[4,8−10] carbohydrates,[11] and enzymes.[12−16] The high sensitivity of CPE assays takes advantage of the intrinsic fluorescence signal amplification that results from the superquenching behavior of CPEs.[1,2,17−22] Such behavior is attributed to a combination of several factors, including delocalization and rapid diffusion of the singlet exciton along the CPE backbone to the quencher "trap site", as well as the electrostatic ion pairing of quenchers and with oppositely charged CPE side chains.[1,17,19,23−25]

It has also been demonstrated that water-soluble CPEs interact with surfactants resulting in significant but reversible changes in the photophysical properties[17,20,26,27] of the polymer. Studies indicate that the CPE and surfactants form a polymer−surfactant complex due to a combination of electrostatic interaction between the polymer ionic side groups and the surfactant head groups, as well as hydrophobic interactions between the surfactant tails and the conjugated polymer backbone. Formation of the polymer−surfactant complex induces the CPE chains to become more extended, and more importantly, polymer−polymer interaction (aggregation) is disrupted.[17,20,26] The changes in the microenvironment of the polymer in the polymer−surfactant complex result

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10.1021/ac701672g CCC: $40.75
Published on Web 11/29/2007

in a narrowing and blue-shift of the fluorescence, coupled with a significant increase in the fluorescence quantum yield.[17,20,26]

The earlier studies of CPE−surfactant complexes provide the basis for the study presented herein, where we explore the interactions between an anionic CPE and phospholipids. Phospholipids are naturally occurring amphiphiles that serve as the major component of biological membranes.[28] In the present study, we demonstrate that phospholipids interact strongly with CPEs, eliciting significant changes in the fluorescence properties of the polymer. The effects are reversible, and consequently, the CPE−lipid complex provides a platform for the development of a fluorescence turnoff assay for the lipase enzyme phospholipase C (PLC).[29]

PLC catalyzes the hydrolysis of the phosphate ester in a phospholipid selectively at the glycerol side,[29] yielding a diacylglycerol (DAG) and a phosphate-containing head group. The ability to quantitatively monitor PLC catalytic activity and inhibition is important as DAGs play a critical role in cell function and the signal transduction cascade in mammalian systems.[30−33] Several PLC assays have been developed based on turbidimetric,[34,35] pH-stat titration,[36] radiometric,[37,38] and continuous fluorometric[39−48] methods. However, the turbidimetric and titration assays suffer from low sensitivity,[34−36,43] and the inherent disadvantage that large quantities of enzyme and substrate are required. Although the radiometric assay attains the lowest detection limit for PLC that has been reported, ^{3}H-,[49] ^{14}C-,[50] ^{32}P-.[51] or ^{125}I-[38] labeled phospholipids are required as substrates making the approach expensive, laborious, and time-consuming. Fluorometric assays based on the determination of choline[46] or inorganic phosphate[45] offer high sensitivity and are continuous. Although they are advantageous when carrying out studies on enzyme kinetics,[43] most of the fluorometric assays require synthetic fluorogenic substrates,[39−44,46−48] which results in low catalytic turnover of enzymes and reduced reaction rates.[46,47]

We now introduce a sensitive and specific fluorescent turnoff assay for PLC. Like other fluorescence assays, the method is convenient, but it has the additional advantage of being based on natural lipid substrates. The assay is based on the reversible change in fluorescence properties of an anionic CPE induced by the formation of a polymer−phospholipid complex. In particular, the fluorescence of the CPE is enhanced and blue-shifted upon complexation with phosphatidylcholine. Incubation of the polymer−phospholipid complex with PLC results in a decrease of the fluorescence, which is due to the enzyme-catalyzed hydrolysis of the phospholipid. The PLC enzyme assay conditions are optimized, and the effects of the addition of an activator (Ca^{2+}) and several inhibitors of PLC are studied. The assay is calibrated for substrate concentration, allowing the determination of the catalytic kinetic parameters, K_m and V_{max}.

MATERIALS AND METHODS

Materials. $BpPPESO_3$ (an anionic PPE-type CPE, structure shown in Figure 1a), was synthesized according to a literature method.[19] All substrates, enzymes, and proteins were purchased from Sigma-Aldrich and used as received, unless otherwise noted. Calcium chloride and ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA) were purchased from Fisher Chemical. Sodium fluoride was obtained from Mallinckrodt Chemical Works, and 1,2-didecanoyl-*sn*-glycerol (DAG) was obtained from Cayman Chemical. All solvents were obtained from Fisher and used without further purification. Water was distilled and then purified by using a Millipore purification system.

Solution Preparation. Buffer solutions (pH 7.4) were prepared with Tris base and hydrochloric acid. A concentrated aqueous solution of $BpPPESO_3$ was diluted with buffer solution to a final concentration ranging from 0.1 to 15 μM. The stock solutions of substrates, enzymes, and proteins were prepared immediately before their use in the fluorescence assay. The enzyme substrate, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine (10CPC) was dissolved in methanol and adjusted to 20 mM as stock concentration. Phospholipase C from *Clostridium perfringens* (*Clostridium welchii*) (PLC) was dissolved in 50 mM Tris-HCl buffer solution and adjusted to 10 μM as stock concentration, and the assays were conducted in the same buffer. Control enzymes and proteins include phospholipase A_2 from bovine pancreas (PLA_2), phospholipase D from *Arachis hypogaea* (peanut) (PLD), bovine serum albumin (BSA), avidin from egg white (AVI), and peptidase from porcine intestinal mucosa (PEP). They were used in place of phospholipase C in control experiments. Calcium chloride (1.0 M), sodium fluoride (0.2 M), and EDTA (0.2 M) were dissolved in water as stock solutions. Sodium deoxycholate (SDC) was dissolved in methanol as 0.2 M stock solution.

