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ORIGINAL PAPER

Enhancing the Emission of Polydiacetylene Sensing Materials Through Fluorophore Addition and Energy Transfer

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Abstract Enhancement of the environmentally responsive fluorescent properties of polydiacetylene (PDA) by combination with lipophilic fluorophores was demonstrated and properties of the PDA/fluorophore systems were explored. Liposomes containing PDA and fluorophores exhibited enhanced Stokes shift and increase in emission as a result of energy transfer from PDA to fluorophore. The effects of fluorophore variation, degree of PDA polymerization and diyne placement in the diacetylene lipid tails on the emission enhancement were studied. It was determined that signal generation was optimized at a relatively low extent of PDA polymerization with the optimal degree of polymerization dependent on the other parameters. Energy transfer was used as a tool to detect fluorophore exchange between polymerized and unpolymerized liposomes and to study the effects of fluorophore structure on exchange from unpolymerized to PDA liposomes. Fluorophores that locate at the aqueous interface with alkyl anchors were slow to transfer while fluorophores that partition into the alkyl regions of the liposomes transfer quickly.

Keywords Polydiacetylene · Fluorescence · Liposome · Sensor · Biosensing · FRET · Energy transfer · Transfer between liposomes

Introduction

The environmentally responsive optical properties of the conjugated polymer polydiacetylene (PDA) have been used for signal generation for bio-assay and detection applications [1]. Both the absorbance and emission of the material change in response to stimuli; the chromic changes have been used extensively in the development of PDA sensing materials, with the emissive properties more recently gaining attention [2–6]. PDA emission can be excited directly or through energy transfer from other emissive species [1, 2, 7]. PDA has also been used as an environmentally sensitive quencher for other fluorescent species [8–10]. This paper presents results from studying the enhancement of PDA emission through the addition of fluorophores that accept energy from excited PDA and emit, increasing the total light output of the system and the Stokes shift.

PDA has two great advantages for forming bio-sensing materials: it has a conjugated backbone that gives it absorbance and fluorescence properties for signal transduction, and diacetylene surfactants will self-assemble in water to form biomimetic films and liposomes that can be photopolymerized to generate PDA in situ (Fig. 1) [1]. PDA liposomes have been the most popular PDA material format for assay and bioanalyte detection applications. PDA liposomes can be prepared with lipophilic enzymatic substrates and biological ligands incorporated, or the bio-reactive species can be conjugated to the surfaces. The majority of the efforts developing PDA liposomes for sensing have focused on using the chromic properties for signal generation. Changes in the conjugation state of the polymer backbone due to external environmental disturbances lead to shifts in the absorption maxima causing a gradual change in the color from blue to purple or red. The change in the absorption properties is usually quantified by calculation of the "colorimetric ratio" (CR); the ratio of the intensity of the "blue" maximum absorbance peak (650–620 nm) over the sum of the intensities of the "blue" and

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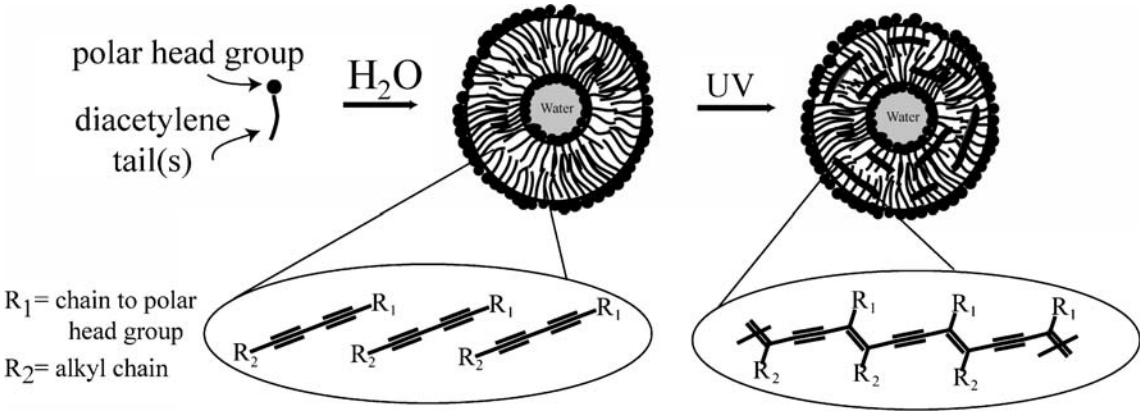

Fig. 1 PDA liposome formation cartoon

"red" (550–500 nm) absorbance peaks [11]. The emissive behavior of PDA parallels the chromic; red PDA is fluorescent while blue PDA is non-fluorescent [12, 13] with a negligible quantum yield estimated at $<10^{-5}$ by femtosecond spectroscopy [14, 15].

The quantum yield of fluorescent PDA materials is not large and decreases with increased temperature [16]. Olmsted measured quantum yields of 1–3% for multilayered red PDA films at room temperature [13]. Fluorescent PDA films and liposomes can be excited with wavelengths above 450 nm and emit two broad fluorescent peaks approximately 560 and 635 nm [3]. The relative heights of the peaks can vary considerably with some materials showing only the 560 nm peak and others having more intense emission at 635 nm than at 560 nm. The ratio of the two emission peaks is dependent on the extent of polymerization; controlled polymerization of 10,12-pentacosadiynoic acid (10,12-PCDA) liposomes showed that the 560 nm peak dominated the emission after polymerization with low UV doses and the relative height of the 635 nm peak increased with UV exposure (Fig. 2). The absorbance profile of the emissive liposomes did not change significantly with UV dose, though the total absorbance increased as more polymer was formed. The change in the emission of PDA liposomes can be much greater than the change in absorbance [1, 3]. For example, for poly(10,12-PCDA) liposomes exposed to base the emission change at 640 nm was 70 times greater than the calculated CR (Fig. 3a). This is easily explained by considering the different spectra for absorbance and emission of red and blue poly(PCDA) liposomes (Fig. 3b). The blue liposomes have no appreciable emission while the red are quite emissive. By contrast there is considerable overlap between the red and blue absorbance spectra of the same samples. The greater response of the emission vs the absorbance shows the advantages of using the changeable PDA fluorescence properties for signal generation, particularly in cases where the color change is not complete.

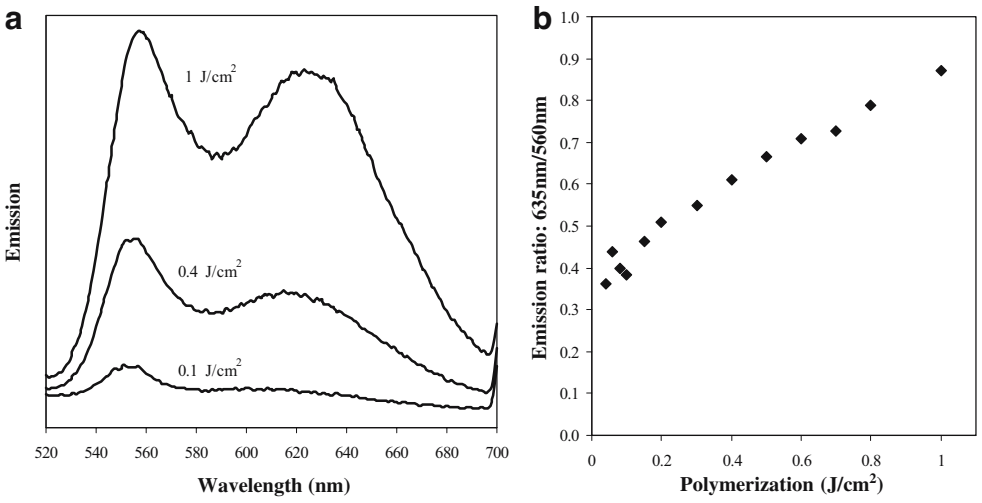

Fig. 2 10,12-PCDA liposomes polymerized with increasing doses of UV. **a** Emission spectra of heated liposomes exposed to 0.1, 0.4 and 1.0 J/cm^2 UV, excitation 475 nm. **b** Chart of UV dose vs ratio of 635/560 nm emission

