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Insight into the Mechanism of Antimicrobial Poly(phenylene ethynylene) Polyelectrolytes: Interactions with Phosphatidylglycerol Lipid Membranes†

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The interactions of antimicrobial poly(phenylene ethynylene) (PPE)-based cationic conjugated polyelectrolytes (CPEs) with lipid membranes were investigated to gain insight into the mechanism of their biocidal activity. Three model membrane systems comprising negatively charged phosphatidylglycerol (PG) lipids were used to mimic the bacterial cell membrane, including unilamellar lipid vesicles in aqueous solution, lipid bilayer coated silica microspheres, and lipid monolayers at the air−water interface. Two PPE CPEs, one containing a thiophene moiety on the PPE repeat unit and the second containing a diazabicyclooctane (DABCO) moiety on the pendant side chain, were chosen, since the former exhibits distinct dark biocidal activity and the latter shows strong light-activated antimicrobial activity but little dark biocidal activity. The interactions of these two PPE polymers with lipid membranes were characterized in detail by CPE fluorescence spectral changes, fluorescence resonance energy transfer (FRET), fluorescence quenching, monolayer insertion, and dynamic light scattering assays. Both PPE polymers exhibit affinity for the anionic lipid membrane systems. Their concomitant association and insertion into the membrane leads to conformational changes of the PPE polymer from an aggregated state to a more extended state, as evidenced by the polymer's enhanced fluorescence and FRET between the polymer and rhodamine incorporated in the lipid membrane. In comparison, the thiophene polymer exhibits stronger interactions with PG lipid membranes than the DABCO-containing polymer. The former induces a larger fluorescence enhancement, shows faster transfer across the lipid membrane, and inserts more readily and to a higher extent into lipid monolayers. The observed differences between the two PPE polymers in their interactions with the lipid membrane may stem from their structural differences, as the DABCO-containing polymer has a much bulkier and larger pendant group on its side chain. The higher degree of membrane interaction and insertion, and subsequent membrane disorganization, of the thiophene polymer may account for its dark biocidal activity.

1. Introduction

In order to combat the increasing threat from pathogenic bacteria, various antimicrobial agents have been developed over the past decade, such as cationic peptides,[1−3] long chain quaternary ammonium compounds,[4,5] and polymers containing functional pendant groups (quaternary ammonium groups or alkyl pyridinium moieties).[6] Besides the development of novel and efficient biocides, it is important to understand the mechanism by which these materials interact with and kill bacteria. It is generally accepted that the cationic functional groups on the biocides are responsible for their toxicity. For example, it has been proposed that the selective interaction between the amphipathic secondary structures of cationic peptides, e.g., α-helices and β-sheets, with anionic bacterial membrane via electrostatic interaction serves as the mechanism for their antimicrobial action.[1] The biocidal activity of organic monolayers containing quaternary ammonium compounds is attributed to the exchange of counterions between the functionalized cationic surface and the bacterial membrane.[7] The quaternary amines in antimicrobial polymers and surfactants are believed to be responsible for causing cell death by disrupting the cell membrane, thus allowing the release of intracellular contents.[8]

Cationic polymers containing pendant quaternary ammonium groups have been among the most promising candidates as effective antimicrobials and biocides.[6] Earlier, we have found a number of fluorescent poly(phenylene ethynylene) (PPE)-based conjugated polyelectrolytes (CPEs) with pendant quaternary ammonium groups to be highly efficient light-activated biocides against a number of Gram negative bacteria such as *Escherichia coli*,[9] *Cobetia marina*,[10] and *Pseudomonas aeruginosa* strain PAO1 (PAO1)[10,11] as well as some Gram positive bacterial spores such as *Bacillus anthracis, Sterne*.[9] The light-activated biocidal activity of these PPE-based CPEs (for example, diazabicyclooctane

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(DABCO) containing PPE (Scheme 1)) may be attributed to the generation of singlet oxygen species or more corrosive reactive oxygen species by these photoactive polymer materials.[10] However, we have discovered very recently that a similar PPE-based CPE containing a thiophene group in the PPE repeat unit instead of a phenyl ring (Scheme 1) shows distinct biocidal activity against PAO1 bacteria in the dark, but has very little light-activated activity.[12] The difference in the biocidal activity of the PPE-based CPEs shows that a fundamental understanding of the mechanism of this relatively new class of biocides is needed.

Utilization of model lipid membrane systems to mimic the bacteria cell membrane to investigate their interactions with antimicrobial molecules has been extensively applied to understand the mechanism of biocidal activities for antimicrobial molecules.[13−17] However, the interactions of our PPE-based antimicrobial molecules with lipid membranes have yet to be elucidated. Therefore, a detailed investigation of the polymer−lipid interactions was carried out in the present study to gain insights into the biocidal mechanism of PPE CPEs.

It is known that anionic lipids, such as phosphatidylglycerol (PG) and cardiolipin, along with zwitterionic lipids such as phosphatidylethanolamine, are abundantly present in most bacterial membranes and give the membrane an overall negative charge.[14,18] In this study, we used two PG lipids to mimic the bacterial cell membrane, the unsaturated 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG) lipid and the saturated 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DMPG) lipid. Two PPE-based CPEs (Scheme 1) were chosen, as the thiophene containing PPE (**1**) displays distinct dark biocidal activity and the DABCO-containing PPE (**2**) has pronounced light-activated antimicrobial properties.[11,12] Studying the interactions between the two PPE-based CPEs and PG lipids not only helps to elucidate the antimicrobial mechanism of the PPE polymers but will also help us to understand the structural basis of their activities. To probe the polymer−lipid interactions, three model membrane systems were used, unilamellar liposomes in aqueous solution, lipid bilayer coated silica microspheres, and lipid monolayers at the air−water interface. The PPE-based CPEs are intrinsically fluorescent, and their fluorescence is sensitive to their microenvironment.[19,20] To characterize lipid−polymer interactions, a number of fluorescence-based techniques have been employed to investigate the fluorescence changes of the PPE polymers upon their interaction with lipid membrane. The insertion of the polymers into lipid monolayers as well as the changes in liposome size upon interacting with polymers was also measured. The results of these studies provide a better understanding of the structure−activity relationship of biocidal polymers.

2. Experimental Section

2.1. Materials. The two cationic PPE CPEs used in the present study (**1** and **2** in Scheme 1) were synthesized according to methods reported elsewhere.[19,21] The structures of these PPE CPEs are shown in Scheme 1. The PPE polymers were dissolved in Milli-Q water (18.2 MΩ cm^{-1}) and the concentration of stock PPE solutions was between 1.3 to 1.5 mM in polymer repeat unit (PRU). Nonporous borosilicate glass microspheres (5 μm diameter) were purchased as a dry powder from Duke Scientific (Palo Alto, CA). 1,2-Dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DMPG), and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (DMPE-Rh) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The structures of the phospholipids used are shown in Scheme 2. DOPG was dissolved in chloroform and DMPG was dissolved in chloroform containing 10 vol % methanol. The concentration of DOPG used to prepare liposomes was 2 mM and the concentration of DMPG for Langmuir monolayer spreading was ca. 0.4 mg/mL. The quencher, 9,10-anthraquinone-2,6-disulfonic acid (AQS, Scheme 3), was purchased from Sigma-Aldrich and was dissolved in Milli-Q water to a concentration of 5 mM. 10× PBS (phosphate buffered saline) was prepared as stock buffer solution containing 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4 and 2.4 g KH_2PO_4 in 1 L pure water. 1× PBS was obtained by diluting 10× PBS in pure water and adjusted by NaOH to a pH of 7.4 and used in the present study to prepare samples.

