Conjugated Polyelectrolyte Supported Bead Based Assays for Phospholipase A_2 Activity†

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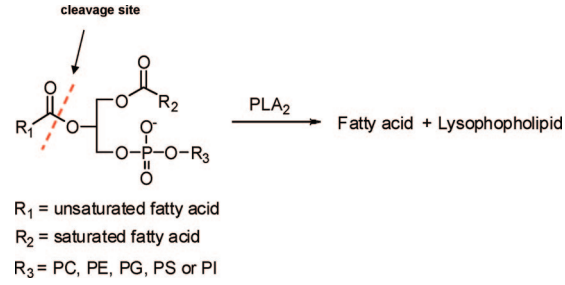
A fluorescence based assay for human serum-derived phospholipase activity has been developed in which cationic conjugated polyelectrolytes are supported on silica microspheres. The polymer-coated beads are overcoated with an anionic phospholipid (1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)) (DMPG) to provide "lipobeads" that serve as a sensor for PLA_2. The lipid serves a dual role as a substrate for PLA_2 and an agent to attenuate quenching of the polymer fluorescence by the external electron transfer quencher 9,10-anthraquinone-2,6-disulfonic acid (AQS). In this case quenching of the polymer fluorescence by AQS increases as the PLA_2 digests the lipid. The lipid can also be used itself as a quencher and substrate by employing a small amount of energy transfer quencher substituted lipid in the DMPG. In this case the fluorescence of the polymer is quenched when the lipid layer is intact; as the enzyme digests the lipid, the fluorescence of the polymer is restored. The sensing of PLA_2 activity has been studied both by monitoring fluorescence changes in a multiwell plate reader and by flow cytometry. The assay exhibits good sensitivity with EC50 values in the nanomolar range.

1. Introduction

Conjugated polyelectrolytes (CPEs) are a versatile class of materials that show potential in a variety of applications including the detection of biomolecules such as proteins, antibodies, and DNA.[1−7] Water-soluble CPEs based on poly(phenyleneethynylene) (PPE) backbones exhibit strong photoluminescence and rapid transport of the singlet excitation throughout the conjugated backbone. An important characteristic of these materials is the efficient quenching of the CPE fluorescence that can be achieved by ion pairing of the CPE chains and quenchers (either energy transfer or electron transfer) of opposite charge. This amplified quenching ("superquenching") can be attributed to a combination of Coulombic and hydrophobic interactions in aqueous media (enhanced association between the polymer and counterion) and to rapid migration of excitons through the polymer following photoexcitation. The amplified quenching is generally retained for PPE CPEs when they are transferred from solution to supported formats. For example, in recent work by Zeineldin et al.[8] a bead-based (physisorbed) conjugated cationic polymer based system was developed to provide an assay for mellitin, a membrane-lysing peptide. In this case the surface layer of CPE on the beads was overcoated with a lipid bilayer which was removed by the action of mellitin. In other studies it has been shown that CPEs can be grown covalently from the surface of a bead or planar surface to provide a surface composed of polymer brushes. The surface grafted layers are stable and highly fluorescent; the fluorescence of the surface grafted conjugated polyelectrolytes (SGCP) may be quenched with varying efficiency by small molecule electron transfer and energy transfer quenchers. In the present paper we report the use of assemblies of CPEs and phospholipids for the measurement of enzyme activity of phospholipase enzymes.

SCHEME 1: PLA_2 Catalysis of the Hydrolysis of Phospholipids, Releasing Fatty Acids and Lysophospholipids[a]



[a] In the present study R_3 used is PG.

Hydrolytic enzymes such as proteases and lipases play a crucial role in many physiological and pathological processes, thus making it attractive to develop high throughput screening assays, which are simple, easy to use and require minimum or no purification steps for their real time employment. Human serum derived phospholipase A_2 (PLA_2) is of special interest to researchers. Recent investigations have shown that PLA_2 can be a potential biomarker for diagnosing the degenerative disease atherosclerosis.[9−15] Scheme 1 illustrates the catalytic action of PLA_2 on its natural substrates. PLA_2 activity can be assessed by measuring its digestive activity or by measuring protein concentration using an immunoassay. Most reported assays determine the level of PLA_2 by following its catalytic activity.[16] Prestwich et al.[17,18] reported a fluorogenic assay for the detection of PLA_2 activity in real time with a continuous readout, using fluorescence resonance energy transfer (FRET) as a detection technique. In their assay fluorogenic phospholipids with different head groups (PA, PC, PE, PS and PG) were used as substrates.

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The fluorogenic substrates have a common backbone of a diacylglycerol moiety, in which the *sn*-1-acyl chain is labeled with Dabcyl (p-methyl red). The attachment of a BODIPY fluorophore to the *sn*-2-acyl position is assisted by PLA_2. The close proximity of the BODIPY fluorophore to the Dabcyl (fluorescence quencher) in the intact phospholipid ensures FRET between the two fluorophores. The enzyme activity is measured by the enhancement in BODIPY fluorescence because of the increase in the intermolecular distance between BODIPY and Dabcyl, which is achieved by the cleavage of the *sn*-2-acyl chain by PLA_2 present in the samples being measured. Perez-Gilabert et al.[19] described a spectrophotometric assay for the continuous and indirect measurement of PLA_2 activity. In this assay, PLA_2 catalyzes hydrolyses of dilinoleoyl phosphatidylcholine (substrate for PLA_2) releasing linoleic acid which is then oxidized by lipooxygenase. The activity of PLA_2 is measured by the increase in absorbance at 234 nm due to the formation of hydrogen peroxide from linoleic acid. Radioimmunoassays,[20] an enzyme-linked immunosorbent assay[21] and a time-resolved fluoro-immunoassay[22] are all based on the use of antibodies to PLA_2 and hence measure protein concentration. Most of the assays developed for PLA_2 so far exhibit low-to-moderate sensitivity consequently requiring high concentrations of the enzyme or the substrate.