Instrumentation. UV−visible absorption spectra were obtained on a Perkin-Elmer Lambda 25 UV/vis spectrophotometer, with a scan rate of 960 nm·min^{-1}. Fluorescence spectra were recorded on a Jobin Yvon-SPEX Industries Fluorolog-3 model FL3−21 spectrofluorometer and corrected by using correction factors generated in-house with a primary standard lamp. The fluorescence cuvette was placed in a custom-built thermostated

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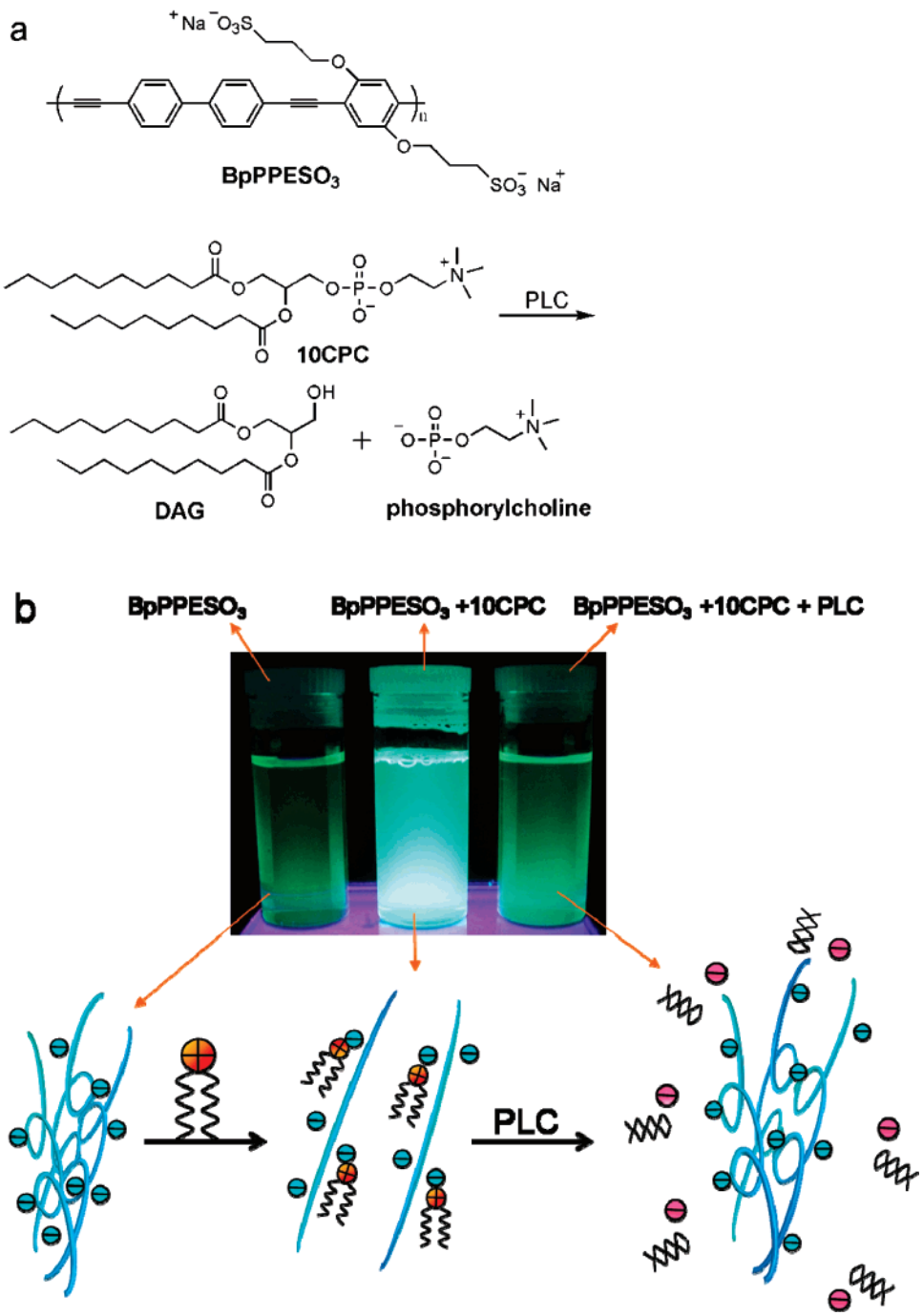

Figure 1. (a) Structures of polymer, $BpPPESO_3$ and substrate, 10CPC, and reaction scheme for hydrolysis of 10CPC by PLC. (b) Mechanism of PLC turnoff assay.

cell holder, which was maintained at 37 °C during the assay and was equipped with a microsubmersible magnetic stirrer.

Fluorescence Turnoff Assay Procedure. The PLC enzyme assays were carried out at 37 °C in a fluorescence cuvette with continuous stirring. For kinetics studies, the fluorescence intensity was measured with excitation and emission wavelengths of 400 and 460 nm, respectively. A typical assay procedure was carried out as follows. First, a 2.0-mL aliquot of the $BpPPESO_3$ solution was allowed to thermally equilibrate at 37 °C, and then the initial polymer fluorescence intensity (I_p) at 460 nm was measured. The substrate (10CPC) was added to a second 2.0-mL aliquot of $BpPPESO_3$ solution, this mixture was incubated for 15 min, and then the solution was placed in the spectrophotometer, and the fluorescence intensity (I_{bt}) as a function of time was recorded as a blank. (Note that the blank signal I_{bt} decreased slightly with time due to photobleaching of the polymer; see Supporting Information for more detail.) Another freshly prepared 2.0-mL aliquot of $BpPPESO_3$/10CPC solution was incubated at 37 °C, and then it was quickly pipetted into a cuvette containing a 4-μL aliquot of the enzyme solution, and the fluorescence intensity of the $BpPPESO_3$/10CPC/PLC solution was monitored as a function of time (I_t). Subsequently, the sample fluorescence intensity I_t was corrected for photobleaching by using the blank intensity I_{bt} (see Supporting Information for details and validation of the correction

procedure). The sample and blank fluorescence intensity (I_{bt} and I_t, respectively) were measured using the same conditions. After correction for photobleaching, the corrected fluorescence intensity I_{tc} at each time t was derived.

For full wavelength-spectral scans of the assays, the fluorescence intensity (I_p, or I_t) versus wavelength profiles were recorded with excitation wavelength at 400 nm. Because fresh assay solution was used for spectral measurements at each time t, it was not necessary to correct for photobleaching.

