

Fluorescent-Based Thermal Sensing in Lipid Membranes

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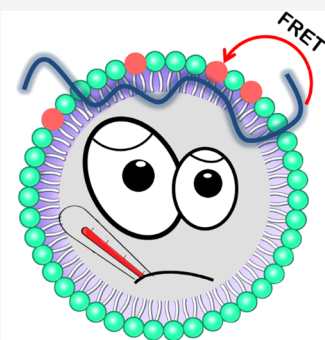


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ABSTRACT: Thermal mapping in biological membranes could unlock and help us understand many chemical and physical processes that do not only pertain to localized membrane phenomena but also extend to many other intra- and extracellular pathways. In this manuscript, we report the development of a ratiometric thermal fluorescent probe based on the Förster resonance energy transfer between a lipid-embedded conjugated polyelectrolyte and a lyophilic acceptor dye. We showed that the Förster resonance energy transfer (FRET) pair is sensitive within the relevant physiological temperature window (20.0–50.0 °C). The signal was also shielded from an external pH and stable when cycled multiple times. The probe was also sensitive to the membrane composition and could, therefore, be further developed to probe the membrane composition and viscosity.



A FRET-based Lipid Thermal Probe

INTRODUCTION

Temperature is a critical parameter that controls and drives many physical and chemical events in cells.^{1,2} Despite its fundamental role, we still lack a robust tool to accurately measure it at the cellular scale. More specifically, mapping thermal changes in cellular membranes is still challenging and could help us understand many chemical and physical processes that do not only pertain to localized membrane phenomena but also extends to many other intra- and extracellular pathways.³

For example, physical damages in the biological lipid membrane at low temperatures are still elusive.⁴ The general observation is that the lipid components of most membranes are heterogeneous and undergo phase transitions from gel phase to liquid-crystalline lamellae, and some to a nonlamellar, over a range of temperatures.⁵ Plants and microorganisms have developed sophisticated mechanisms for acclimation and survival at low temperatures in response to the subsequent decrease in the membrane fluidity. This is achieved by altering the biochemistry of the lipid membrane; this is achieved by increasing the amount of unsaturated fatty acid^{6,7} and changing the composition of the glycerolipids⁸ and proteins.^{9,10} Membranes are not only responsive to low temperature but also to any fever-type conditions, which also result in a constitutional modification of the plasma membrane affecting the signaling department.¹¹ Membrane permeability is another property that is temperature-dependent since heat controls the opening of ionic channels as a result of the change in the interaction of lipid domains as they approach their phase transition temperature.¹²

Given all that was presented earlier, it is crucial to develop a tool that would allow an accurate reporting of thermal changes in lipid membranes. A common method has been to rely on

the temperature dependence of the resonant frequency shift of protons in water, which is approximated to be 0.01 PPM/°C.^{13,14} This method is however technically challenging. Alternatively, lyophilic fluorescent probes were developed as a simpler approach to estimate the local temperature in biological lipid membranes. For instance, Chapman et al. reported the use of 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) and 6-dodeca-noyl-2-dimethylamino-naphthalene to record the indirect effect of microenvironmental changes, which occur during bilayer heating.¹⁵ The monochromatic fluorescent signal readout is, however, prone to many external factors including, but not limited to, quenching, photodegradation, and excitation source fluctuations. Ratiometric measurements, on the other hand, negate these effects.^{16,31} Our group has reported on the use of conjugated polyelectrolytes (CPEs) in complexation with poly(vinylpyrrolidone) (PVP) as a ratiometric probe for the detection of thermal fluctuations in solution, hydrogels, and solid films.^{17–20}

In this manuscript, we also relied on the use of a conjugated polyelectrolyte as a donor in a Förster resonance energy transfer (FRET) pair thermal sensor where the acceptor is a lyophilic fluorescent dye. The photophysics of conjugated polyelectrolytes highly depends on the solubility of the polymer backbone.^{21–23} A collapsed state leads to efficient energy transfer between its different chromophores and overall low quantum yield.^{24,25} An extended backbone hinders the