In this paper, we report a convenient fluorescence assay for PLA_2 by measuring its catalytic activity using the phenomenon of attenuated superquenching. We have examined several different cationic PPE derivatives either physically adsorbed or surface grafted onto solid nonporous borosilicate microspheres. These polymer-coated beads are subsequently coated with 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG), an anionic phospholipid, presumably as a bilayer. The lipid bilayer serves the dual purpose of acting as a substrate for PLA_2 and shielding the fluorescence of PPE from quenching by 9,10-anthraquinone-2,6-disulfonic acid (AQS). These coated beads are termed "lipobeads". These "lipobeads" can provide a sensor for PLA_2 because, as the DMPG is digested, the amplified quenching of the polymer fluorescence by AQS is restored. A second FRET-based assay has been developed using a quencher (Rhodamine) labeled phospholipid wherein the intact lipobeads show near-complete quenching of the polymer fluorescence prior to treatment with PLA_2. Digestion of the phospholipid by the enzyme restores the fluorescence of the polymer in this case. The sensing has been studied both by monitoring fluorescence changes in a multiwell plate reader and by flow cytometry.

2. Experimental Section

Reagents. The cationic polyelectrolyte poly(*p*-phenyleneethynylene) derivatives **1** and **2** were synthesized according to methods described elsewhere,[23] and the anionic quencher 9,10-anthraquinone-2,6-disulfonic acid (AQS) was obtained from Sigma-Aldrich (St. Louis, MO). Borosilicate glass microspheres (5 μm diameter) were purchased in dry form from Duke Scientific (Palo Alto, CA). 1,2-Dimyristoyl-*sn*-glycero-3-[phosphor-*rac*-(1-glycerol)] sodium salt (DMPG) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). ProLong Gold antifade used in confocal microscopy experiments was purchased from molecular probes (Carlsbad, California). Phosphate buffer saline (PBS) and Triton X-100 were obtained from (Sigma-Aldrich (St. Louis, MO). Human serum Phospholipase A_2 was purchased from Cayman Chemicals (Ann Arbor, Michigan).

General Synthetic Procedures and Source of Starting Materials for Polymer 3. All chemicals used for synthesis were reagent grade and purchased from Aldrich. Chemicals and reagents were used without purification unless otherwise noted. ^{1}H and ^{13}C NMR spectra were recorded on either a Varian VXR 300 or Mercury-300 spectrometer and chemical shifts are reported in ppm relative to TMS. Diiodohydroquinone (**4**),[24] 1,4-bis(3-bromopropoxy)-2,5-diiodobenzene (**5**)[25] and 3,3′-[(2,5-diiodo-1,4-phenylene)bis(oxy)]bis[*N*,*N*,*N*-trimethyl-1-propanaminium] bromide salt (**6**)[25] were synthesized according to the literature procedures.

2,5-Bis((trimethylsilyl)ethynyl)thiophene (7). 2,5-Dibromothiophene (4.00 g, 16.53 mmol), CuI (0.38 g, 1.98 mmol), $Pd(PPh_3)_2Cl_2$ (0.69 g, 0.98 mmol), and 120 mL of isopropylamine were placed in a Schlenk flask and the solution was degassed with stirring for 30 min under ice-bath by bubbling argon gas. To this solution was added (trimethylsilyl)acetylene (6.49 g, 66.12 mmol). The solution was stirred under an ice-bath for 1 h. The temperature was raised to room temperature and mixture was kept stirring for an additional hour. The resulting solution was heated to 75 °C and stirred for 20 h. The solvent was removed and the solid was purified by flash chromatography on silica gel with hexane to yield a yellow solid **7** (2.54 g, 55.5%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.24 (s, 18H), 7.04 (s, 2H).

2,5-Diethynylthiophene (8). To a suspension of compound **7** (0.4 g, 1.45 mmol) in deoxygenated methanol (20 mL) was added 0.1 mL of 0.5 M aqueous KOH solution. The mixture was stirred at room temperature under argon for 40 min. The solution was diluted with water (50 mL) and extracted with *n*-pentane (2 × 50 mL). The combined organic solution was dried over Na_2SO_4 and the solvent was removed at reduced pressure to yield **8** as a viscous oil (0.14 g, 73%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.32 (s, 2H), 7.09 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 132.6, 123.6, 82.1, 76.2.