2.2. Preparation of Polymer-Coated Microspheres. Microspheres coated with PPE polymers (Si-**1** and Si-**2**) were prepared according to methods reported elsewhere.[21,22] The amount of polymer used was chosen to achieve 1.2× monolayer coverage. Specifically, the concentration of stock PPE-coated microspheres is ca. 3.0×10^8 beads/mL. The polymer-coated beads were washed 10 times to ensure that there was no free polymer present in the supernatant. This was confirmed by fluorescence measurements.

2.3. Preparation of Phospholipid Liposomes. DOPG liposomes were prepared using the following procedure. First, DOPG and DMPE-Rh lipids were dissolved in chloroform and diluted to 2 mM for the former and 0.002−0.02 mM for the latter and stored in aliquots (1 mL) at −20 °C until further use. Second, one such aliquot of pure DOPG or DOPG-DMPE-Rh mixtures (one aliquot of each other) was taken and dried under vacuum overnight. The dried lipids were then resuspended in one aliquot amount of PBS buffer and incubated at room temperature with shaking for 30 min, and finally extruded to close to optical clarity using a mini-extruder (Avanti Polar Lipids, Alabaster, AL) through a polycarbonate membrane filter with 100 nm pore size for 11 passes.

2.4. Preparation of PPE-Liposome Mixtures and Microsphere-Supported Bilayers. PPE polymer and liposome mixtures were prepared by mixing specified volumes of the prepared liposome and PPE stock solutions (1.3 mM in PRU) using a vortex mixer for 30 min. The final PRU concentration of PPE polymer in the mixtures was ca. 1.3×10^{-2} to 3.9×10^{-2} mM, which was about 1−3 mol % of the DOPG lipid. Microsphere-supported bilayer beads were prepared by suspending 100 μL stock solutions of PPE-coated microspheres in 1 mL of the prepared liposome solution and then mixing at room temperature using a vortex mixer for 30 min. The lipid-coated microspheres were centrifuged, and the supernatant containing extra liposomes was decanted and discarded. The cycle of resuspension and centrifugation was repeated four additional times, and then the bilayer-coated microspheres were resuspended in 1 mL PBS buffer.

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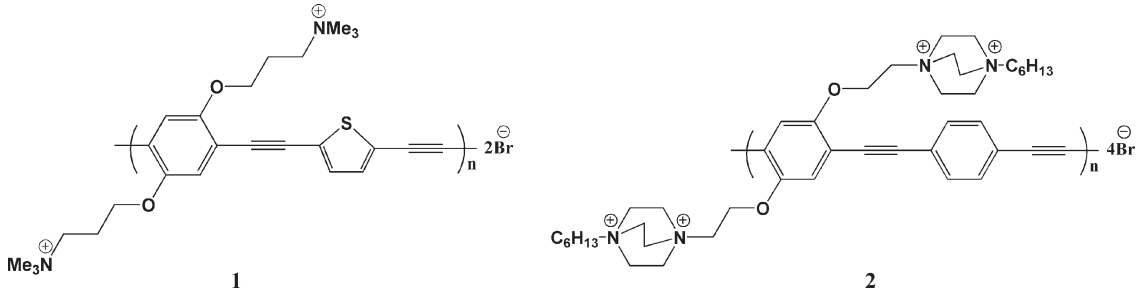
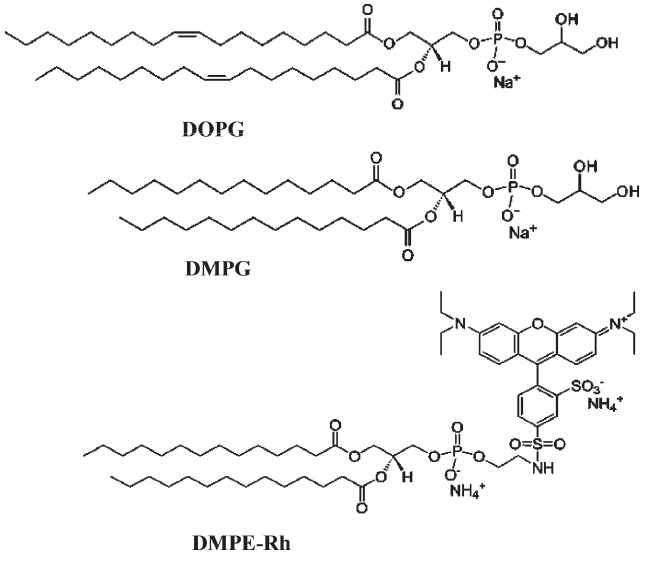
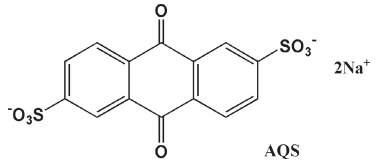
Scheme 1. Structures of the Two Cationic Conjugated Polyelectrolytes Studied



Scheme 2. Structures of Anionic Phospholipids



Scheme 3. Structure of 9,10-Anthraquinone-2,6-disulfonic Acid (AQS), Disodium Salt



2.5. Fluorescence Assays. Fluorescence measurements of microsphere suspensions and PPE-liposome mixtures were carried out using a SpectroMax M-5 microplate reader (Molecular Devices). A 96-well plate (BD Falcon, white/clear bottom) was used and 100 μL of each sample was analyzed. The excitation wavelengths for polymers **1** and **2** were 427 and 394 nm, respectively, and the emission spectra were collected from 460 to 700 nm for polymer **1** and from 430 to 650 nm for polymer **2**.

2.6. Dynamic Light Scattering Experiments. The hydrodynamic radii of DOPG liposome and its mixtures with PPE polymers were measured by using the WyattQELS system in a DAWN multiangle laser light scattering instrument (DAWN HELEOS II, Wyatt Technology Corporation, Santa Barbara, CA). A linearly polarized gallium arsenide laser was used as the light source (658 nm at 130 mW). Data were collected in batch mode using a microcuvette that contained 40 μL of sample. The DOPG liposome samples and its mixtures with PPE polymers were diluted to 1/50th of their original concentrations for the dynamic light scattering measurements. For each sample, three sets of data were collected, and the sampling time for each data set was 10 min. ASTRA V software was used to analyze the dynamic light scattering data. The hydrodynamic radius was extrapolated using a cumulant analysis, assuming that the scattering species are spherical and monodisperse.

2.7. Monolayer Insertion Assay. The insertion experiments for PPE polymers into a lipid monolayer were carried out using a Langmuir trough equipped with a Wilhelmy plate and two identical mobile Delrin barriers (KSV Instruments, Monroe, CT). The water subphase volume used was 50 mL, and the maximal working surface area is 100 cm^2. The polymer insertion was carried out at either constant surface pressure or constant surface area. Initially, the lipid monolayer was deposited at the air−water interface and left undisturbed for at least 15 min to ensure the complete evaporation of the organic solvent. Then, the lipid monolayer was compressed to a target surface pressure of 25 mN/m or a target surface area with surface pressure at ca. 25 mN/m. The surface pressure or the surface area was then kept constant via a builtin proportional-integral-derivative control feedback system by adjusting the surface area or surface pressure. A PPE CPE solution (50 μL) was then injected into the water subphase underneath the monolayer using a microsyringe and allowed to equilibrate with the monolayer. The final polymer concentration in PRU in the subphase was ca. 1.3 μM. If the polymer favorably interacts with the lipids, it will insert into the monolayer. As a result, the surface area or the surface pressure will increase when the surface pressure or the surface area is held constant. The monolayer surface area and the monolayer surface pressure were recorded as a function of time.