Calculation of Initial Rate of Reaction (v_0). I_{tc} was converted to substrate concentration $[10CPC]_t$ as a function of time by using eq 1, which is derived from the calibration plot of fluorescence intensity ratio (I/I_p) versus concentration of substrate [10CPC].

$$[10CPC]_t = [10CPC]_0 \frac{I_{tc}/I_p - 1}{I_{0c}/I_p - 1} \quad (1)$$

In eq 1, $[10CPC]_0$ is the initial substrate concentration, $[10CPC]_t$ is the substrate concentration at time t, I_p is the fluorescence intensity of the polymer solution before addition of substrate, I_{0c} is the initial corrected fluorescence intensity at $t = 0$, that is, the fluorescence intensity after addition of substrate but before the addition of enzyme and I_{tc} is the corrected fluorescence intensity at time t after enzyme addition. A plot of $[10CPC]_t$ vs time was then derived and the v_0 was calculated from the slope of the plot by using data for the region where hydrolysis of 10CPC is less than 5 μM.

RESULTS AND DISCUSSION

Overview of Turnoff Assay. The structure of the conjugated polyelectrolyte used in the assay, $BpPPESO_3$, is shown in Figure 1a. The synthesis and characterization of the polymer is described in the Supporting Information. Previous studies on structurally similar, anionic PPE-type CPEs have shown that in water these polymers exist as aggregates. The characteristic feature of the aggregated state of CPEs is that the fluorescence emission appears as a broad, structureless band that is Stokes shifted significantly from the absorption band. Like the previously studied CPEs, in water, $BpPPESO_3$ exists in an aggregated state, as is clearly signaled by the broad, structureless fluorescence band that is Stokes shifted from the absorption band ($\lambda_{max}^{flr} = 503$ nm and $\lambda_{max}^{abs} = 419$ nm).

The cartoon shown in Figure 1b illustrates the mechanism of the PLC turnoff assay. Although 10CPC is a zwitterion, electrostatic (ion−dipole) and hydrophobic forces induce the formation of a polymer−lipid complex between the phospholipid and $BpPPESO_3$. As a result, the backbone of $BpPPESO_3$ is more extended and aggregation of the polymer is reduced. The lipid-induced changes in polymer conformation and aggregation state are signaled by a blue-shift and enhancement in the polymer's fluorescence intensity. Introduction of PLC to the polymer−lipid complex induces hydrolysis of 10CPC. The PLC catalyzes hydrolysis of the zwitterionic head group from the hydrophobic tail, disrupting the amphiphilic structure of the lipid, which is a key factor allowing 10CPC to complex with the polymer. One of the 10CPC hydrolysis products, DAG, is hydrophobic and charge neutral while the second product, phosphorylcholine, has a net negative charge. Apparently neither of these species interacts with $BpPPESO_3$ to disrupt its aggregation. As a result, the polymer's fluorescence reverts to its original (aggregated) state, in which the spectrum is red-shifted and less intense. As shown by the photograph in Figure 1b, the changes in $BpPPESO_3$ fluorescence that accompany the assay are easily observed by eye. After addition of 10CPC to the solution of $BpPPESO_3$ the fluorescence is considerably brighter (compare middle vial with left vial), and the subsequent addition of PLC causes the fluorescence intensity to decrease again (right vial). The 10CPC/PLC assay is rendered quantitative by applying a calibration (eq 1), which relates the $BpPPESO_3$ fluorescence intensity to 10CPC concentration. This allows one to determine the enzyme reaction kinetics from changes in polymer fluorescence intensity.

Effect of 10CPC on Fluorescence of $BpPPESO_3$. A series of titrations was carried out to quantify the effects 10CPC and polymer concentration on the polymer's absorption and fluorescence. Upon addition of 10CPC to an aqueous $BpPPESO_3$ solution at room temperature, the absorption of the polymer blue-shifts by only 2 nm without a significant change in the shape of the absorption band (see Supporting Information). However, as shown in Figure 2a, the fluorescence spectrum changes dramatically. In particular, addition of 10CPC (0−15 μM) to a solution of $BpPPESO_3$ ($c = 1$ μM) induces a blue-shift of the fluorescence maximum from 503 to 436 nm combined with a 50-fold increase in the fluorescence intensity at 436 nm. The significant change of the fluorescence intensity suggests that 10CPC inhibits the aggregation of the polymer as has been reported for the addition of other surfactants to CPEs.[20,27]

Figure 2b illustrates the plots of the relative fluorescence intensity at 436 nm as a function of concentration of added 10CPC at different $BpPPESO_3$ concentrations. For each plot, the fluorescence intensity increases gradually at low 10CPC concentration. After the concentration of 10CPC reaches a certain point, the intensity increases sharply and linearly until reaching a plateau, at which point further addition of 10CPC causes little additional change in the intensity. (The plot for 5 μM $BpPPESO_3$ in Figure 2b only shows the effect of added 10CPC below the concentration range where the sharp increase is observed.) Note that as the $BpPPESO_3$ concentration increases, more lipid is needed to induce the fluorescence change. This is consistent with the involvement of a polymer−lipid complex in the observed fluorescence intensity enhancement. Importantly, over the concentration range in which the fluorescence intensity increases sharply, the relative increase in fluorescence intensity (I/I_p) is nearly linearly proportional to 10CPC concentration. This suggests that is should be possible to create a calibration curve that relates fluorescence intensity to 10CPC concentration.