Polymer 3. A solution of compound **6** (100 mg, 0.15 mmol), CuI (4 mg, 0.02 mmol), and $Pd(PPh_3)_4$ (10 mg, 0.01 mmol) in 8.5 mL of DMF/H_2O/^{i}Pr$_2$NH (v/v/v = 9/6/2) was deoxygenated with argon for 30 min. Then, compound **8** was added to the solution under argon. The resulting solution was heated at 70 °C for 22 h. The reaction mixture was poured into 200 mL of acetone. The precipitate was dissolved in small amount of Millipore water and treated with NaCN, filtered using 25 μm glass filter and followed by dialysis against deionized water using 6−8 kD MWCO cellulose membrane. The polymer solution was lyophilized to yield a yellow-tan solid (46 mg, 51%). ^{1}H NMR (300 MHz, CD_3OD): 2.38 (br), 3.21 (br), 3.63 (br), 4.22 (br), 7.23 (br), 7.33 (br). ^{13}C NMR (75 MHz, $CDCl_3$) spectrum was not obtained due to the limited solubility of the compound.

Preparation of the Polymer Beads. ***Surface Grafted Beads.*** The preparation and characterization of surface grafted beads with the same polymer repeat units as 1 has been previously reported.[26]

Physisorbed Beads. Borosilicate glass nonporous beads (60 mg, 5 μM average diameter as specified by the manufacturer) were weighed out into an Eppendorf tube. They were then suspended in 1.2 mL of ultrapure water. The beads were then counted using a hemacytometer. There were approximately 1.8×10^8 beads/mL. A certain amount of polymer (polymer **1** or **2** or **3**) was weighed and dissolved in deionized water, and the absorption spectrum was obtained by using a spectrophotometer (Spectramax M5). The concentration of the polymer solution (polymer in consideration) was calcu-

lated using the Beer−Lambert law, using extinction coefficient of 35100 M^{-1} cm^{-1}.[27] The cationic PPE has an approximate surface area of 280 square angstroms per polymer repeat unit (PRU).[27] Calculations were done using 5 μm diameter beads, assuming a smooth spherical shape when calculating their surface area and it was determined how much polymer was needed to provide monolayer coverage to a given amount of beads. The final coating solution that was added to the beads contained enough polymer such that it formed 1.2 monolayer's on the bead surface. After the addition of the polymer solution to the beads, they were vortexed for 30 min, washed 5 times in PBS buffer (20 mM PBS + 150 mM NaCl) until the supernatant was no longer fluorescent. The beads were finally suspended in 1.2 mL of PBS buffer and stored at 4 °C until further use.

Coating of DMPG (Negatively Charged Lipids) on Polymer (Positively Charged) Beads (Both Physisorbed and SGCP). DMPG was obtained from Avanti lipids in a powder form. It was dissolved in chloroform and diluted to 2 mM and stored in aliquots at −20 °C until further use. When the lipobeads were prepared, one such aliquot was taken, vaccuum-dried until all the chloroform evaporated. The dried lipids were rehydrated by adding PBS buffer at room temperature and shaking then for 10 min and later sonicating in a hot water bath for 1 min until the cloudy suspension, turned optically clear to achieve single unilamellar vesicles. The temperature of the hot water bath was maintained steadily above the transition temperature of the DMPG lipids which is 17 °C.[28] The required number of beads (1×10^6 beads/200 μL) were added to the DMPG liposome suspension, shaken for 30 min and allowed to rest for 5mins after which they were washed 5 times with water to remove the unbound liposomes.

Confocal Microscopy. 10uL from a bead stock of 1.8×10^8 beads/mL was aliquoted onto a microscope glass slide. To this 25 μL of ProLong Gold antifade mounting media was added. A coverslip was put on and the slide was left in dark for 24 h. During this process the mounting media solidifies the bead suspension, thus helping to eliminate problems such as rapid Brownian motion, which hinders the image capturing process. The antifade in the mounting media also alleviates in part the photobleaching of the polymer's fluorescence due to constant exposure to the confocal microscope laser. A Zeiss confocal microscope (LSM 510) with 488 nm argon laser and a band-pass filter of 505−530 was used to acquire the confocal images.

Fluorescence Quenching Measurements. A plate reader was used for the fluorescence quenching measurements for polymers **1**, **2** and **3** physisorbed on beads. In fluorescence quenching experiments aliquots of the quencher solution of different concentrations were added directly to the samples in the wells with an accurate laboratory micropipette. The 96 well plate was vortexed, and the fluorescence quenching was measured immediately. The samples were excited at 380 nm using a xenon lamp. The emission was collected at 530 nm. The initial emission of the polymers on solid supports was taken before and after the addition of the quencher. The Stern−Volmer equation was fitted for each set of data collected at the emission wavelength. The quenching experiments for the polymer **1** surface grafted on beads have been described elsewhere.[26]

Flow Cytometry Measurements. All bead based sensors were examined for fluorescence using a FACS flow cytometer (Becton-Dickinson, Sunnyvale CA) with excitation at 488 nm. Fluorescence signals were acquired on the FL-1 channel (525 nm) and displayed using log amplification. The data were analyzed with CellQuest software. This included the three physisorbed polymers supported on beads, the same beads following their coating with DMPG lipid bilayer and these beads following treatment with Triton X-100. All the fluorescence measurements were made using 1×10^5 lipobeads suspended in 200 μL of PBS buffer. AQS (50 μM) was then injected into the sample and vortexed for 10 s, and the fluorescence intensity was immediately measured. PLA_2 (0.25 μM) or Triton X100 (0.25% w/v) was added to the above lipobead suspension and vortexed for 30 s and the fluorescence intensity measured. The phospholipase activity was quantified by optimizing the EC50 curve experiment. EC50 is defined as "the molar concentration of an enzyme, which produces 50% of the maximum possible response for that enzyme". Under the same conditions of experiments as mentioned above, different concentrations of PLA_2 derived from human serum were added to the lipobead suspension and incubated for 50 min. AQS was added to the samples at the end of incubation and the readings measured. The same procedure was repeated for surface grafted beads coated with the DMPG lipid bilayer.