3. Results and Discussion

3.1. Interaction of Thiophene Polymer and Liposomes in Aqueous Solution. Unilamellar lipid vesicles, or liposomes, have been widely utilized as a model biomembrane system.[23,24] Interactions between antimicrobial molecules and liposomes have been extensively studied to mimic how biocides interact with biomembranes and to fundamentally understand the mechanism of their biocidal activities.[14,15,25] Since PPE-based CPEs are intrinsically fluorescent, their photophysical changes can be used to probe variations in their microenvironment.[19] The interaction of the thiophene polymer (polymer **1**) with DOPG liposomes in aqueous solution was examined by several fluorescence-based techniques, including fluorescence emission spectra, fluorescence resonance energy transfer (FRET), and fluorescence quenching.

3.1.1. DOPG Liposomes Enhance the Intrinsic Fluorescence of Polymer 1. The fluorescence emission spectra of DOPG liposomes, polymer **1**, and the polymer **1**−DOPG liposome mixture were recorded on a microplate reader (excited at 427 nm, Figure 1). Interestingly, the presence of DOPG liposomes increases the fluorescence intensity of the polymer by almost an

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order of magnitude, even though DOPG alone does not show any fluorescence. At the same time, the emission maximumof polymer **1** blue-shifts from 524 nm as observed in PBS buffer to 484 nm in the presence of DOPG liposomes. Such spectral changes have been found for PPE polymers when they are transferred from a poor solvent (e.g., water) to a good solvent (e.g., methanol).[19,26] These phenomena have also been reported when such PPE polymers were mixed with oppositely charged surfactants[27,28] or phospholipids.[29] For both cases, the spectral changes have been attributed to the conformational changes of PPE polymers from an aggregated state to a more extended state. This is because PPE CPEs, as amphiphilic molecules, have a strong tendency to form aggregates in poor solvent.[19,26] The presence of good solvent or oppositely charged surfactants or phospholipids facilitates PPE CPEs to disaggregate by improved dissolving[19,26] or forming polymer−surfactant complexes.[27−29] Therefore, the present spectral variations for polymer **1** suggest that both the microenvironment and the conformation of the polymer have been substantially changed. Since the only difference between the polymer solution and the polymer−liposome mixture is the presence of DOPG liposomes, the liposomes are responsible for modifying the microenvironment and inducing conformational changes. This is a reasonable conclusion, since the DOPG lipid bilayer bears negative charges which can attract the positively charged polymer to associate with the bilayer and consequently alter the microenvironment for polymer **1**, as the interior of the lipid bilayer presents a much more hydrophobic environment than the exterior aqueous solution. The association of polymer on the lipid membrane may involve further interactions such as hydrophobic forces between them and cause conformational changes for polymer **1** as observed for other PPE CPEs.[27,29]

However, the use of small unilamellar vesicles instead of free surfactant molecules presents a more complicated system, where the interactions between polymer and liposomes may be different from that of polymer and surfactants. There are issues such as the location of PPE polymers within liposomes (on the surface or entrapped in the bilayers) and the possibility of disruption of liposomes into small fragments or not, which need to be considered in the present case. We have explored the former issue by FRET and fluorescence quenching techniques as described in the following sections and the latter issue by dynamic light scattering investigating the changes of liposome sizes after mixing with PPE polymers, which will be addressed in section 3.5.

*3.1.2. FRET from Polymer **1** to Rhodamine Incorporated in DOPG Liposome*. FRET refers to a process during which a donor chromophore, initially in its excited electronic state, transfers energy to an acceptor chromophore. The process is dependent on the spectral overlap between the donor emission spectrum and the acceptor absorption spectrum as well as the distance between the donor and the acceptor.[30] Usually, the donor and the acceptor must be in close proximity (typically between 1−10 nm) for an efficient FRET to occur.[30] On the basis of this characteristic, FRET has been frequently utilized to probe the distance between a donor and an acceptor[31] to provide

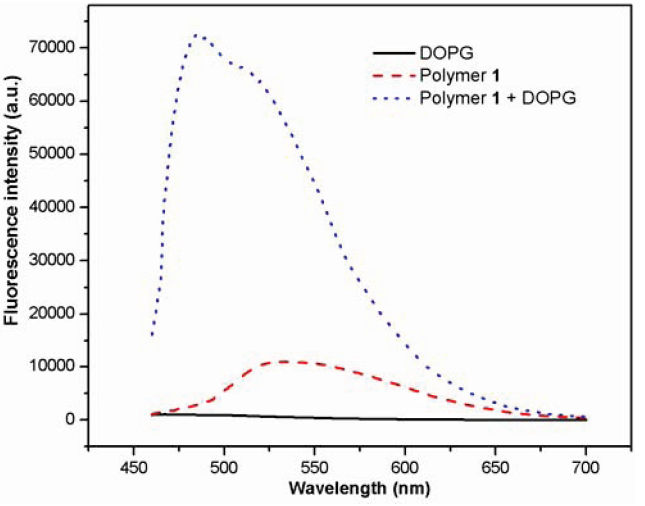

Figure 1. Fluorescence emission spectra of DOPG liposome (black solid), polymer **1** (red dash), and a polymer−liposome mixture (blue dot) (λ_{ex} = 427 nm).

information about the spatial separation and orientation between the donor- and the acceptor-chromophore labeled molecules, such as proteins and lipids.[32] Polymer **1** absorbs energy efficiently in the range 400−450 nm[12] and emits fluorescence in the range 470−600 nm (c.f. Figure 1). The polymer emission spectrum overlaps with the rhodamine B absorption band (540−600 nm centered at 573 nm), rendering polymer **1** and rhodamine an appropriate donor−acceptor pair for FRET. To monitor the association of polymer **1** with the lipid membrane, FRET from the PPE polymer to rhodamine incorporated in DOPG liposomes was investigated.

Rhodamine-labeled DMPE (DMPE-Rh) was incorporated in DOPG liposomes by cosolubilizing DMPE-Rh with DOPG in chloroform before vacuum-drying followed by hydration and extrusion as described in section 2. The ratio of DMPE-Rh in DOPG was kept lower than 1 mol % so as to not influence the structure of DOPG liposomes. Polymer **1** was mixed with DOPG liposomes containing different ratios of DMPE-Rh that ranged from 0.1 to 1 mol %, respectively. All the polymer−liposome mixtures contained the same concentrations of polymer and DOPG lipids. The fluorescence emission spectra of these mixtures were recorded using an excitation wavelength of 427 nm; results are presented in Figure 2a. As shown, the fluorescence emission intensity of polymer **1** at 484 nm continuously decreases when DMPE-Rh content in the liposome increases from 0.1 to 1 mol %; in contrast, the fluorescence intensity of rhodamine centered at ca. 590 nm dramatically increases. It is evident from these data that FRET occurs from polymer **1** to rhodamine chromophores in the lipid membrane.