Prior to developing a calibration allowing one to follow the 10CPC substrate concentration during PLC hydrolysis, a study was carried out to determine the solution conditions under which the PLC activity was optimized, and yet the polymer's response to 10CPC was still acceptable. This survey demonstrated that the optimum solution conditions include the use of Tris-HCl buffer ($c = 50$ mM, pH 7.4) along with added Ca^{2+} as an activator for the PLC.[29] Figure 3 shows the effect of 10CPC on the fluorescence of 15 μM $BpPPESO_3$ in 50 mM Tris-HCl with 2 mM Ca^{2+} at 37 °C. Note that, under these solution conditions, upon addition of 10CPC the fluorescence of $BpPPESO_3$ is blue-shifted and en-

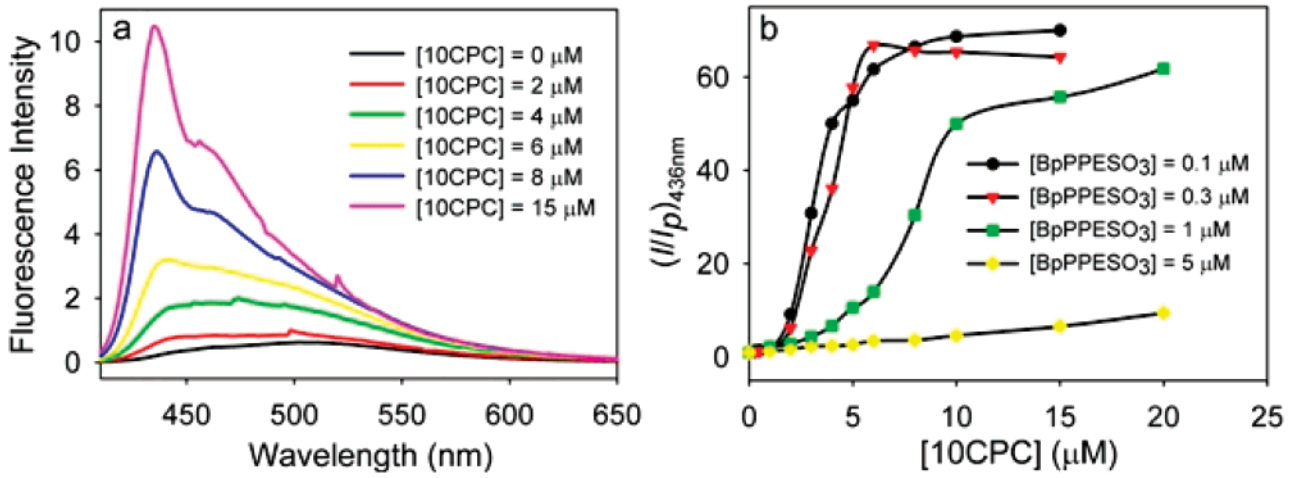

Figure 2. (a) Fluorescence spectroscopic changes of a solution of 1 μM $BpPPESO_3$ in water observed upon addition of 10CPC at 25 °C, λ_{ex} = 400 nm. (b) Fluorescence intensity increase at 436 nm upon titration of 10CPC at different concentrations of $BpPPESO_3$ in water, λ_{ex} = 400 nm.

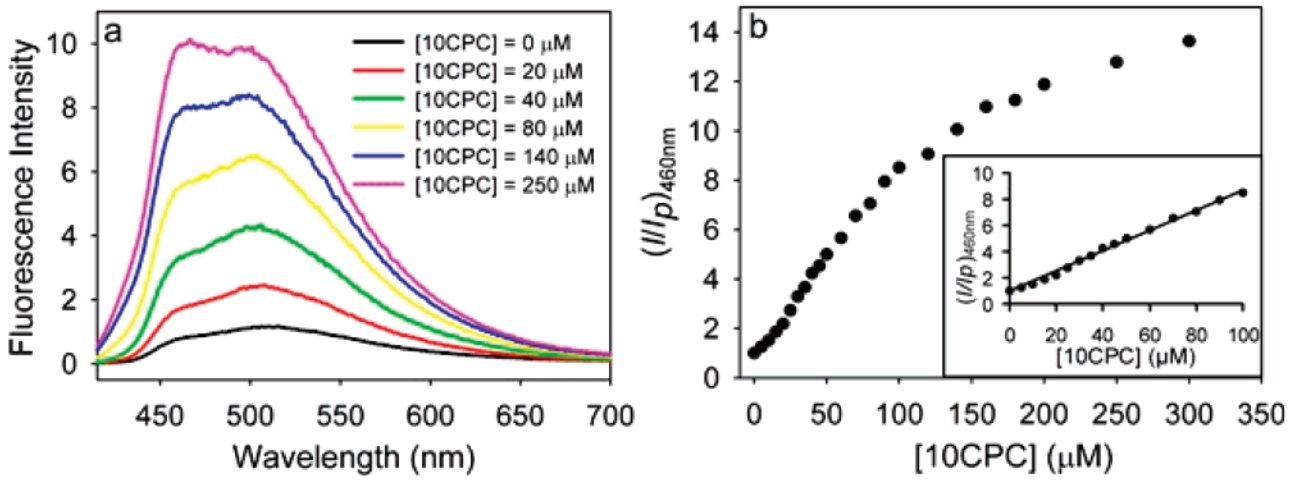

Figure 3. (a) Fluorescence spectroscopic changes for a solution of $BpPPESO_3$ (15 μM) observed upon addition of 10CPC in 50 mM Tris-HCl (pH 7.4) with 2 mM Ca^{2+} at 37 °C, λ_{ex} = 400 nm. (b) Fluorescence intensity increase at 460 nm upon titration of 10CPC, λ_{ex} = 400 nm. Inset: calibration plot of relative fluorescence intensity increase as a function of 10CPC concentration.

hanced to a lesser extent in comparison to the change in fluorescence for the polymer seen in pure water (see Figure 2a). This difference arises because Ca^{2+} induces aggregation of $BpPPESO_3$,[2,52] and this effect partially offsets the ability of 10CPC to influence the extent of $BpPPESO_3$ aggregation. Despite the fact that under these solution conditions more 10CPC is needed to elicit a strong fluorescence response, at [$BpPPESO_3$] = 15 μM, a 10-fold fluorescence enhancement is observed upon addition of 100 μM 10CPC. In particular, Figure 3b illustrates the increase in fluorescence intensity at 460 nm (λ_{ex} = 400 nm) as a function of added 10CPC. This plot features a trend similar to that shown in Figure 2b, with the $BpPPESO_3$ exhibiting a significant and nearly liner intensity increase until reaching a plateau for [10CPC] > 250 μM. The inset in Figure 3b shows a calibration plot of the relative fluorescence intensity increase (I/I_p) as a function of [10CPC]. The plot features a good linear relationship over the concentration range 0−100 μM with a *y*-intercept of 1, and consequently, the [10CPC] is directly proportional to (I/I_p − 1) at any time during a PLC hydrolysis reaction. This calibration is used to derive eq 1, which is used to determine $[10CPC]_t$ during the PLC assay.

