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A reversible colorimetric and fluorescent polydiacetylene vesicle sensor platform

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ABSTRACT

A multi-functional liposome has been developed that demonstrates reversible changes in color and fluorescence intensity in response to changes in pH. Addition of a pendant tryptophan moiety gives the assembly the ability to detect *Escherichia coli*. The sensor platform is made from a polymerized vesicular assembly of hydrazide-terminated diacetylene lipids and a BO558 fluorescent dye. The polymer backbone is responsible for the color change, while the organization induced by hydrogen bonding of the terminal hydrazide groups permits the reversibility of the color change. Fluorescence quenching is due to internal rearrangement of the liposome and occurs in both polymerized and unpolymerized structures.

1. Introduction

One of the major synthetic strategies in supramolecular chemistry is to use the power of self-assembly to build multi-functional structures from simple components. A good example of this is in the design of chemo- and biosensors built from separate sensing and reporting components. It is desirable for such devices to be selective for the analyte of interest, to work rapidly, and to be active at low concentrations [1,2]. It is also useful if the reporter function requires little or no external equipment to convey information, such as reporters constructed from colorimetric or fluorescent materials [3]. Highly conjugated organic materials have been very successful in this regard. For example, in very elegant work, Swager and co-workers have developed a series of conjugated polymers as the sensing materials for a variety of molecular targets [4–6]. Leclerc and co-workers have reported a wavelength-shifting fluorescence measurement with cationic polythiophenes [7,8], while Sparano and Koide have recently described a fluorescent sensor for specific RNA sequences on the basis of a 2′,7′-dichlorofluorescein fluorophore conjugated to two aniline derivatives as quenchers [9]. Nanostructured polymers have also shown promise, including polyaniline nanowires that have been used for detecting specific *Bacillus* strains [10]. Many other examples have also been reported [1,2,11].

The development of sensing systems based on self-assembled derivatives of 1,3-butadiyne (or diacetylenes, DAs) has also attracted interest. Certain DAs undergo a solid-state, topotactic polymerization in the presence of UV, X-ray, γ-ray irradiation or occasionally, by heating. The resulting polymers (PDAs) are highly conjugated, deeply colored and display interesting electronic and optical properties [12]. The possibility of using PDAs as reporter components in sensors has attracted much attention recently, since perturbation of the backbone planarity leads to an abrupt color transition from blue to red [12–15]. External stimuli, including heat, mechanical stress, or analyte/substrate binding of chemical or biological species, may trigger this unique chromic transition and sensing platforms based upon it have been designed to detect a variety of species, including toxins, sugars, and viruses [5,6,16,17].

In addition to the color change, the material becomes fluorescent in the red form. However, since this is a rather weak fluorescence, little effort has been devoted to exploit the effect [18]. Alternative strategies involving the addition of strong fluorophores to the systems have been reported [19]. Fluorophores can be bound to the surface of the liposome, entrapped in the hydrophobic bilayer or encapsulated in the aqueous interior.

The colorimetric shift of PDA liposome systems from blue to red is usually irreversible, and reflects a transition from a thermodynamically metastable blue form to a lower energy red form [20]. Only a few examples of reversible chromism of the PDA have been reported to date [15,20–24]. This behavior requires a complementary interaction (usually hydrogen bonding) that provides a restorative force to the system. Reversible fluorescence is a more difficult challenge, but Cheng and co-workers have developed a series of pH sensitive liposomes that, while not colorimetrically reversible, do display reversible fluorescence [25,26].

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We present here a simple approach for the fabrication of a novel self-assembled vesicle sensor that demonstrates both reversible color and fluorescence switching. Addition of a detector component results in a sensor system for *Escherichia coli*. The liposome features a PDA backbone that provides color switching, a head group that provides the restorative force when protonated, a lipid component bound to the fluorophore BODIPY, and a lipid-bound lectin (tryptophan) for *E. coli* detection.