To verify that the fluorescence of rhodamine incorporated in the lipid bilayer originates from the FRET process, a control experiment was carried out with DOPG liposomes containing 0.1 mol % DMPE-Rh in the absence of polymer **1**. The experimental result is presented in Figure 2b. For direct comparison, the emission spectrum of the liposomes with polymer present is overlaid. The emission of rhodamine with a 427 nm excitation in the control experiment (liposomes alone) only generates a low emission spectrum centered at 590 nm, significantly weaker than that with the presence of the PPE polymer. This confirms that

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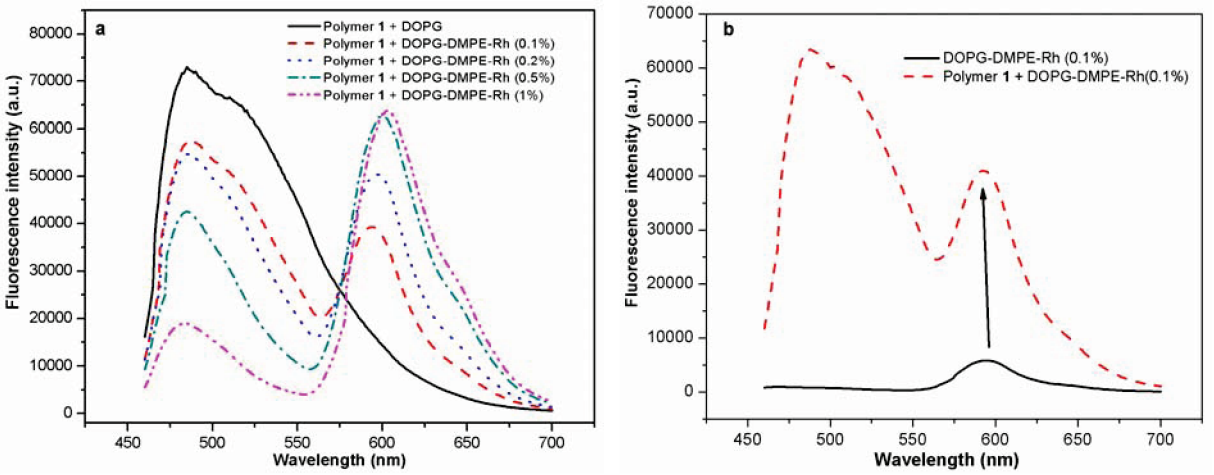

Figure 2. (a) Fluorescence emission spectra of polymer **1**: DOPG liposome mixtures containing 0.1 to 1 mol % of DMPE-Rh. (b) Comparison of the fluorescence emission between rhodamine-containing liposomes with and without polymer **1**. Excitation wavelength used is 427 nm and the concentrations of both polymer **1** and DOPG lipids were the same for all samples.

FRET has indeed occurred from the polymer to rhodamine, revealing that the distance between the polymer and rhodamine is in the range of a few nanometers. Our results suggest that polymer **1** is associated with the lipid membrane so that it can be in close proximity to rhodamine moieties. The location of the polymer on the surface or in the lipid bilayer is further studied by fluorescent quenching techniques in the following section.

3.1.3. Liposomes Screen Fluorescence Quenching of Polymer. The fluorescence of the cationic PPE CPEs can be efficiently quenched by low concentrations of an electron transfer quencher that contains an anionic charge such as AQS (Scheme 3). Compared to FRET, electron transfer quenching requires a closer proximity of the electron donor to the electron acceptor.[19] Fluorescence quenching therefore is greatly influenced by the accessibility of the quencher to the polymer; quenching experiments were carried out to determine the accessibility of the AQS quencher to the PPE polymer. The goal is to obtain information about the location of polymer **1** within the polymer−liposome mixtures.

The fluorescence emission intensities of polymer **1** at 524 nm and mixtures of polymer **1** and DPOG liposomes containing different molar percentage of rhodamine at 484 nm were monitored before and after the addition of the AQS quencher. For each measurement, the fluorescence of each sample containing the same amount of polymer **1** (100 μL of 1.3×10^{-2} mM polymer) was first measured. Then, 2 μL of 5 mM AQS was added, and the fluorescence intensity of the sample was measured to compare to that of polymer alone. The results are shown in Figure 3. As shown, the fluorescence of the polymer solution without liposome is almost completely quenched by AQS, indicating that the polymer is fully accessible to AQS. However, there is less than 10% quenching in all the polymer−liposome mixtures tested irrespective of the percentage of DMPE-Rh in the liposome. These results indicate that the lipid membrane efficiently blocks the access of the quencher AQS to the polymer, suggesting that most of polymer **1** becomes entrapped, either within the lipid bilayer or in the interior of the liposomes, and effectively protected from the anionic quencher.

Combining our observations from fluorescence enhancement, FRET and fluorescence quenching studies, it is reasonable to conclude that polymer **1** first associates with the oppositely charged liposomes and then inserts into the bilayers probably due to the hydrophobic interactions between the PPE polymer and the lipid membrane[33] and finally becomes incorporated or entrapped in the liposomes.[34,35] We believe that, for small liposomes, the effect of curvature and the possible concomitant higher negative charge density on the inner leaflet may also provide a driving force for the migration of polymers from the outer to the inner surface of the bilayer.[36] DOPG liposomes were also prepared in the presence of AQS, and the fluorescence of polymer **1** was measured under these conditions. There is ca. 40% of PPE polymer unquenched, indicating that a certain amount of polymer **1** remains in the nonpolar region of liposome bilayer, with the rest of the incorporated PPE polymer being on the surface of the inner leaflet. The incorporation of the positively charged polymer into the biomembrane can induce alterations in lipid packing and organization, potentially disrupting membrane function and resulting in cell death. A similar process of binding, insertion, and destabilization of phospholipid bilayers has been proposed to explain the biocidal activity of α-helical antimicrobial peptides.[37] Thus, the ability of the polymer to efficiently incorporate into the lipid membrane may explain the polymer's remarkable "dark" biocidal activity.

3.2. Interaction between Polymer 1 and Microsphere-Supported Lipid Bilayer. The development of antimicrobial coatings on surfaces holds great potential in practical applications.[7] Therefore, studying the interactions between surface-confined PPE polymers with lipid membranes is very important. Microsphere-supported bilayers were prepared by mixing cationic PPE-coated nonporous silica microspheres with DOPG liposomes.[22,38] A lipid bilayer forms spontaneously on the polymer-coated microspheres, due to the electrostatic attraction between the cationic polymer and negatively charged lipid bilayer. The fluorescence emission spectra of polymer **1** coated microspheres

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(34) The results for the present system (cationic CPE and anionic liposomes) are quite different from those reported in a recent study of an anionic CPE with liposomes formed from a zwitterionic phospholipid (ref 35.). In this study, it is indicated that the CPE may associate with the surface of the liposome or become entrapped within the liposome, there is very little crossing of the bilayer by the polymer.
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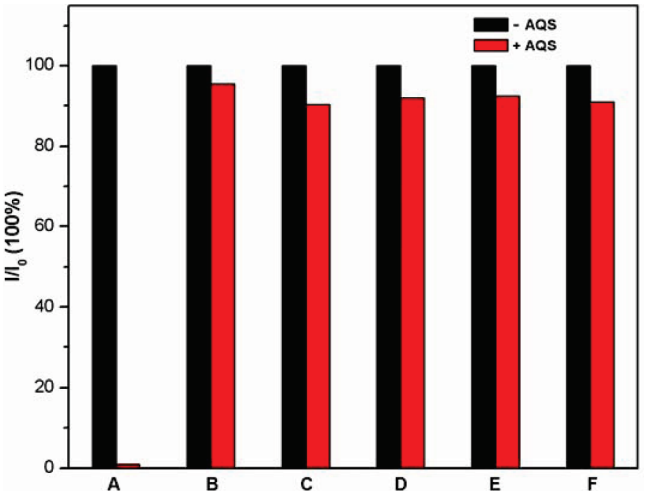


Figure 3. Fluorescence quenching of polymer emission intensity by AQS (0.1 mM): (A) polymer **1**; (B) polymer **1** plus DOPG liposome; (C−F) polymer **1** plus rhodamine-labeled liposome (the molar percentage of DMPE-Rh in the liposome is 0.1, 0.2, 0.5, and 1, respectively).

with lipid bilayers containing different ratios of DMPE-Rh were collected using an excitation wavelength of 427 nm (Figure 4a). As a control, the emission spectrum of the polymer-coated microspheres alone was also recorded.