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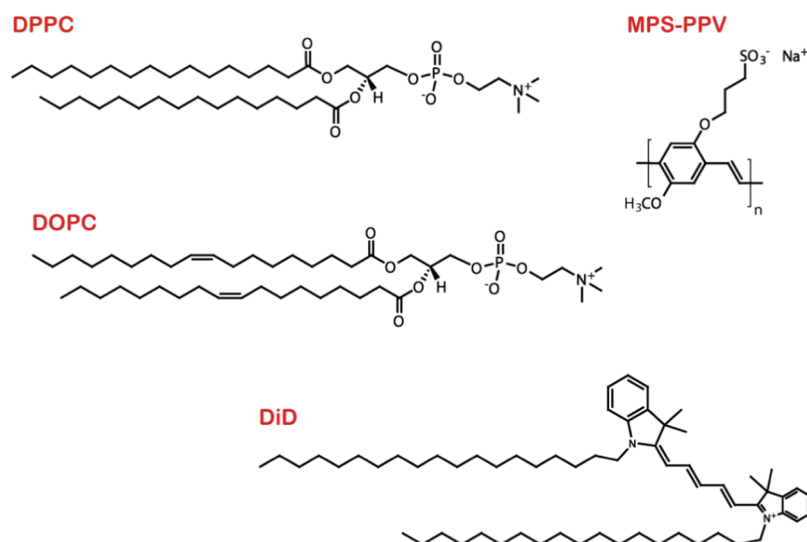


Figure 1. Chemical structures of poly[5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenevinylene] potassium salt solution (MPS-PPV), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC).

exciton migration to quenching sites, leading to an enhanced fluorescent emission.

CPEs have been widely studied in complexation with lipid membranes.^{26–28} Given their hydrophobic backbone, they tend to embed themselves in the hydrophobic core of the lipid membrane. As such, they have been used in many sensing platforms to detect lipase activity and differentiate between different cell lines and microorganisms.^{29–31} We argued that the change in the membrane fluidity with temperature should influence the solubility of the polymer backbone, leading to a change in the recorded fluorescent signal. Poly[5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenevinylene] potassium salt solution (MPS-PPV), a negatively charged conjugated polyelectrolyte ($E_m = 550$ nm) was embedded in a 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) lipid membrane with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD), a lyophilic fluorescent dye ($E_m = 640$ nm). Upon increasing the temperature, the recorded FRET signal decreased and was stable under different pH conditions. The reported lyophilic thermal sensor could be instrumental in unraveling the mechanism of many biochemical reactions in cellular membranes.

RESULTS AND DISCUSSION

MPS-PPV as a Thermal Sensor. Our group has developed a ratiometric fluorescent thermal sensor using conjugated polyelectrolytes, specifically, poly-(phenylene ethynylene) (PPE-CO₂-7) in complexation with poly(vinylpyrrolidone). Using this probe, we successfully measured temperature fluctuations in solution, soft gels, and thin films.^{17–20} However, PPE-CO₂-7, a stiff polymer, was not a good candidate to be used in lipid membranes. We have previously shown that it embeds itself in the hydrophobic core as a single polymer unit and would not lead to a good thermal response.³² As such, we turned our attention to poly[5-methoxy-2-(3-sulfopropoxy)-1,4-phenylene-vinylene] MPS-PPV, which has been previously developed by us and others into many sensing schemes, especially in complexation with lipid membranes.^{21,24,25} Its spectroscopic properties are greatly influenced by its interactions with the lipid membrane. For instance, when a

single MPS-PPV polymer is embedded within the lipid membrane, its backbone is extended and shows an exponential decrease in intensity over time with a broad red-shifted peak. This behavior is characteristic of the photobleaching of a collection of multiple independent chromophores.³³

We, therefore, argued that any changes in temperature in the lipid membranes would change the local microviscosity, which should, in return, affect the fluorescent response of MPS-PPV. DPPC was first selected as the lipid membrane of choice given its high transition temperature ($T_m = 41$ °C) and its abundance in some cellular membranes (Figure 1).³⁴ At room temperature, the membrane is gel-like and would transition into a liquid state when heated.³⁵ Indeed, the Fresnel normalized reflectivity for DPPC shows a total bilayer thickness of 5.6 nm at 25 °C, which decreases to 4.8 nm at higher temperatures (55 °C). Close to the transition temperature, the electron density reveals an increase in roughness at the lipid–water interface and the bilayer is in the liquid disordered state.³⁶