2. Experimental

2.1. Materials and synthesis

10,12-Pentacosadiynoic acid (PCDA) was purchased from GFS chemicals (Powell, OH). The PCDA-HY [20] and PCDA-TR [14] were synthesized according to the published procedures, and characterized by ^{1}H NMR and ^{13}C NMR. 4,4-Difluoro-5-(2-thienyl)-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid (BODIPY 558/568C_{12}, abbreviated as BO558) was purchased from Molecular Probes. Solvents were dried before use. All other reagents were from commercial houses and used as received.

2.2. Vesicle preparation

Vesicles for pH studies: a chloroform solution containing PCDA-HY (1 mg) and BO558 (6 μg, 0.5 mol%) was added into a 15 ml vial. The solvent was removed by nitrogen stream and 3 ml of DI water were added into the vial. The sample was sonicated (probe sonicator) for 20 min, and then placed in a refrigerator at 5 °C for 2 h. The milky solution was then passed through a 0.45 μm syringe filter, which gives a clear, colorless solution. DI water (2 ml) was added into 0.2 ml of this stock solution for spectroscopic studies.

Vesicles for E. coli detection: liposomes were prepared as above, except PCDA-TR (5 mol%) was also added to the original solution.

2.3. Polymerization

HCl (0.04 ml, 1 M) was added to 1.5 ml of the above solution. After 5 min, the solution was irradiated with 0.3 J/cm^2 UV light (254 nm) for 1 min to yield a dark blue, clear vesicle solution.

3. Results and discussion

Liposomes were prepared from combinations of the lipids shown in Fig. 1. The hydrazine- (PCDA-HY) [20] and tryptophane-terminated (PCDA-TR) [14] compounds were prepared from 10,12-pentacosadiynoic acid using literature procedures. BO558 is a shorter-chain lipid featuring a carboxylic acid head group and the lipophilic BODIPY fluorophore as a tail group. The compound is commercially available and was chosen because the fluorophore is resistant to photobleaching, insuring good performance even after the photopolymerization of the diacetylenes. Combinations of these lipids could be assembled into vesicles by sonicating highly dispersed aqueous suspensions of the lipids.

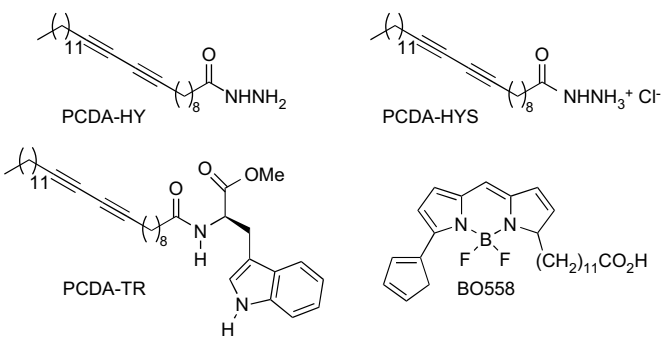

Fig. 1. Molecular structures of the vesicle sensor components used in this study.

Lipid assemblies containing the diacetylene moiety mid-way along the hydrophobic chain and terminated carboxylic acid head groups can be photopolymerized to form deep blue vesicles by exposure to 254 nm light. These have been reported to undergo an irreversible blue to red color change upon heating, treatment with organic solvents or deprotonation with strong base. Deprotonation results in a dramatic increase in the size of the liposome due to repulsion from the anionic head groups and may even result in a collapse of the structure.

In 1999, Jonas and co-workers demonstrated that liposomes with hydrazine head groups behave differently [20]. In the neutral form, there is extensive hydrogen bonding between adjacent amide groups, but the organization of the structure is such that the diacetylene groups are not in position to polymerize. Addition of HCl protonates the terminal amines, but instead of disrupting the assembly because of repulsion of the charged head groups, a slightly different organization arises due to hydrogen bonding between the ammonium ions and the chlorine counter ions. This new assembly is organized such that the diacetylenes are positioned properly for topotactic photopolymerization to occur and ready formation of the highly conjugated blue form of the PDA backbone is achieved [20].