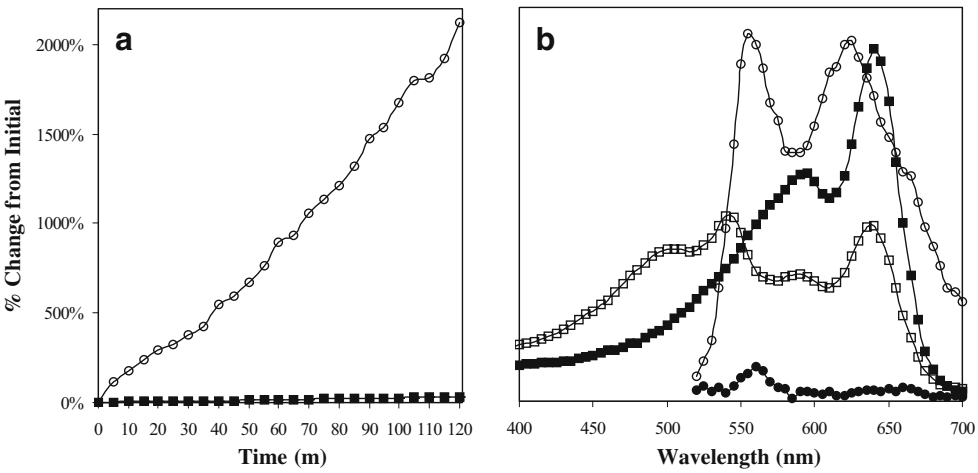

Fig. 3 Absorbance and emission of poly(10,12-PCDA) liposomes diluted 1:9 with 25 μM $NaHCO_3$ measured with Spectromax 250 and Gemini EM plate readers respectively. **a** Kinetic plot showing change in emission at 640 nm (*open circles*) and CR using 640 and 500 nm absorbances (*filled squares*) during base exposure. **b** Spectra of absorbance before exposure to base (*filled squares*), absorbance after exposure to base (*open squares*), emission before exposure to base (*filled circles*) and emission after base (*open circles*)

The emissive properties of PDA can be additionally altered and enhanced through incorporation of lipophilic fluorophores capable of undergoing resonance energy transfer (RET or FRET) with the polymer [3]. FRET between small molecule chromophores is described by the Förster mechanism as an excited donor transferring energy through distant dependent ($1/r^6$) dipole–dipole coupling to an acceptor, which then fluoresces [17, 18]. The Förster mechanism is not completely appropriate for predicting or describing energy transfer in conjugated polymer systems as shown by studies of conjugated polymer films [19, 20] and of conjugated polymer incorporated in silica mesophases [21]. In particular, the Förster model's approximation of chromophores as single point dipoles does not hold for extended polymers and the transfer rate does not drop with distance as quickly as predicted by the Förster model [22]. These studies have also shown that in films energy transfer to chromophores is faster than exciton migration along the conjugated backbone, where the chromophores may be non-contiguous conjugated polymer segments or discrete small molecules such as fluorophores, incorporated in the polymer materials [19, 21]. This means that energy transfer to fluorophores is a feasible method for altering the overall fluorescence properties of conjugated polymer materials.

The results of investigating fluorophore enhancement of emission in PDA liposomes prepared with varied combinations of fluorophore, diacetylene and degree of polymerization are presented in this paper. The interaction of these parameters and their influence on the optimization of the fluorescence enhancement is discussed and the competition between energy transfer between conjugated polymer segments vs energy transfer from the polymer to the fluorophore examined. PDA to fluorophore energy transfer was also used as a means to detect lipophilic fluorophore transfer between unpolymerized and polymerized liposomes and to probe the kinetics of the transfer.

Materials

10,12-PCDA and 10,12-tricosadiynoic acid (10,12-TRCDA) were purchased from GFS Chemicals. 6,8-PCDA [23] was synthesized according to literature procedures [24, 25]. Fluorophores 5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)-acetyl)amino)pentylamine hydrochloride (BODIPY® TR cadaverine, **1**), BODIPY® 630/650 STP ester sodium salt (**2**), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine-5,5′-disulfonic acid (DiIC18 (5)-DS, **3**), 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indodicarbocyanine 4-chlorobenzenesulfonate salt (DiIC18(5), **4**), N-((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl) sphingosine (BODIPY® TR ceramide, **5**) and 1,1′-dioctadecyl-5,5′-diphenyl-3,3,3′,3′-tetramethylindocarbocyanine chloride (5,5′-Ph2-DiIC18(3), **6**) were purchased from Molecular Probes (now Invitrogen). 1,2-disteroyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids. Organic solvents were HPLC or Optima grade from Fisher Scientific.

Methods

Liposome preparation

Dried diacetylene or dried diacetylene and fluorophore (0.5%) mixtures were dispersed by probe sonication at 1 mM in deionized water (18 MΩ) until clear solutions at 70–80 °C were obtained. The hot solutions were filtered through 0.8 μ cellulose acetate and cooled overnight at 10 °C [2]. Photopolymerization was achieved with a Fisherbrand UV Crosslinker capable of delivering calibrated energy doses of UV light at 254 nm; liposome solutions were cooled on ice during UV exposure. Polymerized liposome solutions were stored at 4 °C, and converted to the emissive form by heating at 78–80 °C for 5 min, causing an irreversible conversion.

DSPC liposomes with fluorophores were prepared by bath sonication of dried lipids and fluorophores (0.5%) (1 mM in deionized water) at 35–45 °C for 30 m, stored at 4 °C under argon and re-sonicated in a bath sonicator at 50 °C under argon for 15 min then cooled under argon to room temperature before being used in fluorophore transfer experiments.

Fluorescence and absorbance spectra

Fluorescent spectra of unpolymerized, polymerized and thermally converted polymerized liposomes with and without fluorophores, diluted to 0.1 mM lipid in water, were acquired in 250 μl quartz cuvettes with a SPEX Fluoromax-2 using diffraction gratings for wavelength selection. UV–Vis spectra were measured with a Molecular Devices Spectramax-250 plate reader using 100 μl per well of diluted liposome solutions in a Corning UV–Vis transparent 96-well plate. The relative amount of PDA in different liposomes solutions was determined by adding the absorbances from 400 to 700 nm of polymerized liposomes. Prior to summation, the absorbances were adjusted by subtraction of the absorbances of the corresponding unpolymerized liposomes to remove contributions from fluorophore absorbances and scattering.

DLS

Dynamic light scattering measurements were performed with a Precision Detectors PDDLS/PD2000DLS detector with a 800-nm light source. Liposome solutions were diluted 10-fold with deionized water and centrifuged at 1.3 g for 2–5 min. Data were collected on 200 μl samples at room temperature and the Stokes Einstein equation used to calculate the liposome diameter using the first cumulant from each run. Diameter values from four runs were averaged.