DPPC liposomes with an average hydrodynamic size of ca. 225 nm were prepared in the presence of MPS-PPV (Table S1). The initial fluorescence intensity of the CPE increased in the presence of liposomes consistent with previously reported work.²⁴ It reflects the extension of the polymer backbone as a result of its embedment inside the hydrophobic core. To probe the thermal response, the fluorescence intensity signal was measured between 20.0 and 50.0 °C in 5.0 °C increments. The complex emission showed a steady decrease with the increase in temperature (Figure 2). We believe this thermal actuation is the response to the change in the polymer microviscosity. In fact, the quantum yield temperature dependency on viscosity is approximated by $\phi = b(\eta/T)^x$, where b is a constant and x is the viscosity sensitivity of the conjugated polymers. DPPC viscosity has been estimated to decrease from 92 cP at 20.0 °C to 25 cP at 47 °C, which would explain the observed fluorescent signal change.³⁷

The thermal response was not ideal, (i) the signal change was minimal to allow any meaningful thermal mapping, and unlike our previously developed thermal sensor, (ii) the

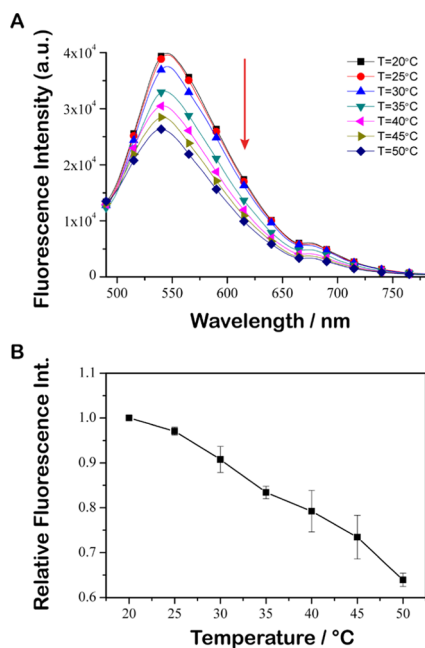


Figure 2. (A) Fluorescence emissions of DPPC/MPS-PPV measured between 20.0 and 50.0 °C at a 5.0 °C increments. (B) Normalized integrated fluorescent intensity extracted from (A) at different temperatures. The lines connecting the experimental points are for visual aid. Measurements were done in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH = 7.3 and 150 mM NaCl and emission spectra were acquired upon excitation at 457 nm.

response was not ratiometric, which can potentially impact the signal dependability.

FRET-Based Thermal Sensor. To overcome these two problems, we stained DPPC with DiD, a lipid-soluble dye. DiD absorption overlaps with the emission of MPS-PPV allowing an efficient Förster resonance energy transfer (FRET) with a Förster radius (R_0) of 4 nm (Figure S1). At room temperature, the calculated initial FRET was 0.55. As the temperature increases, the CPE emission increased concurrently with a decrease in acceptor emission (Figure 3). This resulted in a decrease in the calculated energy transfer, especially between 30 and 45 °C, before it stabilizes at a FRET value of 0.15 at high temperatures. At the highest temperature, the fluorescent intensity of the conjugated polyelectrolyte (2.2×10^4 a.u.) is comparable to that at the same temperature for DPPC/MPS-PPV alone (2.5×10^4 a.u.). We, therefore, believe that the observed thermochromic change is not only due to the individual photophysical thermal response of the donor and acceptor (quenching of their signal; Figures 2 and S2) but also to structural changes.

As such, we speculate, as the temperature increases, the distance separating MPS-PPV and DiD changes as a result of the change in the DPPC viscosity, roughness and membrane thickness, which might affect the position of the CPE within the membrane. To better understand and support our claims, we prepared liposomes with different viscosities and looked at the FRET-thermal response and how MPS-PPV embeds itself in the membrane as the solution temperature changed.