3.1. Reversible color and fluorescence switching of PDA vesicle sensors

In order to expand the functionality of these hydrazine-terminated liposomes, a small amount (0.5 mol%) of the lipid fluorophore BO558 was added to the matrix lipid PCDA-HY. Fig. 2 shows the evolution of the electronic spectrum of the vesicle solution under various conditions. The as-formed liposome (Fig. 2a) showed absorptions at 530 and 567 nm due to the BO558 (the matrix lipid does not absorb above 300 nm). Irradiation of this solution resulted only in very slow formation of a blue color due to polymerization of the diacetylene moiety and very poor overall conversion. Treatment of the solution with dilute HCl produced no significant change to the absorption spectrum of the initial assembly, but the liposomes become highly sensitive to 254 nm irradiation and deep blue within 1 min (Fig. 2b and c, respectively).

After 1 min of irradiation with 254 nm light, the vesicle sensors showed typical PDA absorption spectra, having a distinct maximum at 640 nm and a shoulder at 575 nm. This is characteristic of "blue form" PDAs. When the irradiation time is extended to 5 min, it was

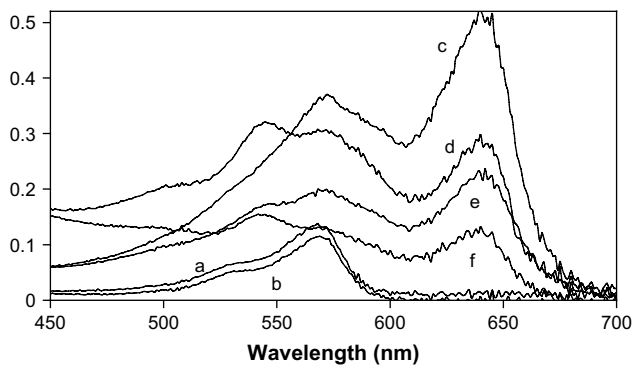

Fig. 2. UV–visible absorption spectra of liposomes composed of PCDA-HY and BO558: (a) as prepared; (b) solution 'a' treated with 0.02 ml 1 M HCl; (c) solution 'b' irradiated at 254 nm for 1 min; (d) solution 'c' treated with 0.02 ml 1 M NaOH; (e) solution 'd' treated with 0.02 ml 1 M HCl; (f) solution 'e' treated with 0.02 ml 1 M NaOH.

observed that the amount of "red form" PDA increased. Continuing irradiation resulted in conversion to the red form of the PDA (not shown).

Neutralization of the blue liposomes with NaOH (Fig. 2d) resulted in rapid conversion to the red polymer form, with absorption maximum at 544 nm. The NaOH eliminates the hydrogen bond network from the terminal amines, while leaving the amide hydrogen bonding intact [23]. When the basic vesicle sensor is acidified with HCl again, the color reverted to the blue form (Fig. 2e) and then back to the red form on additional base treatment (Fig. 2f). With each cycle, there is a decrease in overall intensity. Some of this is due to the dilution of the liposomes with addition of the aqueous acid and base, but some is also due to aggregation and precipitation of the liposomes during the acidification stage. This is also evidenced by the increase of the baseline at 450 nm due to scattering from the aggregated liposomes.

The fluorescence spectra of the liposome excited at 475 nm were then collected under the same set of conditions, as were electronic spectra (Fig. 3). Emission was observed at 585 nm in the neutral vesicle. Unlike BO558-containing liposomes made from PCDA, the fluorescence intensity was not significantly quenched compared to that of the free fluorophore in solution. However, addition of HCl, and the resulting reorganization of the lipid assembly, caused a dramatic drop (~ 70%) in emission intensity and a red shift (Fig. 3, curve b). Photopolymerization to the blue form of the liposome resulted in further quenching to about 10% of the original intensity (curve c).

Addition of NaOH resulted in dramatic recovery of the fluorescence intensity as the blue color of the vesicle quickly changed to red. The recovered intensity (Fig. 3d) was approximately 75% of that of the unpolymerized, neutral liposome, and nearly three times of that observed after addition of HCl (Fig. 3b). The emission also displayed a blue shift after addition of base, from around 584 to 577 nm. Very interestingly, subsequent addition of 0.02 ml 1 M HCl results in a substantial decrease in fluorescence, reducing the intensity to below curve c (Fig. 3e), while the color of the vesicle changes from red to blue as shown in Fig. 2e. Addition of NaOH again resulted in the recovery of fluorescence as the vesicles again turned red. This process is then reversible in both the diacetylene absorbance and the BO558 fluorescence upon acid and base cycling. To the best of our knowledge, this reversibility of both color and fluorescence in a single system has not been previously reported.