PLC Turnoff Assay. In order to demonstrate the feasibility of using $BpPPESO_3$ as the basis for a PLC turnoff assay, we initially examined the effect of added 10CPC and PLC on the polymer's fluorescence intensity in water without added buffer or Ca^{2+}. The results of this initial experiment are shown in Figure 4. In particular, the initial fluorescence from a solution $BpPPESO_3$ (c = 1 μM) is blue-shifted and significantly enhanced in intensity by addition of 10 μM 10CPC (change indicated by arrow 1). After introduction of PLC (c = 2.3 nM) to the solution, hydrolysis of 10CPC causes a red shift of the fluorescence band and a decrease in intensity with increasing incubation time (indicated by arrow 2). The $BpPPESO_3$ fluorescence decreases ~50% within 1 min even in the unbuffererd solution and without added Ca^2 activator, which demonstrates the potential for the PLC turnoff assay.

In order to optimize the conditions for the PLC assay, the effect of the Ca^{2+} activator was investigated when assays were conducted in 50 mM Tris-HCl buffer (pH 7.4). Calcium ion serves as an activator for PLC because Ca^{2+} interacts with the substrate

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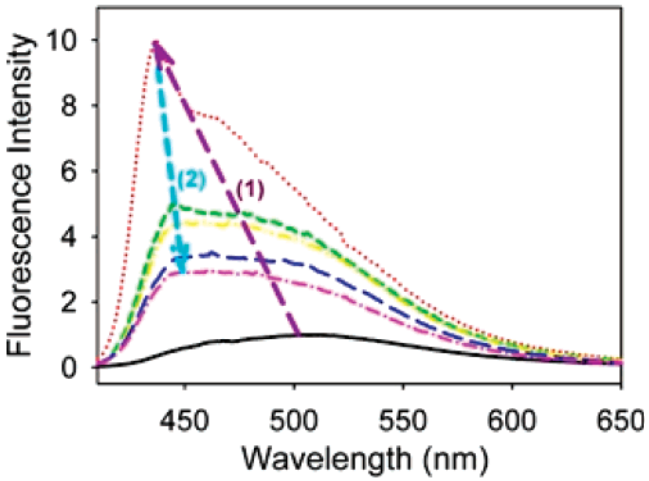

Figure 4. Fluorescence spectroscopic changes observed in the PLC turnoff assay. (—) Initial fluorescence of 1.0 μM $BpPPESO_3$ in water at 25 °C; (••) fluorescence after step 1: addition of 10 μM 10CPC; fluorescence intensity as a function of time after step 2: addition of 2.3 nM PLC and incubate for 1 (— —), 5 (- -), 20 (— —), and 45 (— · —) min, λ_{ex} = 400 nm.

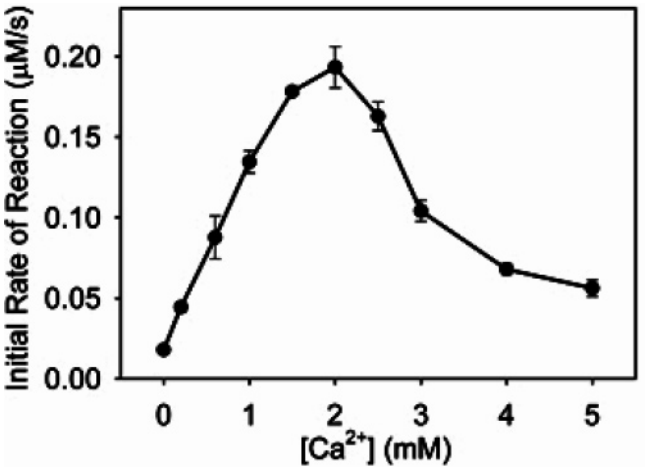

Figure 5. Effect of concentration of activator, $[Ca^{2+}]$, on the initial rate of hydrolysis (v_0) of 10CPC by PLC. Experiment conditions: 15 μM $BpPPESO_3$, 80 μM initial 10CPC, and 20 nM PLC in 50 mM Tris-HCl (pH 7.4) at 37 °C, λ_{ex} = 400 nm.

providing charge density, which enhances binding of the enzyme with the substrate.[53] The dependence of the initial enzyme-catalyzed rate (v_0) on $[Ca^{2+}]$ is shown in Figure 5. The experiment was conducted with a solution containing $BpPPESO_3$ (c = 15 μM), 10CPC ($c_{initial}$ = 80 μM), and PLC (c = 20 nM). Note that v_0 increases almost linearly until it reaches a maximum rate when $[Ca^{2+}]$ = 2 mM, and at higher concentrations, v_0 decreases. The observed dependence of v_0 on $[Ca^{2+}]$ is similar to that observed in previous kinetic studies of PLC activity.[41,54]

In a series of investigations, we examined the kinetics of the PLC-catalyzed hydrolysis of 10CPC at varying PLC concentration. These real-time kinetic assays were carried out with a solution containing $BpPPESO_3$ (c = 15 μM) and 10CPC ($c_{initial}$ = 30 μM) in Tris-HCl buffer (50 mM, pH 7.4) in the presence of 2 mM Ca^{2+} at 37 °C. Figure 6a illustrates plots of the $BpPPESO_3$ fluorescence intensity monitored at 460 nm as a function of time for solutions containing PLC at concentrations ranging from 0 to 50 nM. Note that the rate of decrease in the $BpPPESO_3$ fluorescence intensity increases as the concentration of PLC increases. Figure 6b illustrates a plot of v_0 as a function of PLC concentration for the range 0–75 nM, and it is evident from this presentation that the initial catalyzed reaction rate varies linearly with enzyme concentration. The analytical detection limit for PLC is 0.5 nM (22 ng·mL^{-1}, 6.6 × 10^{-4} unit·mL^{-1}, one unit is defined as the amount of enzyme hydrolyzing 1.0 μmol of 10CPC/min at pH 7.4 at 37 °C) and was obtained from the calibration curve in Figure 6b. This sensitivity is comparable to values obtained by using different assays (see Discussion section).[55,56]