Interestingly, similar changes were observed from these supported-bilayer experiments (Figure 4a) to those observed in liposomes (Figure 1), including a dramatic enhancement of the polymer's fluorescence intensity and a distinct blue shift in the emission maximum from ca. 512 nm to ca. 484 nm. Our results indicate that the PPE polymer exists as aggregates on the surface of the microspheres as evidenced from the weak and broad fluorescence emission, which is consistent with our previous findings.[10] The DOPG bilayer deposited on top of the polymer serves to disaggregate the underlying polymer as evident from the large increase in the polymer's fluorescence emission intensity and the blue shift of its emission maximum. As both PPE polymer and DOPG bilayer are confined on the microsphere surface, the disaggregation of the PPE polymer could result from its penetration into the surface-supported DOPG bilayer and thereby induce conformational changes from aggregated state to more extended state.

The significant FRET between polymer **1** and lipid-incorporated rhodamine is also observed in the microsphere-supported bilayer system, as evidenced by the decrease of the polymer's fluorescence and the increase of the rhodamine emission with increasing amounts of DMPE-Rh (Figure 4a). These observations indicate again that the distance between the polymer and rhodamine is in the range of a few nanometers, suggesting that the polymer intercalates into the lipid bilayer.

The coverage of lipid bilayer on the microsphere-coated polymer was confirmed by fluorescence quenching studies. The same amount of quencher, AQS, was added to polymer-coated microspheres in the absence and presence of DOPG bilayer. The results (Figure 4b) show that less than 10% of the polymer fluorescence is quenched when it is covered by a bilayer. In contrast, the fluorescence of the polymer alone coated on microspheres is almost completely quenched (inset in Figure 4b). These results indicate that a contiguous DOPG bilayer is formed on top of the coated polymer and that the bilayer can effectively prevent the AQS quencher from accessing the PPE polymer.[38]

Results obtained from studying the microsphere-coated polymer **1** interaction with the overcoated bilayer once again indicates that the polymer−lipid interactions can induce conformational rearrangement of the polymer and result in the insertion of the polymer into the lipid membrane, possibly causing alterations in the membrane structure. The similarities in the results obtained from polymer **1** dissolved in aqueous solution interacting with liposomes and surface-coated polymer **1** interacting with a confined lipid bilayer indicate that the polymer−lipid interaction is robust and that using PPE coatings as an antimicrobial material holds great potential for the application of the antimicrobial polymer.

3.3. Kinetics of Interactions between Polymer 1 and DOPG Liposomes. The fluorescence changes of polymer **1** in the polymer−liposome mixtures were measured as a function of mixing time so as to obtain information about the kinetics of polymer−lipid interactions. Polymer **1** was added to DOPG liposomes and mixed using a vortex mixer. At different mixing times, an aliquot of the mixture was taken and the fluorescence emission of the aliquot was measured using a microplate reader before and after addition of 2 μL of AQS solution. Polymer was added to the liposomes at time zero. As shown in Figure 5a, a large increase in the fluorescence emission of polymer **1** was observed in the first 5 min after adding polymer to the liposomes. With increasing mixing time, the fluorescence intensity continually increased, indicating that the PPE polymers became less aggregated as they continuously incorporate into the DOPG liposomes. As a control, fluorescence intensity of the polymer alone in PBS buffer was also measured as a function of time. Contrary to the increasing trend observed in the polymer−liposome mixture, fluorescence intensity of the polymer alone did not change over time (data not shown). This confirms that the observed fluorescence increase results from the interactions between the cationic polymer and the anionic lipid, which induces polymer **1** to associate and then insert into the lipid bilayer, transferring from an aqueous solution to a more hydrophobic environment. The continuous changes in fluorescence intensity could be the result of further conformation adjustments of polymer **1** once it is incorporated into the liposome, for example, from a more aggregated state to a more extended state. Fluorescence quenching studies carried out using AQS (Figure 5b) showed that 5 min mixing time was sufficient in preventing the AQS from quenching the polymer, as only 10% of the original fluorescence was quenched, indicating that polymer **1** efficiently and quickly inserted into the DOPG liposomes. The quenching decreased to ca. 5% at 30 min mixing time and reached a plateau at ca. 30 min.

3.4. Comparison of Interactions of Polymer 1 and Polymer 2 with Lipid Membrane. Polymer **2** (Scheme 1), which contains a DABCO-based alkyl-ammonium group on the pendant chain, has been previously reported to exhibit dramatic light-induced biocidal activity while showing little or no dark biocidal activity against Gram negative bacteria.[10,11] The interactions of polymer **2** with lipid membranes, including liposomes, microsphere-supported bilayers, and lipid monolayer at the air−water interface, were investigated, and the results are compared to those obtained from polymer **1**, which exhibits efficient dark biocidal activity but little light-activated antimicrobial property.[12] The comparison helps us to better understand the mechanism(s) of antimicriobial activities of PPE CPEs and sheds light on CPE structure−activity relationships.

*3.4.1. Interactions of Polymer **2** with Liposomes in Aqueous Solution.* Identical experiments were conducted for polymer **2** to compare its interaction with DOPG liposomes to those observed for polymer **1**. Figure 6 shows the fluorescence changes (Figure 6a) and AQS quenching efficiency (Figure 6b) of polymer **2** with the addition