Fluorophore transfer experiments

Spectra Unpolymerized liposomes (30 μl) were combined with thermally converted poly(10,12-PCDA) liposomes and water to a final volume of 200 μl. Each mixture was prepared immediately before measurement with the unpolymerized liposomes added to the diluted PDA liposomes. Each mixture was vortexed briefly, transferred to a 250-μl quartz cuvette and the emission spectra scanned in the Fluoromax-2 with 475 nm excitation. After scanning, the mixture was returned to a storage tube and held at room temperature in the dark for measurements at later time points. Contributions of the PDA liposome emission to the emission at the fluorophore's emission were subtracted when comparing the fluorophore emission response to increased amounts of PDA liposomes in the mixtures.

Kinetics Kinetic emission data were collected in a Molecular Devices Gemini-EM plate reader with dual monochromators for wavelength selection. Wells of a black polystyrene 96-well plate were charged with PDA liposomes and water at a total volume of 185 μl. Initial single point emission measurements were made at 625 nm (excitation at 475 nm), then unpolymerized liposomes (15 μl; quadruplicate samples) with **1** incorporated were added to appropriate wells (water was added to the control wells) and the plate read at 5 min intervals with 3 s shaking before readings. The emission data were averaged and control values, i.e., from wells with only PDA liposomes, subtracted to remove contributions to the 625 nm emission from the PDA.

Results and discussion

Initial experiments were performed to assess the affect of different fluorophores on the emission of the PDA liposomes, and in particular their effect on the change in emission upon the liposomes converting from the non-emissive PDA state (blue) to the emissive state (red). The energy of the excited PDA must be able to excite the fluorophores; this is best predicted by examining the overlap between the fluorophores absorbance spectrum and PDA emission [17, 18]. Lipophilic BODIPY™ (**1**, **2**), and cyanine (**3**, **4**) fluorophores were selected and incorporated into liposomes (Fig. 4). The BODIPY™ **1** and **2** fluorophores insert into the hydrophobic tail region of the liposomes while the cyanine fluorophores sit at the polar surface, anchored by 18-carbon alkyl tails. Absorbance curves of the chosen fluorophores with the emission of fluorescent poly(PCDA) liposomes superimposed to show the overlap between donor (PDA) and acceptors, are shown in Fig. 5.

To enhance the overall emission of the PDA liposomes for detection applications, the fluorophores must remain unexcited by direct exposure to the excitation wavelength used for PDA in addition to accepting energy efficiently from the polymer. When a fluorophore is significantly excited by the wavelength used to excite the polymer, the blue "non-fluorescent" PDA liposomes show a high fluorescent background arising from the fluorophore emission. This background fluorescence leads to an overall decrease in the extent of the change in emission upon the liposomes converting to the PDA fluorescent form and hence reduces the signal. Emission curves of poly(10,12-PCDA) liposomes with the fluorophores incorporated, both as prepared in the non-fluorescent blue form, and after heating to the fluorescent red form are shown in Fig. 6. These figures show that when incorporated in unpolymer-

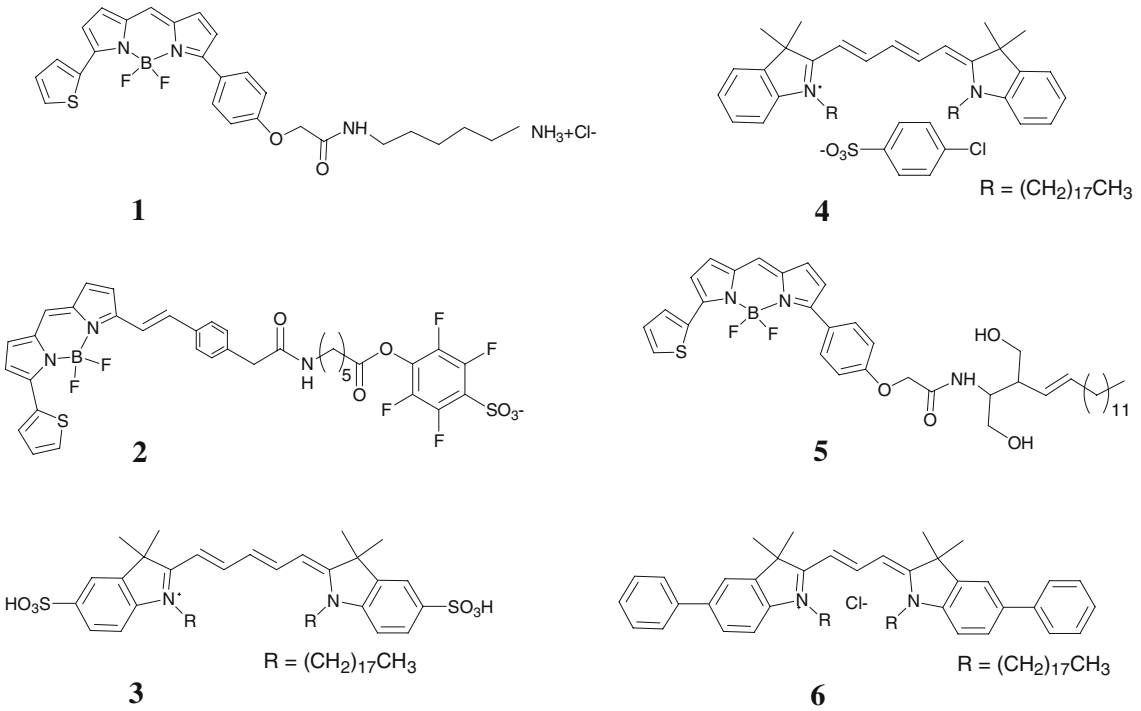

Fig. 4 Fluorophore structures

ized liposomes and in non-emissive poly(10,12-PCDA) liposomes the selected fluorophores (**1**–**4**) do not emit, or have relatively low emissions. After the liposomes have been thermally converted the fluorophores show significant emission, increasing the total light output of the system. The changes in the emissions were calculated for the non-emissive to emissive transition of liposomes with different fluorophores at several wavelengths (Table 1). The identity of the added fluorophore clearly influences the extent and wavelength of the emission gain, with the largest change seen in liposomes with BODIPY® TR cadaverine (**1**) incorporated, at 635 nm. Incorporation of BODIPY® 630/650 (**2**) did not increase the liposome emission at 635 nm, but approximately matched the performance of **1** at higher wavelengths. This may be because **2** has less overlap with the lower emission band of PDA than **1** (Fig. 5). Similarly, the sulfonated cyanine DiIC18(5)-DS (**3**) did not increase the emission compared to the plain liposomes at any wavelength, while liposomes incorporating the non-sulfonated version of DiIC18(5) (**4**) was significantly more emissive than the plain liposomes at 665 nm and higher wavelengths. Though **3** and **4** have very similar structures, the absorbance of **3** is slightly red shifted from **4**, in a region where the emission intensity of PDA is decreasing quickly (Fig. 5), reducing the effectiveness of energy transfer from the PDA to the fluorophore.