An interesting observation is that the $BpPPESO_3$/10CPC/PLC solution becomes turbid as the reaction proceeds when solutions containing ≥35 μM 10CPC undergo PLC-catalyzed hydrolysis. Under these conditions, at longer reaction times, a greenish-yellow precipitate forms and the fluorescence of the polymer decreases sharply. This observation suggests that the product DAG produced by PLC-catalyzed hydrolysis of 10CPC coprecipitates with $BpPPESO_3$ and this effect interferes with the sensor response. In order to prove that DAG is the origin of the precipitation, its effect on the assay was investigated. Thus, various concentrations of DAG were added to a solution containing $BpPPESO_3$ and 10CPC (c = 15 and 50 μM, respectively). It was found that DAG dissolves with a negligible effect on the fluorescence intensity when added at low concentration (c < 10 μM). However, with increasing DAG concentration, the fluorescence intensity gradually decreases followed by a coprecipitation of the DAG/polymer complex at higher concentration of DAG (c > 30 μM). The precipitation results in a significant decrease in the fluorescence intensity. Therefore, quantitative analyses, including the calculations for initial rate of reaction and determination of kinetic parameters, were conducted at a DAG concentration less than 5 μM.

Determination of PLC-Catalyzed 10CPC Hydrolysis Kinetic Parameters. The $BpPPESO_3$-based fluorescence assay was used to determine the kinetic parameters (K_m and V_{max}) for the PLC-catalyzed hydrolysis of 10CPC. The kinetics experiments were carried out using solutions containing $BpPPESO_3$ (c = 15 μM), PLC (c = 20 nM), Ca^{2+} (c = 2 mM), and Tris-HCl buffer (50 mM, pH 7.4) at 37 °C. The initial concentration of 10CPC was varied from 0 to 90 μM, which is the range corresponding to the linear range in the calibration curve shown in Figure 3b (inset), and the v_0 values were obtained and plotted as a function of [10CPC] (see Supporting Information for the plot). Using a nonlinear regression routine, the plot of v_0 versus [10CPC] was fitted with the Michaelis–Menten equation (eq 2, where v_0 is initial rate of reaction and $[S]_0$ is the initial 10CPC concentration) yielding values for K_m and V_{max} of 28 ± 3 μM and 19 ± 0.7 μmol·min^{-1}·mg^{-1}, respectively. Unfortunately, there are no other reports of the values for K_m and V_{max} with 10CPC as substrate. However, using natural egg lecithin (a mixture of several different phospholipids including phosphatidylcholine), K_m was reported to be 16[57] or 28[41,57] μM and V_{max} was 68[57] μmol·min^{-1}·mg^{-1} by

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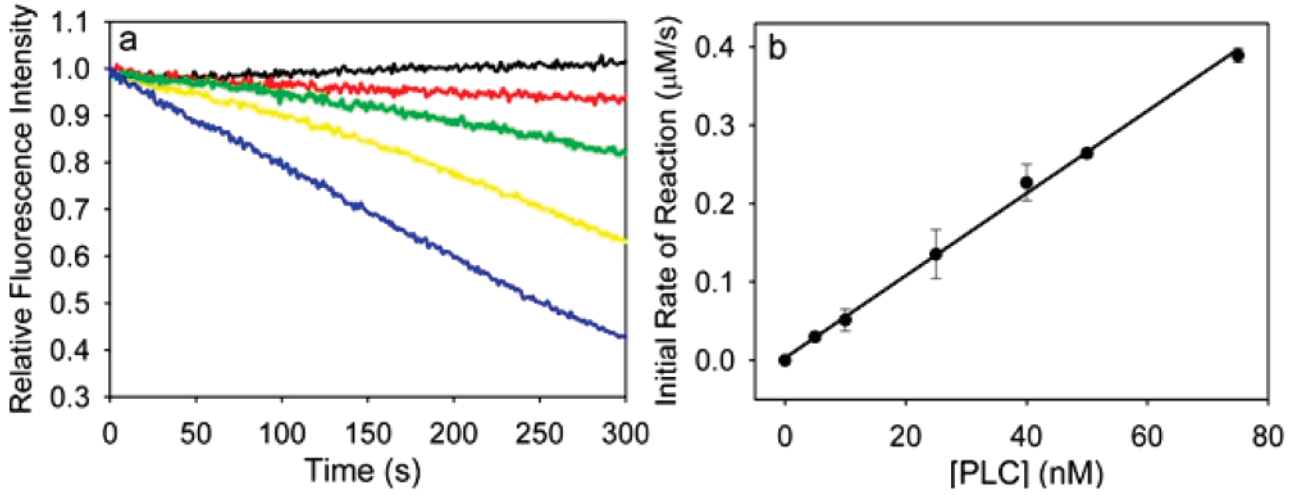

Figure 6. (a) Changes in fluorescence emission intensity (I_{tc}) at 460 nm during the PLC turnoff assay as a function of reaction time for various concentrations of PLC: 0 (black), 5 (red), 10 (green), 25 (yellow), and 50 (blue) nM. I_{tc} was corrected for photobleaching by blank fluorescence intensity I_{bt}. Experiment conditions: 15 μM $BpPPESO_3$ and 30 μM initial 10CPC in 50 mM Tris-HCl (pH 7.4) with 2 mM Ca^{2+} at 37 °C, $\lambda_{ex} = 400$ nm. (b) Dependence of initial rate of reaction (v_0) on PLC concentration.

monitoring the hydrolysis kinetics by phosphorus determination[57] or by a fluorometric method with dye-labeled lecithin as substrate.[41,57] This comparison shows that the K_m value obtained from the $BpPPESO_3$-based fluorescence turnoff assay is in good agreement with values obtained using other assays; however, the V_{max} value is smaller. It is likely that the lower V_{max} arises due to complex formation between 10CPC and the polymer, which effectively decreases the binding of the lipid to PLC.