Thermal Response at Different Lipid Viscosities. The effect of the membrane viscosity on the thermal fluctuation was tested by substituting DPPC with DOPC and introducing cholesterol to the DPPC bilayers. DOPC has a low transition temperature ($T_m = -17$ °C), which makes it liquid at room

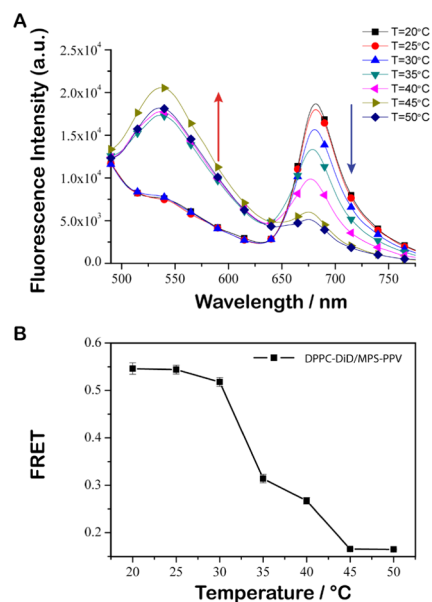


Figure 3. (A) Fluorescence emission of DPPC–DiD/MPS-PPV between 20.0 and 50.0 °C in 5.0 °C increments. (B) Calculated FRET at different temperatures. Error bars are calculated from the standard deviations of three independent measurements. The lines connecting the experimental points are for visual aid. Measurements were done in 10 mM HEPES buffer pH = 7.3 and 150 mM NaCl and emission spectra were acquired upon excitation at 457 nm.

temperature and would mimic the DPPC viscosity at high temperatures. As such, no viscosity-induced change in the MPS-PPV structure is expected with the increase in temperature. As seen in Figure S3, with the increase in temperature, both the emission of MPS-PPV and DiD decrease. The result is consistent with the decrease in the fluorescent signal observed for both probes when individually prepared in DOPC and DPPC liposomes (Figures S2 and S4).

The second experiment was to introduce cholesterol to DPPC bilayers. Cholesterol, in lipid membranes, regulates the membrane fluidity and its mechanical strength by controlling the lipid organization and phase behavior, which results in a decrease in its permeability. When Sanz et al. introduced Chol at a 10% ratio to the DPPC bilayer, they observed a phase separation of two domains with two distinct fluidity. Upon heating, the fluidity corresponding to each phase remained constant for the temperature range of 27–42 °C and merged at temperatures above 46 °C.³⁸ Herein, three DPPC lipid samples were prepared with increasing concentrations of cholesterol 5, 10, and 20%. The liposomes were stained with MPS-PPV and DiD. The FRET was evaluated at room temperature and upon increasing the temperature at 5.0 °C increment. At room temperature, the initial FRET value decreased with the increasing cholesterol concentrations. For pristine DPPC liposomes, the original value was 0.55 and decreased to 0.45 and 0.40 with the increase in cholesterol concentrations from 0 to 10 and 20% (Figure 4). In addition, the change in the thermal FRET response was much smoother with the increase in the percentage of cholesterol. These observations confirm the dependency of the FRET signal on the lipid membrane viscosity.

In addition to validating our hypothesis, the cholesterol plays an essential physiological role in thermal membrane

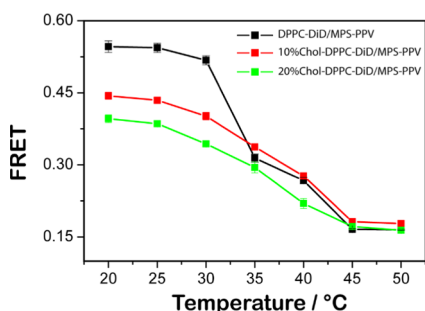


Figure 4. Calculated changes in FRET with increasing temperature as a function of cholesterol percentage in DPPC–DiD/MPS-PPV liposomes. Measurements were done in 10 mM HEPES buffer pH = 7.3 and 150 mM NaCl, and emission spectra were acquired upon excitation at 457 nm. The lines connecting the experimental points are for visual aid.

response and its introduction could be extrapolated to future in vivo studies.^{39,40}

To further validate our hypothesis, we compared measurements recorded in 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC) and DOPC with 10% cholesterol. These lipid films are very close in molecular packing and organization. At room temperature, both lipid films had a low FRET value of around 0.24. With the increase in temperature, POPC and DOPC:Chol FRET values decreased similarly to approximately 0.20 (Figure S5).