Subsequently, BODIPY® TR ceramide (**5**) and cyanine DiIC18(3) fluorophore **6**, were incorporated in 10,12-PCDA liposomes, which were then polymerized and converted. The liposomes were tested for energy transfer from the PDA to the fluorophores, as shown in Fig. 7. Fluorophore **5** has the same fluorescent group as **1** but the fluorescent moiety is adjacent to the polar portion of the molecule, leading to it being closer to the lipid/aqueous interface (Fig. 4). Despite this structural variation, **5** showed comparable acceptance of energy from the PDA polymer to **1**. Cyanine fluorophore **6** accepted energy readily from the PDA; unfortunately, the excitation light also directly excites the fluorophore. As a result, the blue liposomes are already somewhat emissive and the

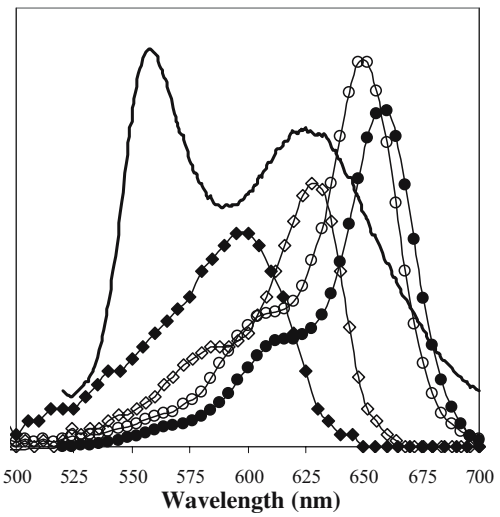

Fig. 5 Comparison showing the overlap of normalized absorbances of fluorophores **1** (*filled diamonds*), **2** (*open diamonds*), **3** (*filled circles*) and **4** (*open circles*) in solution with PDA liposome emission (*line*)

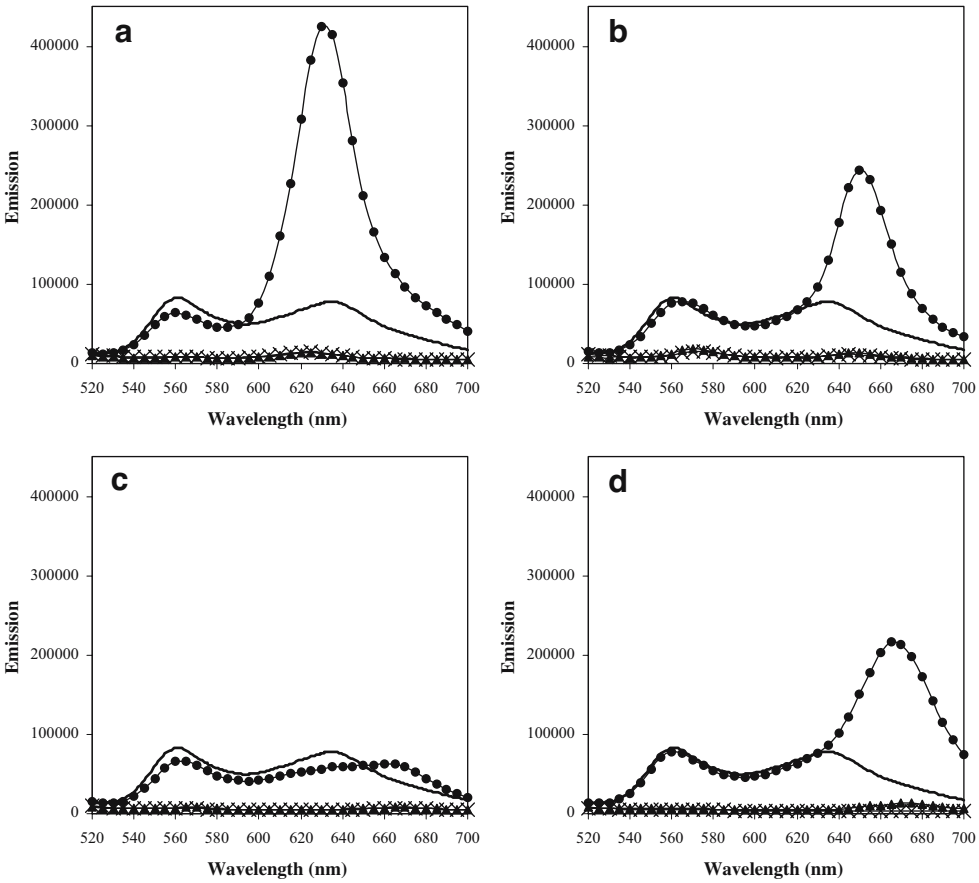

Fig. 6 Emission spectra of poly (10,12-PCDA) liposomes, containing 0.5% fluorophore: unpolymerized (×), polymerized with 0.6 J/cm^2 UV at room temperature (*filled triangles*) and thermally converted (*filled circles*). Emission spectrum of poly (10,12-PCDA) liposomes polymerized with 0.6 J/cm^2 UV and thermally converted (*line*) is also shown. **a** Liposomes with **1**; **b** with **2**; **c** with **3**; **d** with **4**. Excitation at 475 nm

corresponding change in emission upon conversion of the PDA/**6** to the fluorescent form is lower than seen with PDA/**4**. It should be noted that **6** emits approximately four times more strongly when excited at 475 nm in the fluorescent PDA liposome than in the unpolymerized liposome, supporting the hypothesis that most of the emission arises from energy transfer from the PDA.

The excited PDA segments have multiple pathways for returning to the ground state besides emission, including transfer to other polymer segments, transfer to the embedded fluorophores, exciton migration along the backbone until reaching a trap and vibrational relaxation. Exciton migration in other conjugated systems has been shown to be slow relative to transfer between conjugated segments and chromophores [19, 21]. Increasing the density of the polymer, however, may lead to more energy transfer between conjugated polymer segments, and less transfer to the fluorophores. To examine this possibility, 10,12-PCDA liposomes with fluorophores **1** and **4** incorporated were polymerized with increasing doses of UV and thermally converted to the fluorescent form. The emissions at 630 nm (for **1**) and 665 nm (for **4**) were adjusted for contributions from the PDA present in the liposomes, using data from plain PDA liposomes polymerized under the same conditions. The ratios of the fluorophore emission to the PDA emission at 560 nm were calculated for each set of samples and plotted against the UV dose used to polymerize the liposomes (Fig. 8). Both sets of samples showed first an increase in the fluorophore emission/PDA emission ratio at low polymerization extents and then a drop off, with the samples with **1** showing a larger decrease and samples with **4** being less sensitive to increased polymerization. These results support the model that with low amounts of PDA in the liposomes the excited PDA conjugated segments transfer energy to the fluorophore and that within the low UV exposure regime as the amount of PDA goes up more segments are available to be excited and thus to

Table 1 Ratios of the emission of heated liposomes (red) with fluorophores **1**, **2**, **3** and **4**, and without fluorophore (plain), over the emission of the room temperature (blue) liposomes, at specific wavelengths

Wavelength (nm)	1	2	3	4	Plain
635	35.82	11.30	10.25	14.90	13.86
665	17.03	15.76	6.91	16.56	7.59
675	14.44	12.16	5.85	14.59	6.98
690	10.87	8.17	4.39	12.32	5.06