Real time analysis of phospholipase activity was investigated by coating rhodamine labeled DMPG on physisorbed **1** supported beads. A total of 1×10^5 lipobeads in 200 μL were suspended, and their fluorescence was measured in the FL1 channel initially. PLA_2 was then added, vortexed for 30 s, and measured immediately.

3. Results and Discussion

As has been reported elsewhere, polymers **1** and **2** can physisorb onto the surface of anionic microspheres to generate strongly fluorescent particles wherein there is relatively little "self-quenching" of the polymer on deposition on the surface.[23] In the present study we have expanded the polymers to include **3** and some surface grafted conjugated polymers (SGCP) on which we have covalently grown polymers with the same repeat units as polymer **1**.[26] We have previously shown that beads containing **1** as a physisorbed coating can be used as sensors in an assay involving interposition and removal of a lipid bilayer between the physisorbed polymer and the contacting solution. In this study we demonstrate that such beads can be used as sensors to provide a sensitive and useful assay for enzyme activity resulting in membrane disruption.

The structures of the polymers and quenchers used in this work are shown in Figure 1. The 5 μm diameter beads used in this study are initially smooth and have been examined by scanning electron microscopy (SEM). Figure 2a shows an image of a bead prior to coating and Figure 2b shows a bead with a physisorbed coating of polymer **1**.

Figure 2c shows images of beads coated with **2** after the aqueous solution of **2** had been stored for several weeks. Beads coated from fresh solutions of **2** showed images similar to those for **1** and **3**. Interestingly, the beads shown in Figure 2c did not show strong quenching when the quencher AQS was added; we attribute the lack of quenching to the presence of much of the bead coated **2** as possibly nanocrystalline phase, much of which is inaccessible to the quencher. The images of the SGCP show rough surfaces by SEM.[26] Confocal fluorescence images of polymers **2** and **3** and SGCP are compared in Figure 3. It is clear that the physisorbed polymer **2** shows a strong and uniform fluorescence; in contrast, the fluorescence from physisorbed **3** is much weaker, suggesting that there is some self-quenching due to aggregation for this polymer. In contrast, the SGCP beads show strong, but less uniform fluorescence, which may be due to the highly variable length of the appended polymer chains

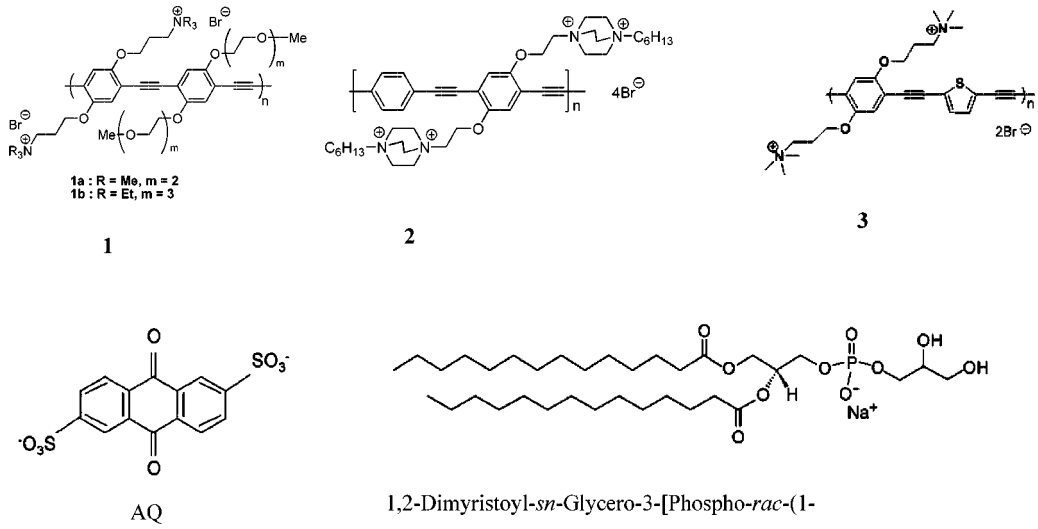


Figure 1. Structures of cationic conjugated polyelectrolytes **1**, **2**, **3**, quencher AQS and DMPG lipid investigated in this study.