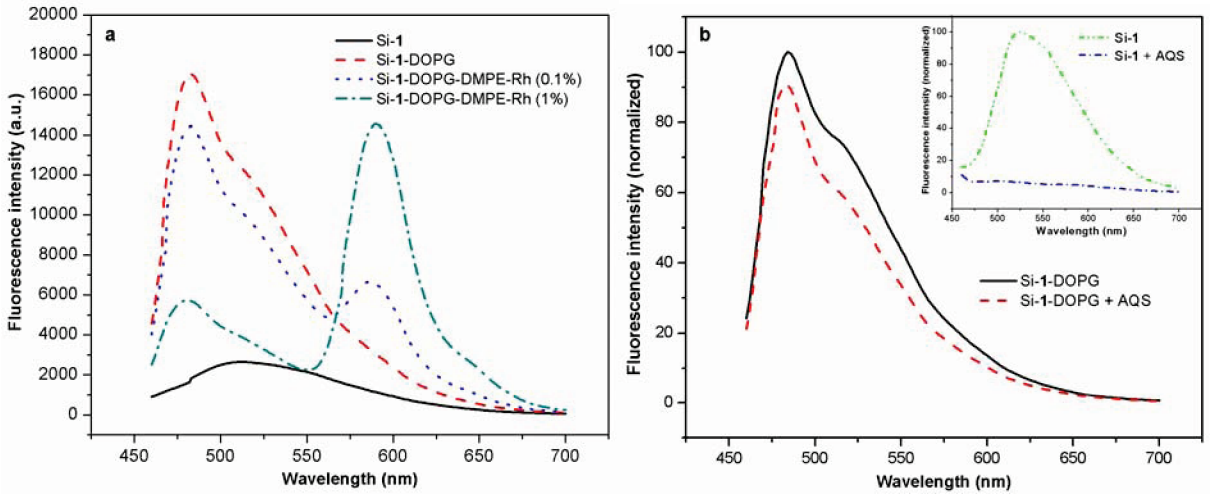


Figure 4. (a) Fluorescence spectra of a series of polymer **1** coated microspheres with DOPG bilayers containing different molar percentage of DMPE-Rh; (b) Fluorescence quenching of polymer **1** coated microspheres in the absence (inset) and presence of DOPG bilayer by AQS. The excitation wavelength is 427 nm and the concentrations of microspheres are the same for all samples.

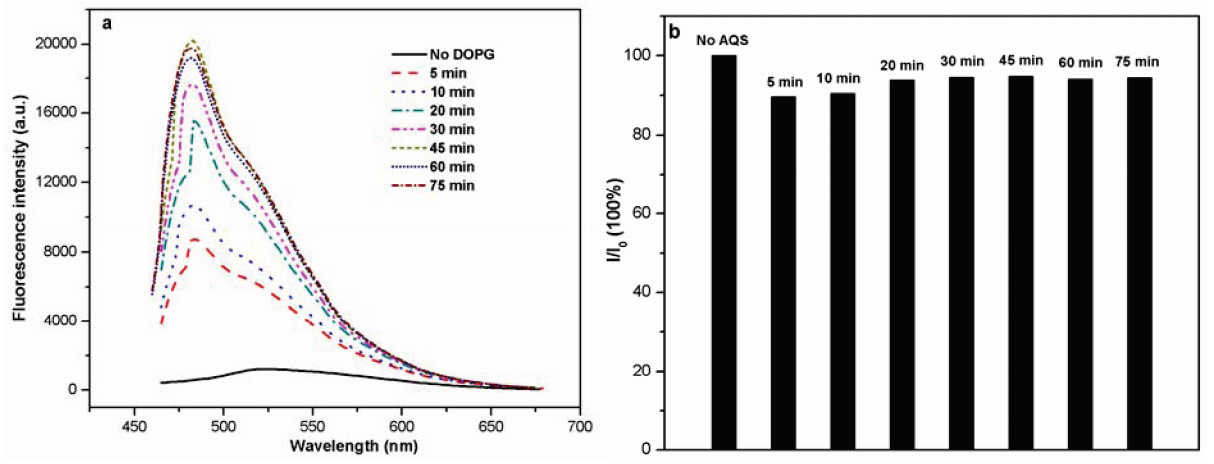


Figure 5. (a) Fluorescence emission spectra of mixtures of polymer **1** with DOPG liposome prepared over different mixing time; (b) AQS quenching efficiency to the same mixtures as prepared in (a).

of DOPG lipsomes as a function of mixing time. Similar to the trends observed in the polymer **1** samples (Figure 5), both fluorescence enhancement and blue shift of emission maximum were observed for polymer **2** when DOPG liposomes were present (Figure 6a), indicating that the lipid membrane has exerted a similar "disaggregation effect" on polymer **2**. When the mixing time is longer than 1 h, the fluorescence emission spectrum of polymer **2** becomes sharp and narrow, indicating that the polymer is now in a "good solvent" environment. However, the magnitude of the fluorescence increase is not as large as that for polymer **1**. Fluorescence only increased by about 2-fold after 5 min of mixing to about 4-fold after an hour of mixing, compared to the 10−30-fold increase observed for polymer **1** during the same time period (c.f. Figure 5a).

As shown in Figure 6b, the fluorescence of polymer **2** is quenched to 20% of its original intensity by AQS when the polymer is mixed with DOPG liposomes for 5 min. This indicates that most of the polymer stays outside of the DOPG liposomes and is easily accessible to the quencher. At longer times, fluorescence emission was increasingly retained from AQS quenching, indicating that more and more polymer **2** becomes inaccessible to the quencher. This could be the result of more polymer inserting into the lipid membrane and becoming shielded from AQS. The DABCO polymer is efficiently protected from the quencher when the mixing time with DOPG liposomes is longer than one hour, where more than 90% of polymer fluorescence is unquenched. This result is consistent with the change in polymer fluorescence with mixing time. In comparison to the larger fluorescence increase and faster shielding of polymer **1** by DOPG liposome, our results suggest that polymer **1** more readily inserts into the lipid membrane and becomes better "dissolved" into the lipid bilayer than polymer **2**. Over a short period of time, e.g., 30 min that is used for preparing most of the samples, polymer **1** is mostly incorporated in the lipid membrane, whereas polymer **2** stays partially on the surface of the liposome as illustrated in Scheme 4.[39]

*3.4.2. Comparison of Interactions of Polymer **2** with Lipid Bilayer on Microspheres.* Fluorescence spectra of polymer **2**-coated microspheres with and without deposited DOPG bilayer

(39) A reviewer has suggested that the results observed in this study may be explained by an alternative possibility that the polymer-liposome interaction may lead to formation of a cylindrical structure where the polymer is "wrapped" by a coat of phospholipid. We concur with the reviewer that this could explain the observed results and are planning experiments to test this possibility. On the other hand, foramtion of such cylindrical structure would probably lead to film collapse or contraction when the polymer is added to phospholipid monolayer at the air/water interface and this is not observed in section 3.4.3 where the results from monolayer insertion experiments are described.

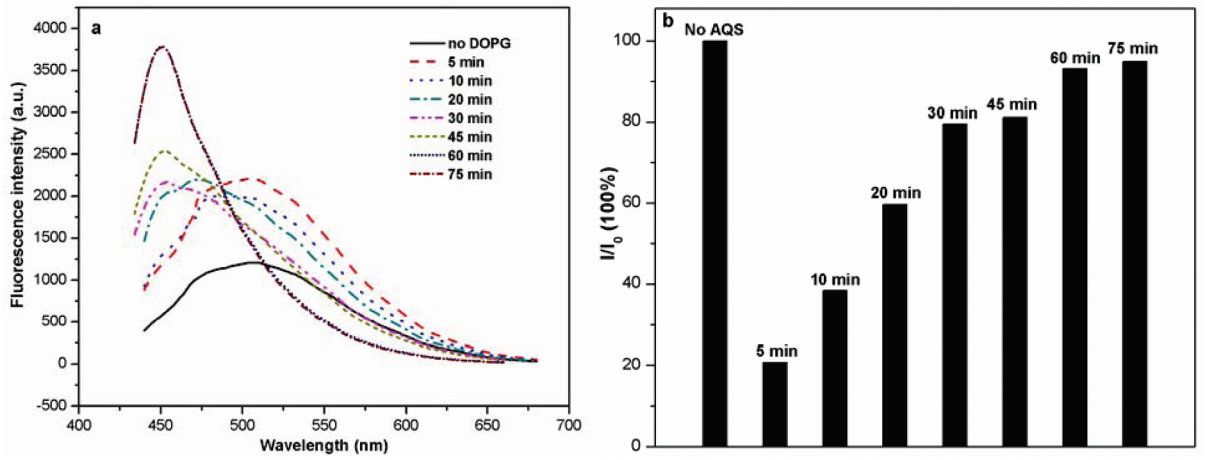
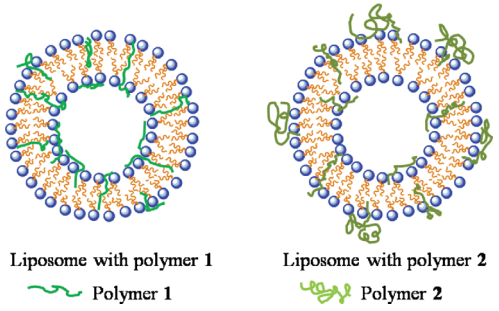