Liposomes polymerized with 0.6 J/cm^2 UV; excited at 475 nm

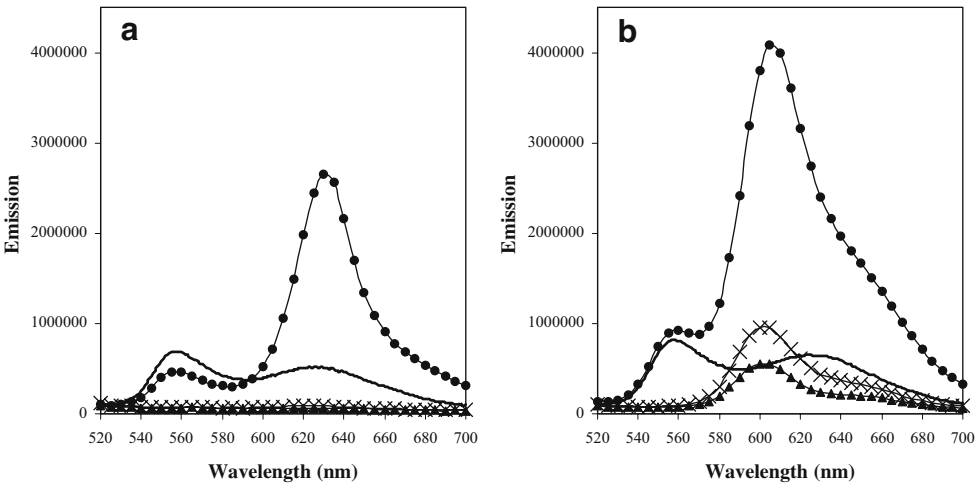

Fig. 7 Emission spectra of poly (10,12-PCDA) liposomes containing 0.5% fluorophore: unpolymerized (×), polymerized with 0.6 J/cm^2 UV at room temperature (*filled triangles*) and thermally converted (*filled circles*). Emission spectrum of poly (10,12-PCDA) liposomes polymerized with 0.3 J/cm^2 UV and thermally converted (*line*) is also shown. **a** Liposomes with **5**; **b** with **6**. Excitation at 475 nm

transfer energy to the fluorophores. With higher doses of UV (i.e., above 0.1 J/cm^2) for polymerization, however, the PDA chains in the liposomes become sufficiently concentrated that PDA segments are in suitable proximity for energy transfer between segments, and this process competes with energy transfer to the fluorophore. It also seemed possible that the drop in emission at higher UV doses reflected bleaching or degradation of the fluorophores by the 254 nm UV light used to polymerize the liposomes; however, additional experiments performed with fatty acid liposomes prepared from other diacetylenes did not support this theory, as discussed below.

A small study was performed to investigate the effects of the diacetylene monomer tail structure on energy transfer to fluorophores. PDA liposomes can be prepared from diacetylenes with different length chains, usually between 22 and 26 carbons, and with the diacetylene at different positions along the chain relative to the head group. The emission profiles of different converted fatty acid liposomes

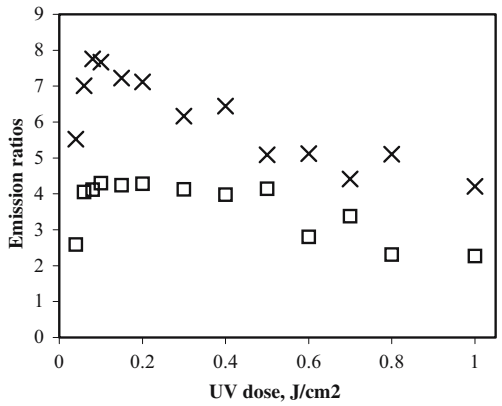

Fig. 8 Chart of the ratios of emission at fluorophore emission wavelength over the PDA emission at 560 nm for poly(10,12-PCDA) with **1** (×, 630/560 nm) and with **4** (*open squares*, 665/560 nm) vs UV dose. Emissions at 630 and 665 nm had PDA contributions subtracted. Excitation at 468 nm

vary as shown in Fig. 9. The emission profiles can be explained by the observation that the extent of polymerization for a given UV dose varies with the structure of the diacetylene tail; this effect has been noted in films and self-assembled monolayers, as well as in liposomes [1]. The relative extent of polymerization is estimated by comparing the total absorbance of the polymer in the liposomes. Liposomes composed of TRCDA polymerize very readily and the emission at 635 nm is greater than the emission at 560 nm as shown in Fig. 9a. On the other hand, liposomes composed of 6,8-PCDA do not polymerize as well as those formed from 10,12-PCDA or 10,12-TRCDA, and the emission spectrum of the polymerized converted liposomes is dominated by the 560 nm emission. Liposomes were prepared from 10,12-TRCDA and 6,8-PCDA with **1** and **4** incorporated and the emission spectra compared to those of the 10,12-PCDA/fluorophore liposomes used in earlier experiments. The spectra of the polymerized liposomes with fluorophores are shown in Fig. 9b–d. The contribution of the PDA fluorescence to the total emission at the fluorophore emission wavelength (630 nm for **1**; 665 nm for **4**) was subtracted and the ratio of the fluorophore emission peak to the 560 nm peak was calculated for the different systems (Table 2). PDA in poly(TRCDA) liposomes does not transfer energy to fluorophore **4**, even when the liposomes are polymerized with a low UV dose. PDA in the poly(10,12-TRCDA) liposomes does transfer energy to **1**, though to a much lesser extent than in poly (10,12-PCDA) liposomes. In contrast, 6,8-PCDA liposomes do not polymerize very well but the PDA formed in these liposomes transfers energy to both fluorophores **1** and **4** very readily. This is consistent with the theory described above that energy transfer to the fluorophores is better at low levels of polymer where competitive energy transfer between polymer chains is less likely due to low polymer density.

These results also show that the differences between energy transfer in liposomes polymerized to different