The mechanism of fluorescence quenching in these systems is not entirely clear. The PDA backbone does absorb strongly at the emission wavelength of the fluorophore and energy transfer between the dye and the conjugated backbone is energetically allowed. Cheng and Ma have used time-resolved fluorescence measurements to argue against Förester energy transfer or some other rate-driven process in BO588-contiaining liposomes composed of glycine-terminated lipids [27]. The fact that our vesicles undergo little volume change during the quenched/unquenched switching supports this thesis. Additionally, the color/fluorescence switching occurs between acidic and neutral conditions, whereas previous systems have operated between neutral and basic conditions. In the latter case, repulsion between the negatively charged head groups not only increases the size of the vesicles, but might also "push" the non-covalently bound BO588 lipid out of the vesicle and thereby move the fluorophore away from the PDA backbone. In our case, fluorescence recovery is observed when the exterior of the liposome is neutral and the system is relaxed.

An important observation here is that the fluorescence switching is driven by a pH-induced reorganization of the vesicle. The switching occurs in both polymerized and non-polymerized vesicles. Note that quenching is not observed in vesicles prepared from saturated lipids, thus the quenching sites do require a π-system, but not necessarily an extended π-system.

3.2. *Color and fluorescence sensing of* E. coli

A primary motivation for investigating PDA-containing liposomes is for their potential use as biosensors and the ease of adding lectins to carboxylic acid PDA vesicles has resulted in a variety of sensor designs customized for many different pathogens. However, PDA biosensors that display reversible colorimetric behavior have not yet been reported [16]. Liposomes prepared from hydrazine-terminated lipids offer a platform that might be suitable for the development of such sensors. One potential concern is that the pH-induced switching behavior of these liposomes takes place near the range required for many pathogens to survive. In these experiments, we carefully monitored the pH to insure that any optical changes were due to the introduction of *E. coli*.

Here, tryptophan was the recognition element for *E. coli* due to the ease of synthesizing PCDA-TR and the known affinity between the amino acid and the pathogen [28]. Vesicles were prepared in the same manner as in the previous section, except that 5 mol% PCDA-TR was added. As before, after treatment with HCl, the vesicles underwent ready polymerization under short UV irradiation. The binding of *E. coli* to the vesicle solution and the effect of the binding were assessed by UV–vis and fluorescence spectroscopies. As shown in Fig. 4c, after 45 s of UV irradiation, the vesicle sensor absorbs mainly at 640 nm with a shoulder at 570 nm and

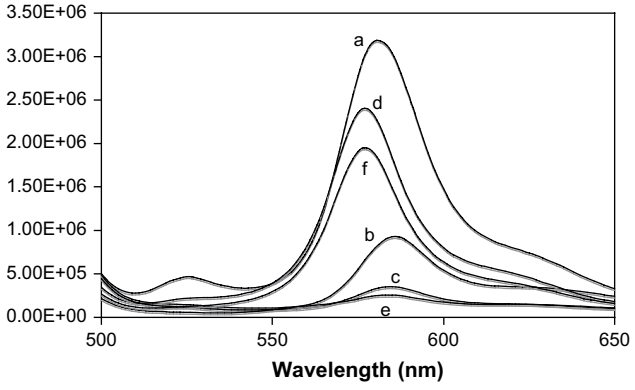

Fig. 3. Fluorescence spectra of liposomes composed of PCDA-HY and BO558: (a) as prepared; (b) solution 'a' treated with 0.02 ml 1 M HCl; (c) solution 'b' irradiated at 254 nm for 1 min; (d) solution 'c' treated with 0.02 ml 1 M NaOH; (e) solution 'd' treated with 0.02 ml 1 M HCl; (f) solution 'e' treated with 0.02 ml 1 M NaOH.