Figure 6. Fluorescence emission spectra (a) and AQS quenching efficiency (b) of mixtures of polymer **2** with DOPG liposome at different mixing times.

Scheme 4. Schematic Presentation of Mixtures of DOPG Liposomes with PPE Polymers 1 and 2



containing different molar percentage of DMPE-Rh were recorded and the results are shown in Figure 7. Compared to the results obtained for polymer **1** (Figure 4a), there are some similarities as well as some differences. A similar fluorescence enhancement and blue shift of the emission maximum are observed for polymer **2** as those observed for polymer **1** with the addition of DOPG bilayer on the microspheres. FRET from polymer **2** to rhodamine contained in the bilayer is also observed. These similarities show that the common structural characteristics of the two polymers, such as the conjugated structure and the presence of cationic pendant quaternary ammonium groups, enable both polymers to interact with the negatively charged lipid membranes and subsequently undergo conformational changes. However, the two polymers also exhibit some obvious differences in terms of their interactions with the lipid membrane. A comparison of the fluorescence of the two polymers on the microspheres with the coated DOPG bilayer shows that the fluorescence enhancement of polymer **2** is far lower than that found for polymer **1** under the same condition. This result is in good agreement with the findings for the two polymers in liposome solutions, indicating again that polymer **2** is less well "dissolved" in the lipid membranes. The difference in the insertion of the two polymers into lipid membrane was further investigated using lipid monolayers formed in a Langmuir trough as described in the following section.

3.4.3. Comparison of the Insertion of the Two Polymers into Lipid Monolayers at the Air−Water Interface. Lipid

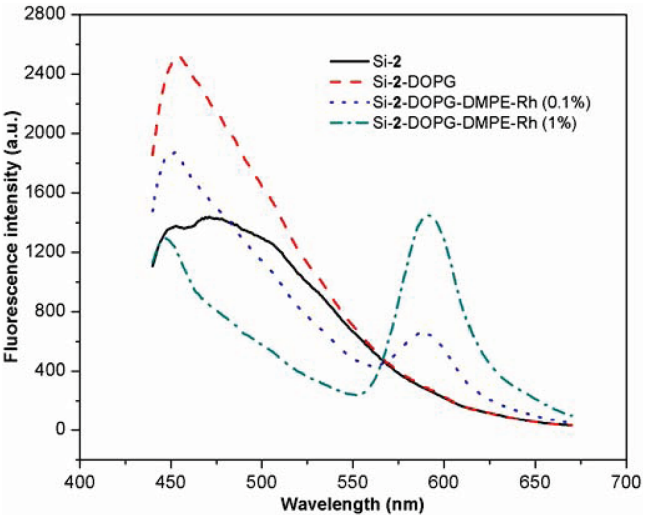


Figure 7. Fluorescence spectra of a series of polymer **2** coated microspheres with absence and presence of DOPG bilayers containing DMPE-Rh with different molar percentage (λ_{ex} = 394 nm; the concentrations of microspheres are the same).

monolayers at the air−water interface have been used to study interactions between polymers or peptides with lipid monolayers, where polymer insertion events can be directly measured and quantified.[40−43] To probe polymer−lipid interactions, the extent of the insertion of the two polymers, **1** and **2**, into lipid monolayers was measured and the results are compared. The measurements were carried out either at a constant surface pressure or at a constant surface area. As unsaturated lipids such as DOPG may be prone to oxidative damage at the air/water interface, a saturated phospholipid with the same headgroup, DMPG, was used for these monolayer studies. Fluorescence studies have shown that DMPG can also induce the dramatic enhancement to the fluorescence of polymer **1**.[44] Therefore, we expect polymer **1** to exhibit similar interactions with DMPG lipids compared to DOPG lipids. We measured the insertion of each of the two

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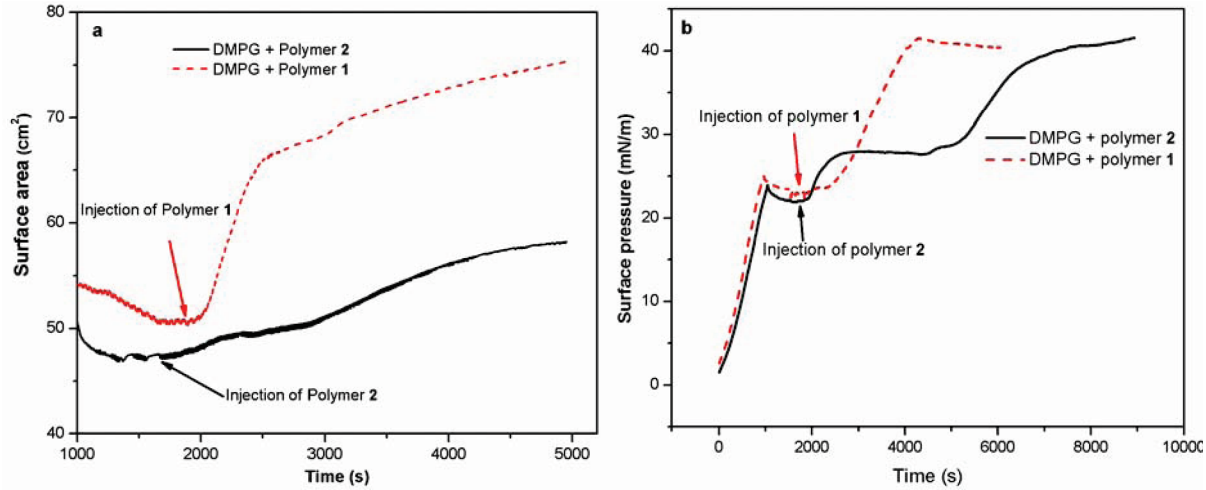

Figure 8. Constant pressure measurements (a) and constant area measurements (b) of DMPG monolayer after PPE polymer injections on a water subphase at room temperature. The surface area increases by 47% for polymer **1** and 20% for polymer **2** at constant surface pressure of 25 mN/m. The surface pressure increases from ~23 mN/m to ~40 mN/m by polymer **1** over 2000 s and by polymer **2** over 6000 s at constant surface area of ~50 cm^2.

polymers into DMPG monolayer held at 25 mN/m or at a constant surface area with a surface pressure close to 25 mN/m, where DMPG is in a well-packed liquid condensed phase (data not shown). From the results shown in Figure 8, the injection of both PPE polymers into the aqueous subphase resulted in increases of the monolayer surface area or the surface pressure when surface pressure or surface area was held constant, respectively. This indicates that both cationic PPE polymers could insert into the DMPG monolayer, confirming that the two PPE polymers exhibit affinity to the negatively charged lipid membrane and their polymer−membrane interactions lead to the insertion of cationic polymers into the lipid membrane. These results corroborate our conclusions drawn from FRET and fluorescence quenching studies.