$$v_0 = \frac{V_{max}[S]_0}{K_m + [S]_0} \qquad (2)$$

Inhibition of the PLC Catalysis. In order to further demonstrate that the observed fluorescence intensity decrease that is induced by addition of PLC to the $BpPPESO_3$/10CPC solution arises due to PLC-catalyzed hydrolysis of the 10CPC, we tested the effect of known PLC inhibitors on the fluorescence assay. Two reported inhibitors for PLC from *C. perfringens* are fluoride ion[58,59] and EDTA.[47,60] Both of these species were tested as inhibitors in assays conducted under the same conditions used for the kinetics studies described above using 10CPC at a concentration of 50 μM. As shown in Figure 7, both fluoride ion and EDTA effectively inhibit the PLC activity. While the overall inhibition increases with increasing inhibitor concentration, the inhibition efficiency is largest at low inhibitor concentration. Nonetheless, the inhibition experiments provide very strong evidence that the assay is effectively reporting on the PLC-catalyzed hydrolysis reaction.

Specificity of the PLC Turnoff Assay. Given that an effective biosensor should exhibit specificity for the target enzyme, it is of interest to examine the response of the $BpPPESO_3$/10CPC-based PLC assay to other proteins. In order to accomplish this objective, five proteins were selected, including PLA_2, PLD, peptidases PEP (a commercial mixture of protease), BSA, and AVI. Three of these enzymes, PLC, PLA_2, and PLD, belong to the group of phospholipases. As shown in Figure 8a, PLA_2 catalyzes the hydrolysis of an acyl ester bond exclusively at the 2-acyl position in the glycerophospholipid, affording a free fatty acid and a lysophospholipid.[61] The lysophospholipid has a surfactant-like structure, containing a single hydrophobic carbon chain and a polar head group. Thus, like 10CPC, the lysophospholipid produced by PLA_2 hydrolysis of 10CPC is expected to complex strongly with the polymer preventing aggregation and quenching of the fluorescence. On the other hand, PLD-catalyzes hydrolysis of the phosphate ester bond on the choline side to form choline and phosphatidic acid (Figure 8a).[62] Although phosphatidic acid also has a surfactant-like structure, its overall negative charge might prevent it from interacting with $BpPPESO_3$. In addition, it is reported that 20−100 mM Ca^{2+} is required for maximum activity of PLD, along with a pH of 5.6.[63] Therefore PLD might not achieve

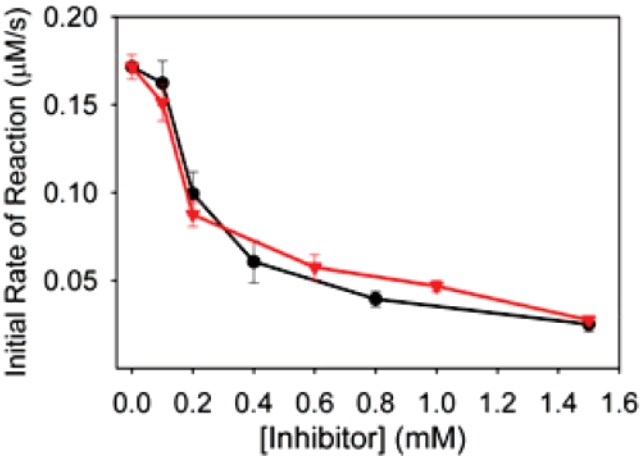

Figure 7. Inhibition of PLC turnoff assay. Plot illustrates the initial rate of reaction (v_0) versus inhibitor concentration: F^- (black ●), EDTA (red ▼). Experiment conditions: 15 μM $BpPPESO_3$, 50 μM initial 10CPC, and 20 nM PLC in 50 mM Tris-HCl (pH 7.4) with 2 mM Ca^{2+} at 37 °C, $\lambda_{ex} = 400$ nm.

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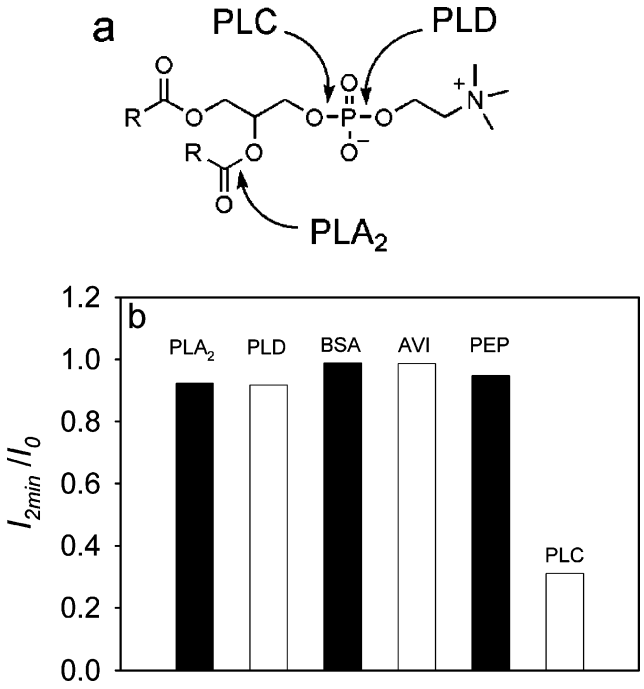

Figure 8. Specificity of PLC turnoff assay. Changes in fluorescence emission intensity at 460 nm following 2-min incubation with PLC and other five control proteins. Experiment conditions: 15 μM $BpPPESO_3$, 50 μM initial 10CPC, and 20 nM PLC or control proteins (except for 0.86 $\mu g/mL^{-1}$ PEP) in 50 mM Tris-HCl (pH 7.4) with 2 mM Ca^{2+} at 37 °C, λ_{ex} = 400 nm.

its full activity in the optimal conditions for the PLC turnoff assay (2 mM Ca^{2+} and pH 7.4).