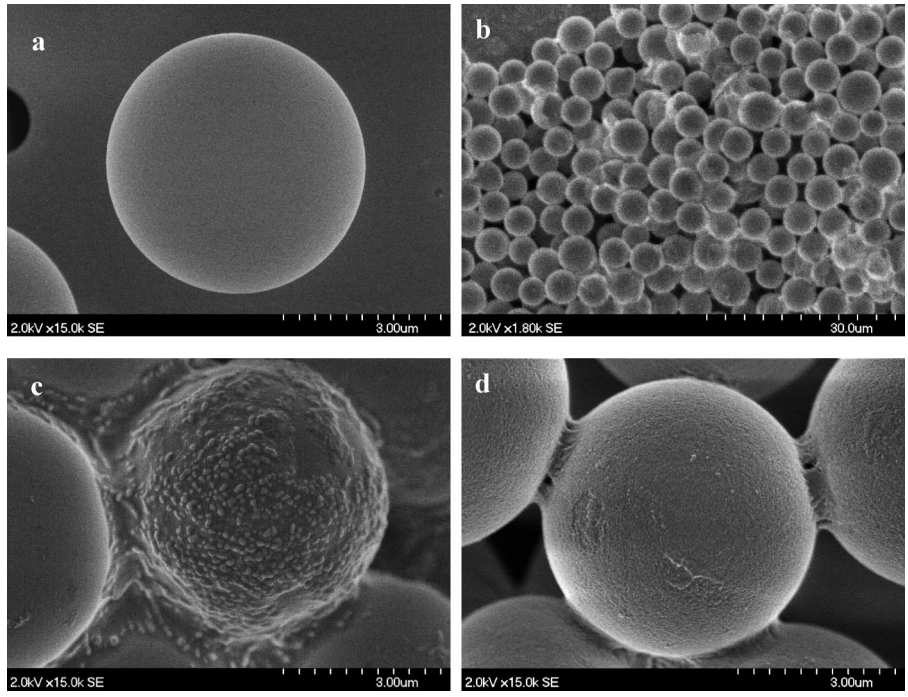


Figure 2. Scanning electron microscopy (SEM) images of 5 μm silica particles: (a) unmodified particles; (b) particles with physisorbed **1**; (c) expanded view of particles with physisorbed **2**; (d) expanded view of particles with physisorbed **3**.

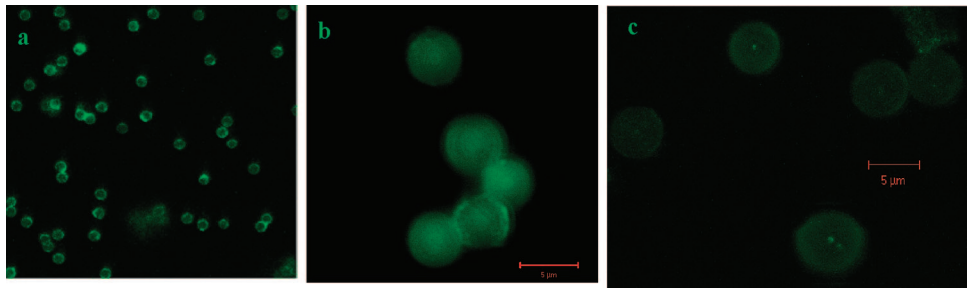


Figure 3. Confocal fluorescence microscopy (CFM) images of 5 μm silica particles coated with polymer: (a) fluorescence image (CFM) of particles with surface grafted **1**; (b) fluorescence image (CFM) of particles with physisorbed **2**; (c) fluorescence image (CFM) of particles with physisorbed **3**.

grown covalently from the surface. The relative fluorescence of the different polymer/bead samples can also be obtained by measuring the fluorescence from a population of beads by flow cytometry (Figure 4). The strongest overall fluorescence is for physisorbed **2** on the beads, followed by physisorbed **3** and the SGCP beads with chemisorbed **1** showing the lowest fluorescence. The fluorescence of the bead supported polymers is strongly quenched by addition of the anionic electron transfer