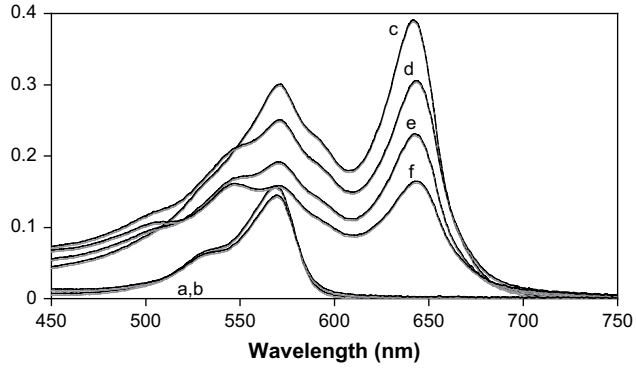

Fig. 4. UV–visible absorption spectra of liposomes composed of PCDA-HY, PCDA-TR and BO558: (a) as prepared; (b) solution 'a' treated with 0.02 ml 1M HCl; (c) solution 'b' irradiated at 254 nm for 45 s; (d) addition of 10 μl solution of *E. coli* to solution 'c' and incubated for 1 h; (e) centrifugation of solution 'd' for 1 m; (f) addition of 10 μl solution of *E. coli* to solution 'e' and incubated for 1 h.

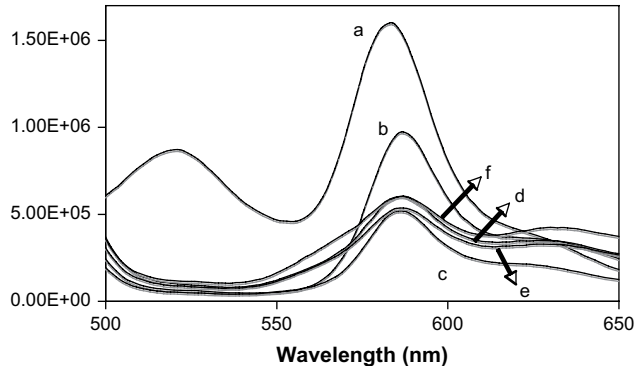

Fig. 5. Fluorescence spectra of liposomes composed of PCDA-HY, PCDA-TR and BO558: (a) as prepared; (b) solution 'a' treated with 0.02 ml 1 M HCl; (c) solution 'b' irradiated at 254 nm for 45 s; (d) addition of 10 μl solution of *E. coli* to solution 'c' and incubated for 1 h; (e) centrifugation of solution 'd' for 1 m; (f) addition of 10 μl solution of *E. coli* to solution 'e' and incubated for 1 h.

gives a blue color. At the same time, the fluorescence is largely quenched (Fig. 5c). With the addition of *E. coli*, a new absorption peak around 550 nm was observed (Fig. 4d) and color changed to purple. Consistent with this, the addition of *E. coli* also resulted in an increase of the fluorescence intensity (Fig. 5d). Centrifugation of the solution resulted in a decrease in both the visible absorption and the fluorescence as some of the *E. coli*/sensor complex was removed. However, the color of the vesicle solution remained purple (Fig. 4e and Fig. 5e). When more *E. coli* was added, the color of the vesicle solution changed to red and the fluorescence emission increased again (Fig. 4f and Fig. 5f).

Clearly, our attempt to reverse the color change by physically removing the *E. coli* from the solution did not succeed. The stability of this system to low pH however suggests that the color change might be reversed if the liposomes were affixed to a surface that could be removed from the bacteria and washed with dilute acid.

4. Conclusions

We have demonstrated a vesicle signaling system with reversible color and fluorescence switching behavior in response to pH change. This is the first example of a reversible color and fluorescence PDA sensing system. The mechanisms of the two processes are related, but not identical. The ability for the system to return to the blue state after being perturbed to the red state is due to the stabilization provided by two different hydrogen bonding motifs, one pH sensitive and the second is not [20]. The quenching of fluorescence is due to the internal organization of the hydrophobic region. The quenching process involves the conjugated π system, but while the extended π system of the polymer may be more efficient for fluorescence quenching, it is not a requirement. Addition of a tryptophan-linked lipid to the ensemble results in a colorimetric/fluorescent biosensor. While this might be transformed into a reversible reporting system, simple physical removal of the bulk of the bacteria is insufficient to reverse the color change.

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