Moreover, the difference in the insertion between the two polymers is clearly shown in Figure 8a,b. At a constant surface pressure, polymer **1** inserts into the DMPG monolayer faster and to a larger extent compared to polymer **2** (Figure 8a). After injection of polymer **1** into the subphase, the lipid monolayer surface area increases from 51 to 66 cm^2 during the first 500 s and results in a 47% increase in the surface area (from 51 to 75 cm^2) over a period of 1 h. In comparison, it takes a longer time (> 1000 s) for polymer **2** to start inserting into the DMPG monolayer and its insertion only caused a 20% increase in the surface area (from 48 to 58 cm^2) over the same 1 h time period. When the surface area was held constant (~50 cm^2), the surface pressure increases from ca. 23 mN/m to ca. 40 mN/m over 2000 s after the injection of polymer **1** (Figure 8b). However, although the surface pressure also reaches 40 mN/m after the injection of polymer **2**, it takes much longer, about 6000 s, indicating that polymer **2** inserts into the DMPG monolayer much more slowly than polymer **1**. The insertion results provide additional evidence for our earlier findings, where polymer **1** appears to associate and become incorporated into the DOPG liposomes more rapidly and more efficiently.

The results from our studies probing the interactions of polymers **1** and **2** with different lipid membrane systems consistently show that polymer **1** exhibits a higher affinity and stronger interactions with lipid membranes and can more readily and efficiently insert into lipid membranes. This difference in the interactions between the two polymers with lipid membrane could stem from their structural difference, where polymer **1** has a shorter and smaller pendant ammonium side chain compared to the longer and bulkier DABCO based alkyl-ammonium groups in polymer **2**. We propose that the difference in the two polymers' interactions with negatively charged lipid membrane may account for the differences in their biocidal activity. The stronger membrane interactions displayed by polymer **1** render it more effective as a biocide in the dark. More detailed investigation elucidating the structure−property relationship of PPE CPEs is currently underway for a number of PPE CPEs with different structures.

3.5. Changes in Liposome Size Induced by PPE Polymers. Dynamic light scattering (DLS) is a technique for measuring the size distribution of particles typically in the submicrometer range. DLS measures the particle translational diffusion coefficient and the size of the particles is calculated using the Stokes−Einstein equation:

$$R_h = K_B T/(6\pi\eta D)$$

where R_h is the hydrodynamic radius, K_B is Boltzmann's constant, T is the absolute temperature, η is the solvent viscosity, and D is the translational diffusion coefficient of the particles.

The hydrodynamic radii of DOPG liposomes and its mixtures with polymers **1** and **2** measured by DLS are summarized in Table 1. It can be seen that the DOPG liposomes alone have a very narrowly distributed size range and a mean radius of 53.5 nm. This size corresponds closely to the pore size (100 nm diameter) of the polycarbonate filter used to prepare the liposomes. The liposome size is unchanged when the sample is stored at 4 °C for 5 days. When the polymers are added to the liposomes, an increase in the size of the liposomes is observed. For the case of polymer **1**, the mean liposome radius is increased to 57.2 nm when 1 mol % of polymer is mixed with DOPG liposomes, and the radius increased further to 60.6 nm when 3 mol % of polymer is added. In all of the three samples, the size distribution remained fairly narrow. However, for the case of polymer **2**, the liposome mean radius is surprisingly increased to 81.2 nm when 3 mol % of polymer is added and the size distribution becomes much wider. It is well-known that the adsorption of polymers on the liposome surface or penetration of polymers into the liposome bilayers will

Table 1. Hydrodynamic Radii (R_h) of Liposomes and Liposome−Polymer Mixtures

samples	concentration of DOPG	concentration of polymer in PRU	$R_{h\ (nm)}$
DOPG	2 mM	0	53.5 ± 1.7
DOPG + Polymer **1**	2 mM	1.3×10^{-2} mM	57.2 ± 2.5
DOPG + Polymer **1**	2 mM	3.9×10^{-2} mM	60.4 ± 2.0
DOPG + Polymer **2**	2 mM	3.9×10^{-2} mM	81.2 ± 5.4

correspondingly change the size of the particle.[45] The results from the DLS measurements further indicate that PPE polymers associate with lipid vesicles and the liposome structures are retained instead of being disrupted into small fragments. The larger increase in liposome size induced by polymer **2** could be attributed to the fact that it mainly adsorbs to the surface of the liposomes instead of being incorporated in the lipid bilayer as in the case of polymer **1** (Scheme 4). This result is in good agreement with the findings from fluorescence quenching studies, which show that polymer **2** remains more accessible to the quencher than polymer **1**, indicating that polymer **2** remains on the exterior of the liposomes.

4. Conclusions

We have studied the interactions between two PPE polymers, a thiophene-containing PPE and a DABCO-containing polymer, with lipid membranes utilizing a number of model membrane systems, including liposomes in aqueous solution, bilayers coated on microspheres, and monolayers at air−water interface, and a wide array of techniques, including fluorescence emission measurements, FRET investigation, fluorescence quenching studies, monolayer insertion assays, and dynamic light scattering measurements. The main findings and conclusions of our study are as follows:

1. The dramatic fluorescence enhancement of the cationic PPE polymers due to the "disaggregation effect" of lipid membranes, the distinct FRET between PPE polymers and rhodamine incorporated in lipid membrane, and the efficient shielding of PPE polymers by lipid membrane from AQS quencher. These results suggest that PPE polymers associate with the lipid membrane, insert into the membrane interior, and possibly migrate across the lipid membrane from the outer to the inner surface. This process may be driven by a combination of the electrostatic and hydrophobic interactions between cationic PPE polymers and the anionic lipids, as well as the possible concomitant higher negative charge density on the inner leaflet of small liposomes due to curvature. The association and incorporation of PPE polymers in the lipid membrane may cause alteration of the packing and organization of membrane lipids, which could partially account for the antimicrobial activity of PPE CPEs.

2. The surprising similarities in the interactions between the free and coated PPE polymers with liposomes and confined lipid bilayers, respectively. This result suggests that physisorption of the PPE polymer films on a solid surface does not affect their interaction with lipid membranes and presents a promising application for using these biocidal polymers as coatings.

3. The distinct differences in the interactions between the two PPE polymers, **1** and **2**, with lipid membranes, where the former can more readily and quickly incorporate into the PG membrane structures compared to polymer **2**. These differences may explain the reason polymer **1** has much more pronounced dark biocidal activity against Gram negative bacteria than polymer **2**.

In an effort to further our understanding of antimicrobial polymer−membrane interactions and to probe the roles of these interactions in their antimicrobial activities and structure−property relationships, the interactions of a number of other antimicrobial PPE-based CPEs containing different pendant structures with various phospholipids containing different headgroups and different alkyl chains are currently under investigation.

Acknowledgment. This research is financially supported by the Defense Threat Reduction Agency (Contract No. W911NF-07-1-0079). Liping Ding would like to thank the Natural Science Foundation of China (No. 20803046) for support.

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