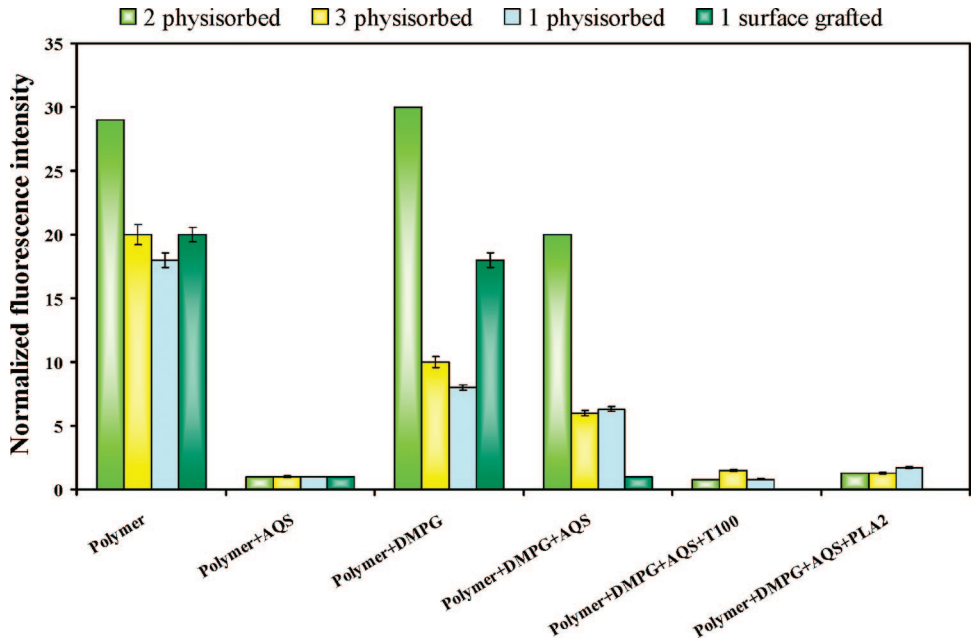


Figure 4. Effect of different types of coating of polymers **1**, **2** and **3** on 5 μm silica beads in the absence (polymer) and presence of AQS (polymer + AQS). Effect of forming and disrupting DMPG lipid bilayers around the polymer supported beads. 1×10^5 lipobeads in 200 μL PBS were examined by flow cytometry in the absence (polymer + DMPG) and in presence of AQS (polymer + DMPG + AQS). Triton X-100 (T100) (a final concentration of 0.25% (w/v)) and PLA2 (lipid bilayer disruptants) were injected after adding the quencher and their effects were observed.

TABLE 1: Stern−Volmer Equation, $I_o/I = K_{sv}[Q] + 1$, Summary of K_{SV} Values[a]

surface grafted on 5 μm Si bead	K_{SV}
1	4×10^4 M^{-1}
physisorbed on 5 μm Si bead	**K_{SV}**
1	6×10^4 M^{-1}
2	8×10^4 M^{-1}
3	**3** $\times 10^4$ M^{-1}

[a] Stern−Volmer quenching of polymer coated 5 μm silica particles suspended in water. Electron transfer quenching by AQS. See Supporting Information for more information.

quencher, anthraquinone-2,6-disulfonic acid (AQS), as indicated by the Stern−Volmer quenching constants reported in Table 1. The quenching constants indicate moderate "superquenching" for the polymers by AQS in the bead suspensions. Interestingly, the quenching constant for polymer **2** by AQS is slightly greater than those for physisorbed **1** and **3**, which may be attributed, at least in part, to the higher charge density for **2**.[29,30]

The beads containing the physisorbed polymers and the surface grafted polymer are both cationic and can be coated by treatment with a suspension of bilayer vesicles formed from the anionic phospholipid, DMPG. Beads with a physisorbed coating of **1** were coated with DMPG and also separately with a mixture of DMPG and DOPE-Rhodamine in a molar ratio of 100:1, respectively. The fluorescence of the samples with DOPE-Rhodamine are compared in Figure 5b. The observation that fluorescence activated by excitation at 488 nm (where primarily **1** is excited) results in near complete quenching of the fluorescence from the polymer and a sensitization of fluorescence attributed to rhodamine suggests that the lipid is in close proximity to the polymer. When the beads containing physisorbed polymers **1**−**3** are treated with DMPG, there is a slight attenuation of the fluorescence for beads with **1** and **3** but a slight enhancement of the fluorescence for beads containing polymer **2**, as shown in Figure 4. The decrease in fluorescence from beads containing **1** and **2** may be attributable to some of the DMPG acting as a detergent to remove polymer from the beads. The lack of a similar effect for polymer **2** may be due to the fact that its higher charge density makes it less prone to removal by the lipid. For all three physisorbed polymers on 5 μm silica beads, there is only a modest amount of quenching when AQS is added to the DMPG coated polymer beads. This "attenuated superquenching" is attributed to the formation of a fairly intact bilayer of DMPG coating, which prevents the close approach of AQS to the polymer. In contrast to the small amount of quenching observed for physisorbed polymer, the SGCP beads show very strong quenching by AQS following treatment with DMPG suggesting that there is very little, if any, intact bilayer "encapsulating" the polymer brushes from the SGCP. As shown in earlier studies, brief exposure of physisorbed polymer beads that have been treated with DMPG to the detergent Triton X-100 results in restoration of quenching by AQS in accord with the expectation that the detergent should disrupt the bilayer.[27]

4. Phospholipase Activity

Beads coated with polymer and DMPG (and DMPG + DOPE-Rhodamine) were treated with PLA_2 in a mixture of PBS buffer and human serum (~1%). AQS (50 μM) was added either before addition of the PLA_2 or after a 50 min incubation of the PLA_2 and sensor beads. The results for all three polymers and AQS are shown in Figure 4. When the beads are exposed to PLA_2 and incubated for 50 min at 25 °C and then treated with AQS, near total quenching of the polymer fluorescence is observed. When the beads are exposed to PLA_2 at 0.025 nM (below specified concentration) and incubated for 50 min, there is little or no decrease in polymer fluorescence, indicating at these concentrations there is probably no removal of polymer from the beads as the DMPG is digested by the enzyme.[31] However, when AQS is added after the incubation, near total quenching of the polymer fluorescence is observed, as antici-