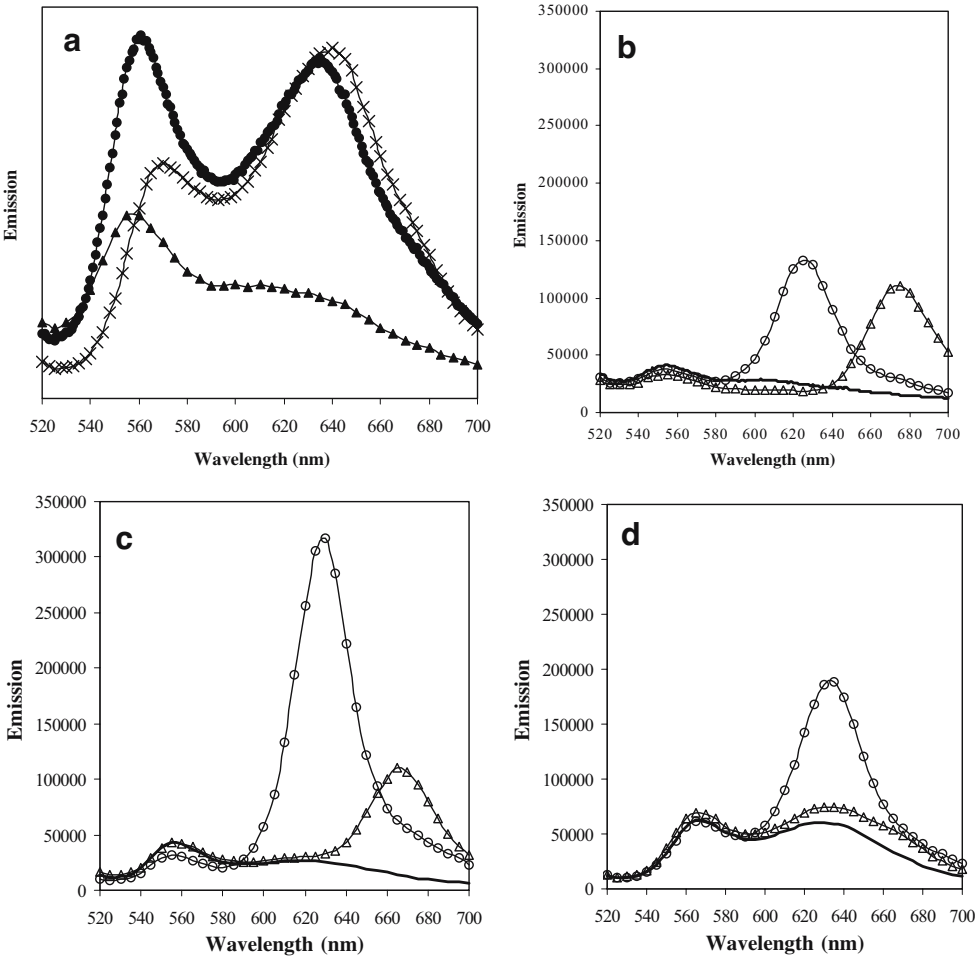

Fig. 9 **a** Emission spectra of heated poly(10,12-PCDA) (*filled circles*, 0.6 J/cm^2 UV dose), poly(10,12-TRCDA) (×, 0.2 J/cm^2 UV dose), and poly(6,8-PCDA) (*filled triangles*, 0.6 J/cm^2 UV dose) liposomes. **b**–**d** Emission spectra of PDA liposomes: with 0.5% **1** (*open circles*), with 0.5% **2** (*open triangles*), and plain (*line*). **b** poly(6,8-PCDA), polymerized with 0.1 J/cm^2 UV; **c** poly(10,12-PCDA), polymerized with 0.1 J/cm^2 UV; and **d** poly(10,12-TRCDA), polymerized with 0.05 J/cm^2. Excitation at 475 nm

extents do not arise from fluorophore exposure to UV during polymerization. As the same UV dose was used for the 10,12-PCDA and the 6,8-PCDA liposomes, the difference in the energy transfer from the PDA to **4** cannot be a function of UV degradation of the fluorophore. Similarly, if UV degradation were the only contributing factor to reduced energy transfer, the 10,12-PCDA liposomes should show less energy transfer to both **1** and **4** than the 10,12-TRCDA liposomes rather than more. Instead, these results suggest that increased amounts of polymer in the liposomes leads to competition between transfer from the excited PDA to other PDA chains vs transfer to the fluorophores. In practical terms, there is a trade-off between polymerizing until there is sufficient polymer for material stabilization and enough emission for a good signal to noise ratio, and optimizing the energy transfer to the fluorophore.

Table 2 Emission ratios of fluorophore emission at 630 or 665 nm over PDA emission at 560 nm for heated PDA liposomes

	630/560 nm		665/560 nm		UV dose	Σ Abs 400–700 nm
	Plain	*w*/1	Plain	*w*/4	J/cm^2	Plain, *w*/1, *w*/4
6,8-PCDA	0.61	10.86	0.33	9.60	0.1	1.2, 1.6, 1.4
10,12-PCDA	0.47	14.81	0.07	3.34	0.1	11.5, 9.9, 13.4
10,12-TRCDA	1.08	3.88	0.50	0.82	0.05	13.0, 12.6, 14.0

Sums of absorbances, adjusted to remove contributions from fluorophores, from 400 to 700 nm of liposomes prior to heating show relative extent of polymerization

A final study used the energy transfer phenomenon to examine the transfer of lipophilic fluorophores from unpolymerized liposomes to PDA liposomes. The ability of PDA liposomes to take up lipophilic species is of interest and has been used in liposome characterization, but has not been systematically studied. Addition of lipophilic species to mixed PDA/phospholipid liposomes has been used to add spin-labeled fatty acids [26] and to incorporate ligands [4, 6]. In a recent report Ma and Cheng used the inhibition of transfer of a BODIPY 558/568 attached to a ganglioside GM1 lipid from micelles into PDA liposomes as a basis for detecting cholera toxin B (CTB) [10]. The energy transfer phenomenon described in this paper provides a method for determining the location of fluorophores. Resonance energy transfer has been used as a tool by other researchers to examine membrane fusion [27, 28] and transfer of species

between liposomes [29]. The emission of the lipophilic fluorophores shows whether they are in unpolymerized liposomes where the energy transfer cannot occur, or in polymerized emissive PDA liposomes where they are excited by energy transfer from the polymer. This provides a probe for measuring transfer of fluorophores (as representative lipophilic species) from unpolymerized liposomes to PDA liposomes.

Unpolymerized 10,12-PCDA liposomes with fluorophores **1**, **2**, **4** and **6** incorporated were mixed with poly (10,12-PCDA) liposomes (without fluorophores) that had been thermally converted into the fluorescent state and the emission of the mixtures was measured immediately after mixing and after 90 minutes. Similar measurements were made using converted polymerized liposomes with fluorophores mixed with unpolymerized liposomes without fluorophores. The emission spectra of PDA liposomes with fluorophores incorporated were also measured and compared to the spectra of the mixtures (Fig. 10). The BODIPY fluorophores **1** and **2** transferred from the unpolymerized liposomes to the PDA liposomes very quickly. Comparison of the spectra of the PDA/fluorophore alone and the mix spectra suggest that almost half of the fluorophores **1** and **2** transferred (approximately 35–40%) after only 1–2 min with an additional 6–7% transferred after 90 m. In contrast, **1** and **2** transferred from the PDA/fluorophore liposomes to the unpolymerized liposomes much more slowly. In the initial mixture measurements the PDA/fluorophore mixed with unpolymerized liposomes and the PDA/fluorophore liposomes alone had very similar spectra (Fig. 10a and b), showing that transfer of fluorophore from the PDA/fluorophore liposomes had not occurred. After 90 m, there was evidence that approximately 15–20% of **1** and **2** had transferred from the PDA/fluorophore liposomes to the unpolymerized liposomes as shown by the drop in fluorophore emission. The cyanine fluorophores **4** and **6** showed no signs of transferring to the PDA liposome from the unpolymerized liposomes (Fig. 10c and d), though **4** transferred the other way, from the PDA/**4** liposomes to unpolymerized liposomes, as shown by 25% and 40% drops vs PDA/fluorophore liposomes in the initial and 90 min emission measurements respectively.