The assays were carried out with the same conditions used for the kinetics studies described above, with 50 μM 10CPC. In each case PLC was not added, but an aliquot containing the other protein was added in to achieve a final concentration of 20 nM (except for PEP, which was added to achieve a concentration of 0.86 $\mu g \cdot mL^{-1}$). Figure 8b compares the fluorescence intensity changes (460 nm) observed 2 min after addition of the protein aliquot to the $BpPPESO_3$/10CPC/buffer solution. As expected, the assay with PLC exhibits ~70% decrease in fluorescence intensity, while the solutions containing the "control" proteins exhibit less than an 8% decrease in intensity. This result demonstrates that the $BpPPESO_3$ assay is specific for PLC. However, note that a small, but significant decrease in fluorescence intensity was observed upon addition of PLA_2, PLD, and PEP to the $BpPPESO_3$/10CPC/buffer solution, suggesting the existence of a nonspecific interaction between the proteins and the $BpPPESO_3$. Although such interaction exists, a negligible decrease of fluorescence intensity (<0.5%) is observed after 2-min incubation of a blank PLC assay (20 nM PLC) in which no substrate, 10CPC, is added. Therefore, the nonspecific effects are very small in comparison to the effect of specific enzymatic hydrolysis of 10CPC by PLC. Thus, it is safe to conclude that the overall interaction between PLC and polymer/phosphatidylcholine complex consists mainly of specific enzymatic activity as well as very small contribution from nonspecific interaction.

Discussion. The assay described herein affords the ability to monitor PLC activity rapidly and in real time. In addition, the method is quite sensitive, with respect to both the amount of enzyme and substrate required (<1 and 5 μM, respectively). The method can also be readily adapted to a fluorescence-based high-throughput screening assay format.[64]

A comprehensive literature survey reveals that many assays for monitoring the enzymatic activity of PLC from *C. perfringens* have been previously reported.[12,35,36,38,39,41,43,44] The previously reported assays are based on a variety of detection methods including turbidimetric, pH-stat titration, radiometric, and continuous fluorometric methods. Concerning the sensitivity, the best detection limit of 0.005 units·mL^{-1} for PLC from *C. perfringens* was achieved by using an ELISA assay.[55] A second assay that is based on the acid-soluble phosphorus method affords a detection limit of 70 ng·mL^{-1} for PLC.[56] (Assuming the molecular mass for PLC is 43 kDa, this corresponds to a detection limit of ~2 nM.) The $BpPPESO_3$-based PLC assay that is described herein clearly affords sensitivity that is comparable to (or better than) that of the earlier reports. With respect to the amount of lipid substrate needed for the assay, substrate concentrations in the millimolar range are required in the previously reported PLC assays.[39,41,47,55–57,59,60,65] By contrast, our method not only allows an assay to be carried out at significantly lower initial substrate concentration but also affords the ability to carry out real-time detection of the enzyme activity. In addition, surfactants such as SDC have been frequently used in the presence of Ca^{2+} ions to disperse water-insoluble phospholipids to enhance the rate of reaction and leading to an effective increase the PLC activity.[29,47,56,60] However, the addition of a surfactant does not afford any improvement in PLC activity if a water-soluble phospholipid is the substrate.[47] The effect of SDC was also examined for the $BpPPESO_3$/10CPC/PLC assay, and it was found that the polymer's fluorescence response was suppressed in the presence of the surfactant. The likely explanation for this effect is that the surfactant influences the fluorescence intensity change by complexing with the polymer (just like 10CPC), effectively counteracting the effect induced by hydrolysis of 10CPC. Nonetheless, since the formation of the polymer/lipid complex facilitates the solubilization of the lipid in water, addition of a surfactant such as SDC is not required for our assay.

While the $BpPPESO_3$/10CPC/PLC assay is relatively easy to implement and has many advantages, nonetheless the method still has some disadvantages. For example, the sensitivity of the assay is affected by various experimental conditions, such as buffer concentration, temperature, or polymer concentration. In addition, nonspecific interactions with various proteins and other solutes (e.g., metal ions, lipids, or surfactants) and the polymer (and lipid substrate) could interfere with the sensor response, especially if quantitative (kinetic) data are needed. Finally, the PLC turnoff assay is limited by one of the hydrolysis products, DAG, which leads to a precipitation of polymer at higher concentration (c > 30 μM). Due to these limitations, when applying this assay with biological samples (e.g., a serum sample), the specific assay conditions will need to be optimized in order to attain the optimal sensor response in the presence of possible interfering species.

CONCLUSION AND OUTLOOK

This paper describes the development and application of a novel fluorescence turnoff assay that affords real-time detection

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of PLC activity with good specificity and that functions at very low substrate and enzyme concentrations. The sensor takes advantage of the interaction between the lipid substrate and a fluorescent, water-soluble conjugated polyelectrolyte. The high sensitivity of the sensor is due to the well-known ability of the fluorescent conjugated polyelectrolyte to exhibit amplified response to aggregation or the presence of charged fluorescence quenchers.[2,17,19,20,22,26]

Although the work demonstrates the turnoff assay with a specific conjugated polyelectrolyte, substrate, and enzyme, this method is quite general and can very likely be extended to other lipases and to different lipid substrates. For example, a similar conjugated polyelectrolyte-based turnoff assay can be designed to detect the activity of sphingomyelinase using sphingomyelin as a substrate. If an anionic phospholipid, phosphatidylinositol biphosphate, is used as a substrate to monitor phosphatidylinositol phospholipase C activity, a cationic CPE can be utilized. It may also be possible to develop a similar assay to monitor DNA transfection, since some natural and synthetic cationic lipids have been utilized in gene transfer.[66,67]

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ACKNOWLEDGMENT

We thank Xiaoyong Zhao for synthesis and characterization of $BpPPESO_3$. We also gratefully acknowledge financial support by the U.S. Department of Energy, Basic Energy Science (Grant DE-FG02-03ER15484).

SUPPORTING INFORMATION AVAILABLE

Details concerning the fluorescence intensity correction for photobleaching; synthesis and structural characterization of $BpPPESO_3$; normalized absorption and emission of $BpPPESO_3$ before and after addition of 10CPC; nonlinear regression of v_0 vs [10CPC] data to derive K_m and V_{max}. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review August 6, 2007. Accepted October 1, 2007.

AC701672G