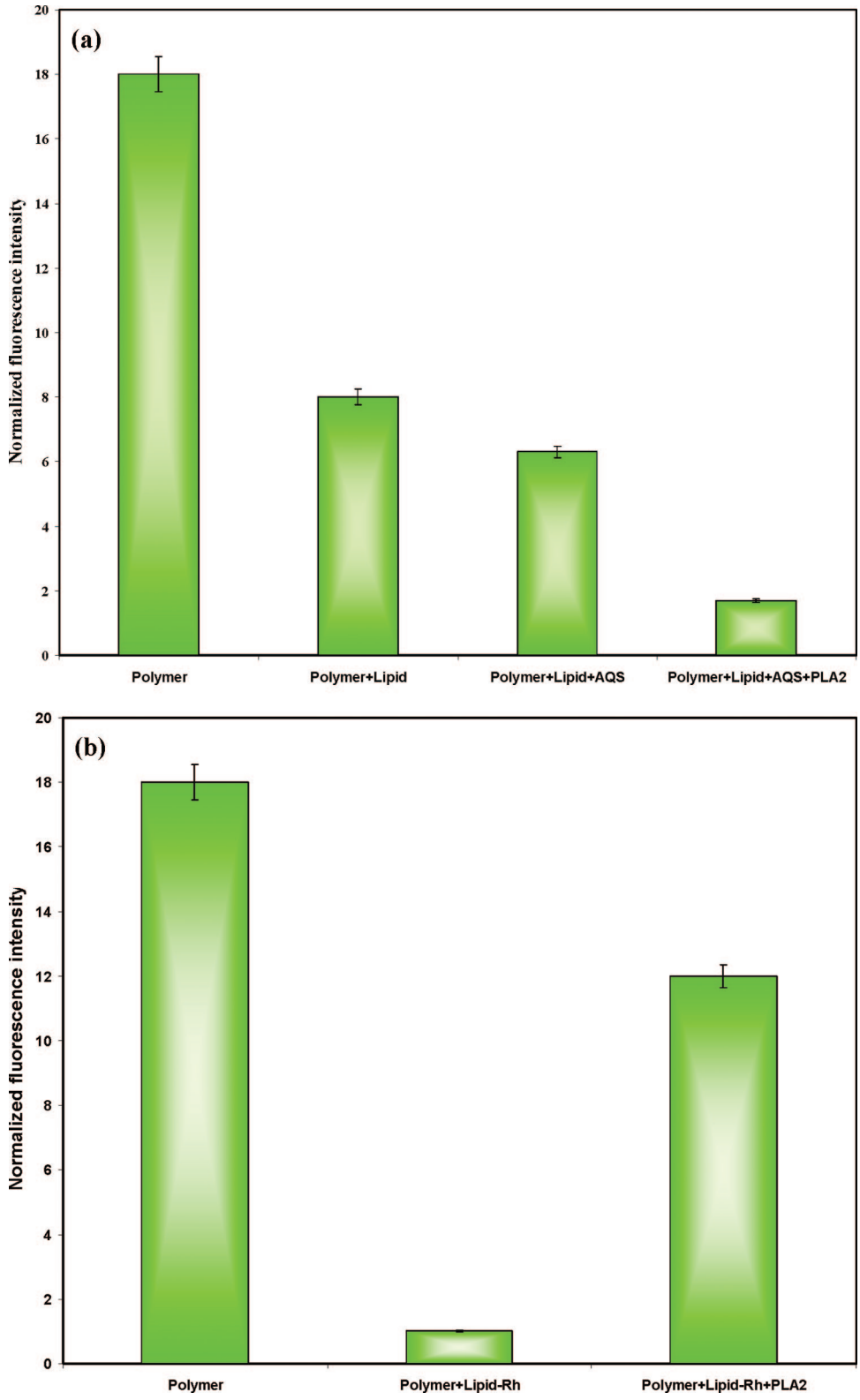
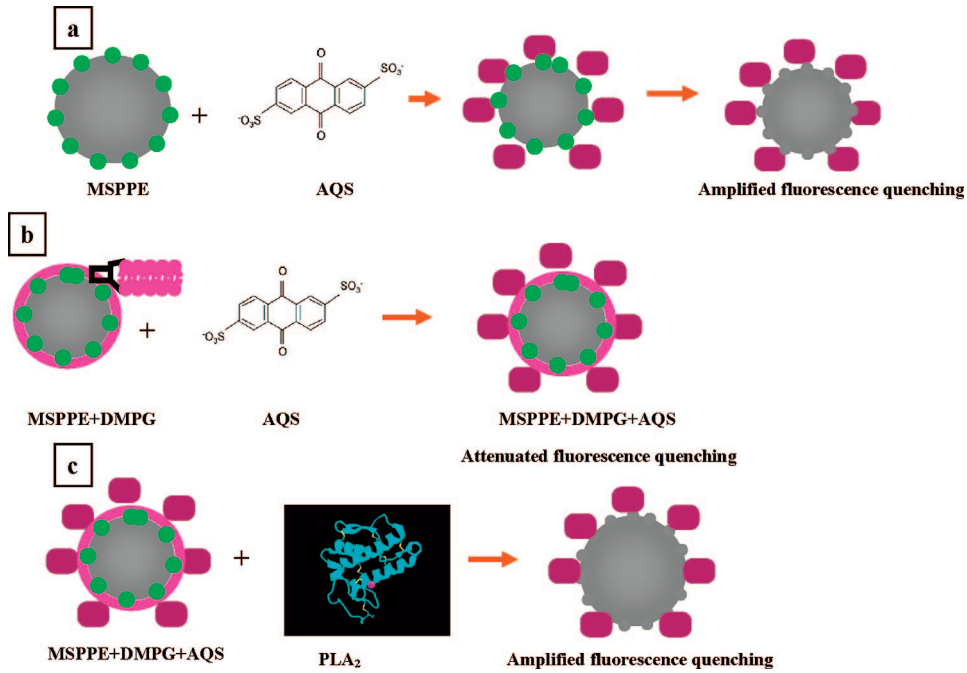