These results showing transfer of fluorophores between liposomes suggest that the technique of mixing PDA liposomes with micelles or other colloidal suspensions of lipophilic ligands or probes in the hope that the species will insert into the PDA liposome has the possible pitfall of an equilibrium existing between the species in the PDA liposomes and the species in a micelle or free in the aqueous solution (i.e., "free" species). In the case of

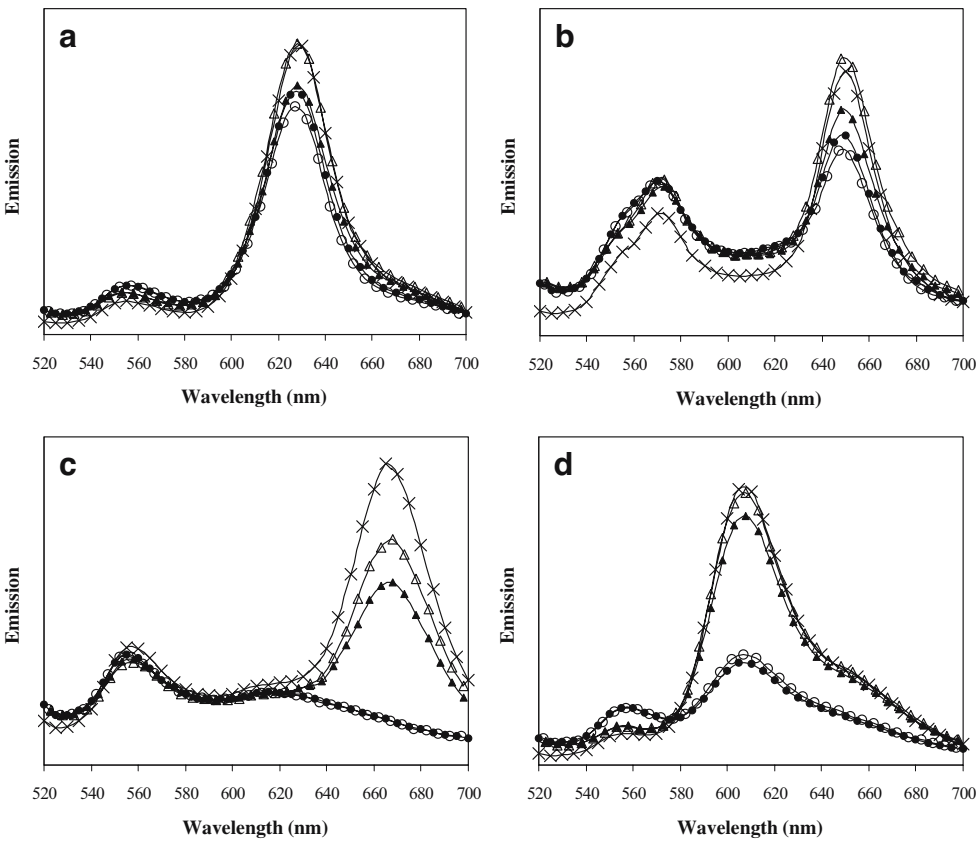

Fig. 10 Emission spectra of 1:1 mixtures of 10,12-PCDA and poly(10,12-PCDA), with 0.5% fluorophores; total lipid concentration constant. Emissive PDA liposomes mixed with unpolymerized 10,12-PCDA liposomes/fluorophore (*open circles*); the same mixture after 90 min (*filled circles*); emissive PDA/fluorophore liposomes mixed with unpolymerized liposomes (*open triangles*); the same mixture after 90 min (*filled triangles*); emissive PDA/fluorophore liposomes alone (×). **a** With **1**; **b** with **2**; **c** with **4**; **d** with **6** (slightly directly excited). Excitation at 475 nm

binding assays the "free" species can compete with the species in the liposome, in the case of fluorescent or spin-labeled probes the probe may report on both the PDA liposome and "free" environments. It is best to incorporate a probe species upon liposome formation if possible, as that leads to more stable adducts.

Further experiments were performed using DSPC liposomes with fluorophores **1** and **5** incorporated as model phospholipid liposomes, mixed with varied amounts of PDA liposomes, and measuring the change in emission at 625 nm over time. The emission of fluorophores **1** and **5** in the mixtures showed a dose dependent response on the amount of fluorescent PDA liposome added (Fig. 11a). The increase in rate of transfer of **1** with increased concentration of PDA liposomes suggests that the transfer has first order kinetics, which is consistent with transfer of lipophilic species between liposomes seen in other studies (Fig. 11b) [30–32]. Fluorophore **5** showed a very low rate and extent of transfer. **1** and **5** have the same fluorescent moiety, however, in **5** it is adjacent to the polar portion of the molecule and in **1** it is at the end of an alkyl tail. The difference in structure affects the fluorophore transfer: fluorophore **1** transfers much more readily than fluorophore **5**. In general it appears that fluorophores that have the fluorescence moiety near the aqueous interface and are anchored with alkyl tails, such as **4**, **6** and **5**, transfer very slowly or not at all, while species with the partially polar fluorophore buried in the alkyl region, such as **1** and **2**, are more prone to transfer. These trends should apply to non-fluorescent lipophilic molecules added to PDA liposomes and can be used to estimate the likelihood of a probe inserting into a PDA liposome.

Conclusions

This paper investigates the phenomenon of energy transfer from PDA to fluorophores in PDA/fluorophore liposomes. The Stokes shift and the emission of PDA liposomes are increased with incorporation of suitable lipophilic fluorophores, amplifying the detection signal in PDA sensing materials. Both BODIPY and cyanine fluorophores were incorporated in PDA liposomes in a series of studies that examined the effect of fluorophore choice, liposome polymerization, and diacetylene tail structure on the energy transfer. The results show that extended polymerization of the liposomes leads to reduction in energy transfer to the fluorophores, presumably because of energy transfer between conjugated polymer segments competing with energy transfer to fluorophores. For each diacetylene/ fluorophore system there is an optimal balance between sufficient polymerization to create PDA for energy transfer to fluorophores and liposome stabilization and excessive polymerization that decreases energy transfer to the fluorophore. The energy transfer phenomenon was used to examine transfer of lipophilic species, specifically lipophilic fluorophores from unpolymerized liposomes to PDA liposomes. Cyanine and BODIPY fluorophores that were anchored with alkyl chains penetrating the alkyl region of the liposome bilayers while the fluorophore moiety stayed near the aqueous interface were relatively resistant to transfer between liposomes. The two BODIPY fluorophores with the fluorescent moiety inserted into the hydrocarbon region were able to transfer swiftly between liposomes. The fluorophores also transferred from PDA liposomes to unpolymerized liposomes. These results show

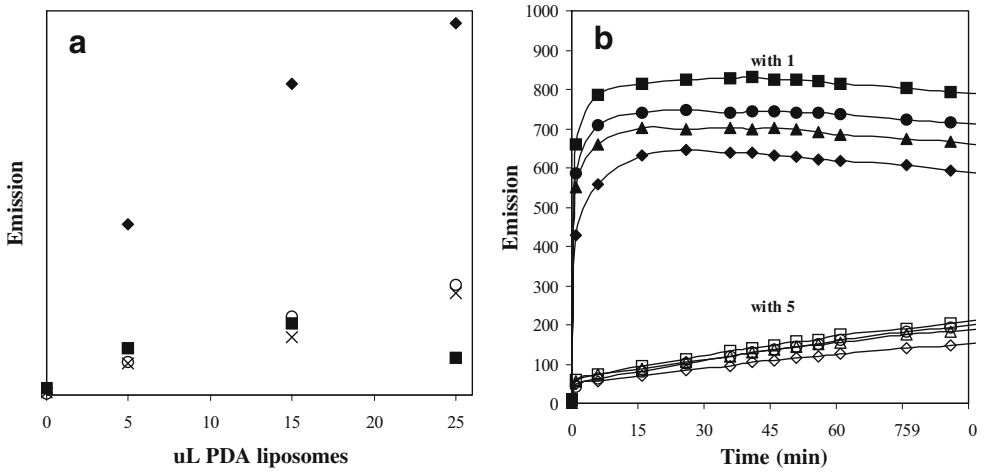

Fig. 11 Charts showing **a** emission at 625 nm of 200 μl mixtures of increasing amounts of heated poly(10,12-PCDA) liposomes with DSPC/1 (15 μl) at 625 nm (*filled diamonds*)and at 560 nm (×), and with DSPC/5 (15 μl) at 625 nm (*filled squares*) and at 560 nm (*open circles*), measured immediately after mixing. **b** Emission at 625 nm vs time of DSPC/1 (*filled symbols*) and DSPC/5 (*open symbols*) mixed with 5 μl (*diamonds*), 10 μl (*triangles*), 15 μl (*circles*) and 20 μl (*squares*) of heated poly(10,12-PCDA) liposomes; 200 μl total volumes. Excitation at 475 nm; 625 nm emissions adjusted to remove contributions from PDA liposomes

that insertion of lipophilic species into PDA liposomes is a complicated process and consideration should be given to the possibility that the lipophilic species will be present outside of the PDA liposomes, whether in micelles, liposomes or other colloidal structures. This is relevant to cases where the inserted lipophilic species is a ligand for a target or is being used to probe the internal environment of the PDA liposome, as the molecules outside the PDA liposomes can compete with the inserted molecules.