Figure 5. (a) Fluorescence "turn off" or amplified fluorescence quenching format ("real time" assay) with physisorbed **1** particles coated with DMPG lipid bilayer (1×10^5 lipobeads in 200 μL PBS). Lipobeads were examined in the presence and absence of PLA2 after the addition of AQS (25 μM). (b) Fluorescence "turn on" with physisorbed **1** particles coated with rhodamine labeled DMPG lipid bilayer (1×10^5 lipobeads in 200 μL PBS). Lipobeads were examined in the presence and absence of PLA2 after the addition of AQS (25 μM).

pated for the bead-coated polymer with no DMPG present. The overall assay with AQS (or other external quenchers) is shown in Scheme 2. For polymer **1**-coated beads, incubation of the beads and PLA_2 in the presence of AQS for 50 min resulted in efficient quenching of the polymer fluorescence and suggesting that the assay may be carried out in either end-point or real time modes. Similarly, as shown in Figure 5b, when the beads coated with Rhodamine labeled DMPG are incubated with PLA_2, the fluorescence of the polymer is regenerated, again consistent with removal of DMPG by digestion with the PLA_2.

Although all three physisorbed polymers exhibit similar behavior as summarized in Scheme 2, in the reaction of the

SCHEME 2: Investigation of Polymer Supported Bead Based Fluorescence "Turn Off" Assay Using Flow Cytometry: (a) 5 μm Silica Microspheres Coated with Polymer;[a] (b) Attenuated Fluorescence Quenching Obtained When a Lipid Bilayer Is Formed around the Polymer Coated Beads and AQS Is Introduced; (c) Upon Introduction of an Enzyme, Lipid Bilayer Digestion and Amplified Fluorescence Quenching Similar to That in (a)



[a] Amplified fluorescence quenching is observed in the presence of the quencher AQS.

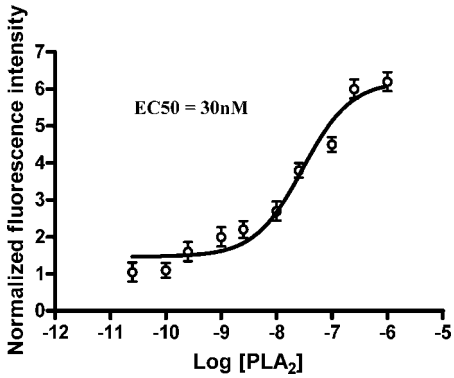


Figure 6. 1×10^5 lipobeads (polymer **3** physisorbed particles coated with DMPG) in 200 μL PBS incubated with different concentrations of PLA2 for 50 min. These were examined using a flow cytometer immediately after the addition of AQS. The EC50 value for PLA2 derived from human serum measured using the amplified fluorescence quenching format with 30 nM.

polymer/DMPG coated beads with PLA_2 there are significant differences in the performance/stability of the different polymer-coated beads. For example, polymer **1** forms well-coated beads that can be used when freshly prepared but undergo a strong decrease in fluorescence on storing for even a few days that render them not useful over time. Solutions of polymer **2** tend to form nanocrystals (as shown in Figure 2 and discussed above) and the **2**-coated bead suspensions, though more stable than those from **1**, may also undergo some crystallization on the surface. Beads coated with polymer **3** are stable over long periods and thus more suitable for a practical sensor and thus we used these beads for carrying out a quantitative assay for PLA_2.

As shown in Figure 6, the reaction of DPMG with PLA_2 was monitored by following the fluorescence of bead-coated 3 after a 50 min incubation with PLA_2 and subsequent addition of AQS (50 μM) as a function of PLA_2 concentration. A typical sigmoidal shape for the fluorescence intensity is observed and a value for EC50 can be estimated as 30 nM. This compares with EC50 values for human serum Phospholipase A_2 reported in the literature.[32]

5. Conclusions

The results obtained in this study indicate the feasibility of a bead-based assay format based on disruption of a lipid bilayer overcoating a surface layer of a fluorescent conjugated polyelectrolyte. In the present case we have monitored the digestion of a lipid coating by PLA_2, an enzyme that is an attractive target for a simple and sensitive assay. We have demonstrated two potentially useful assays, one involving the employment of a potential quencher whose quenching of the polymer fluorescence is attenuated by the presence of the phospholipid overcoating but is restored when the coating is digested by the enzyme. A second format involving a quencher-labeled phospholipid operates in a reverse fashion in that the quencher is removed as the phospholipid layer is digested which is an advantage as it can function in the presence of complex media (human serum). The sensitivity of these assays can be improved by modifying the CPE structure, or possibly by covalent attachment of shorter polymer segments. This assay can be extended for the quantification of other members of the phospholipase family as well as other lipases such as Triglyceride lipases.

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Supporting Information Available: Stern−Volmer quenching diagrams. This material is available free of charge via the Internet at http://pubs.acs.org.

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