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References

1. Reppy MA, Pindzola BA (2007) Biosensing with polydiacetylene materials: structures, optical properties and applications. Chem Comm (42):4317–4338
2. Reppy MA, Sporn SA, Saller CF (2000) Method for detecting an analyte by fluorescence, in *US Patent*. Analytical Biological Services, USA
3. Reppy MA (2002) Signal generation from switchable polydiacetylene fluorescence. Mat Res Soc Symp Proc 723:O5_9_1–O5_9_6
4. Kolusheva S, Molt O, Herm M, Schrader T, Jelinek R (2005) Selective detection of catecholamines by synthetic receptors embedded in chromatic polydiacetylene vesicles. J Am Chem Soc 127:10000–10001
5. Pindzola BA, Nguyen AT, Reppy MA (2006) Antibody-functionalized polydiacetylene coatings on nanoporous membranes for microorganism detection. Chem Commun (8):906–908
6. Kolusheva S, Zadmard R, Schrader T, Jelinek R (2006) Color fingerprinting of proteins by calixarenes embedded in lipid/polydiacetylene vesicles. J Am Chem Soc 128(41):13592–13598
7. Li X, McCarroll M, Kohli P (2006) Modulating fluorescence resonance energy transfer in conjugated liposomes. Langmuir 22 (21):8615–8617
8. Saul T, Der-Balain G, Kenhey P, Mathis H, Johnson S, Ribbi H, Witty T (1993) Fluorescent lipid polymer-macromoledular ligand compositions as detection element in ligand assay, in *U.S. Patent*. Biocircuits Corporation, USA
9. Ma G, Cheng Q (2005) Vesicular polydiacetylene sensor for colorimetric signaling of bacterial pore-forming toxin. Langmuir 21(14):6123–6126
10. Ma G, Cheng Q (2006) Manipulating FRET with polymeric vesicles: development of a "Mix-and-Detect" type fluorescence sensor for bacterial toxin. Langmuir 22(16):6743–6745
11. Okada S, Peng S, Spevak W, Charych DH (1998) Color and chromism of polydiacetylene vesicles. Acc Chem Res 31:229–239
12. Baughman RH, Chance RR (1976) Comments on the optical properties of fully conjugated polymers: analogy between polyenes and polydiacetylenes. J Polym Sci Polym Phys Ed 14:2037–2045
13. Olmsted JI, Strand M (1983) Fluorescence of polymerized diacetylene bilayer films. J Phys Chem 87:4790–4792
14. Yasuda A, Yoshizawa M, Kobayashi T (1993) Fluorescence spectrum of a blue-phase polydiacetylene obtained by probe saturation spectroscopy. Chem Phys Lett 209(3):281–286
15. Kobayashi T, Yasuda M, Okada S, Matsuda H, Nakanishi H (1997) Femtosecond spectroscopy of a polydiacetylene with extended conjugation to acetylenic side groups. Chem Phys Lett 267:472–480
16. Carpick RW, Mayer TM, Sasaki DY, Burns AR (2000) Spectroscopic ellipsometry and fluorescence study of thermochromism in an ultrathin poly(diacetylene) film: Reversibility and transition kinetics. Langmuir 16(10):4639–4647
17. Clegg RM (1995) Fluorescence resonance energy transfer. Curr Opin Biotechnol 6(1):103–110
18. Lakowicz JR (1999) Principles of fluorescence spectroscopy, 2nd edn. Plenum, New York, p 367
19. Beljonne D, Pourtois G, Silva C, Hennebicq E, Herz LM, Friend RH, Scholes GD, Setayesh S, Müllen K, Brédas JL (2002) Interchain vs. intrachain energy transfer in acceptor-capped conjugated polymers. Proc Natl Acad Sci U S A 99(17):10982–10987
20. Schindler F, Lupton JM, Feldmann J, Scherf U (2004) A universal picture of chromophores in π-conjugated polymers derived from single-molecule spectroscopy. Proc Natl Acad Sci U S A 101 (41):14695–14700
21. Nguyen T-Q, Wu J, Doan V, Schwartz BJ, Tolbert SH (2000) Control of energy transfer in oriented conjugated polymer-mesoporous silica composites. Science 288:652–656
22. Wong KF, Bagchi B, Rossky PJ (2004) Distance and orientation dependence of excitation transfer rates in conjugated systems: beyond the Förster theory. J Phys Chem A 108 (27):5752–5763
23. Tachibana H, Yamanaka Y, Sakai H, Abe M, Matsumoto M (1999) Effect of position of butadiyne moiety in amphiphilic diacetylenes on the polymerization in Langmuir–Blodgett films. Macromolecules 32:8306–8309
24. Eglington, G, McRae W (1963) Cadiot-Chodkiewicz coupling-Unsymmetrical coupling of terminal acetylenes with 1-bromoacetylenes. Adv Org Chem 4:252–281
25. Tieke B, Wegner G, Naegele D, Ringsdorf H (1976) Polymerization of tricosa-10,12-diynoic acid in multilayers. Angew Chem Int Ed Engl 15(12):764–765
26. Halvey R, Rozek A, Kolusheva S, Hancock REW, Jelinek R (2003) Membrane binding and permeation by indolicidin analogs studied by a biomimetic lipid/polydiacetylene vesicle assay. Peptides 24:1753–1761
27. Blumenthal R, Gallo SA, Viard M, Raviv Y, Puri A (2002) Fluorescent lipid probes in the study of viral membrane fusion. Chem Phys Lipids 116(1–2):39–55
28. Hilfinker R, Willi A, Isele U, van Hoogevest P (1994) Fluorescence resonance energy transfer measurements as a tool to detect fusion and drug exchange in liposomal suspensions. Proc SPIE 2078:467–470
29. Nicols JW (1985) Thermodynamics and kinetics of phospholipid monomer–vesicle interaction. Biochemistry 24(23):6390–6398
30. Jones JD, Thompson TE (1990) Mechanisms of spontaneous, concentration dependent phospholipid transfer between bilayers. Biochemistry 29(6):1593–1600
31. Sanyal SN, Sadana T, Majumdar S (1987) Kinetics of cholesterol movement between liposomes and intestinal brush border membranes. Indian J Exp Biol 25(10):674–680
32. Storch J, Kleinfeld AM (1986) Transfer of long-chain fluoroscent free fatty acids between unilamellar vesicles. Biochemistry 25 (7):1717–1726