MPS-PPV Signal Dependency on Membrane Positioning. To further validate the previous assumptions, MPS-PPV was added to either preformed DPPC or 20% Chol-DPPC liposome solution. We argued that the cholesterol-containing liposomes will be less permeable to the addition of MPS-PPV, given its effect on the lipid bilayers, and would result in a lower fluorescent signal.

The initial measured intensity of the CPE, when added to pristine DPPC, was significantly higher than the less permeable liposome (Figure 5). In both cases, the fluorescence emission

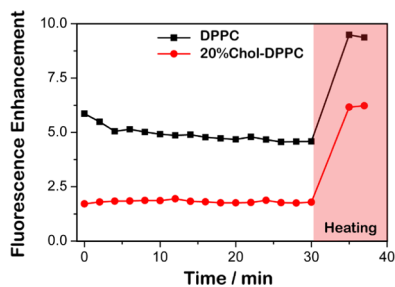


Figure 5. Fluorescence enhancement of MPS-PPV upon the addition of preformed liposomes of DPPC and 20% Chol-DPPC over time. At the 30 min mark, the solution was heated to 50.0 °C above the DPPC transition temperature.

increased over time and was more pronounced in the case of DPPC, which might be due to the diffusion of MPS-PPV into the hydrophobic core. When both mixtures were heated above the transition temperature, a higher fluorescent intensity was recorded in both samples. The cholesterol-containing liposomes signal, however, was significantly less than that of DPPC.

As such, we believe that the actuating thermal response is based on the restructuring of the conjugated polyelectrolytes inside the hydrophobic core with the increase in temperature.

Thermal Response Reversibility. Thermal sensing reversibility is essential to accurately and reliably monitor thermal fluctuations in any given system over time and under different conditions. A liposome solution of DPPC–DiD/MPS-PPV was subjected to a temperature cycle between 20.0 and 50.0 °C. The calculated thermal response was plotted versus the number of cycles and showed high reversibility (Figure 6).

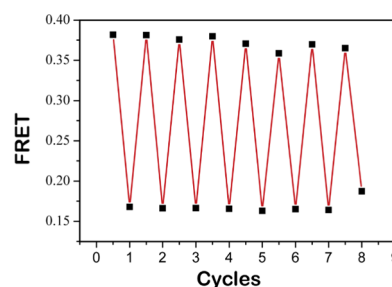


Figure 6. Reversibility of the calculated FRET signal upon heating DPPC–DiD/MPS-PPV liposomes between 20.0 and 50.0 °C. Measurements were done in 10 mM HEPES buffer pH = 7.3 and 150 mM NaCl and emission spectra were acquired upon excitation at 457 nm. The lines connecting the experimental points are for visual aid.

pH Stability of the Thermal Probe. A major limitation that we encountered in our previously reported thermal probes prepared by complexing (poly(phenylene ethynylene) carboxylate) PPE-CO₂-7 and PVP was its pH sensitivity, which limited its applications to experiments under only controlled conditions.¹⁸ In the current reported thermal probe, we argued that the lipid membrane would shield the conjugated polyelectrolytes from external solution conditions. As such, the thermal response of DPPC–DiD/MPS-PPV was tested and compared under acidic, neutral, and basic conditions (pH 5.2, 7.3, and 9.3). No obvious changes in the sensor thermal response were detected (Figure 7). When a solution of only MPS-PPV was tested, a 13.5% difference in signal was detected between pH 7.3 and 5.2, which might be the result of the electrostatic interaction between the protonated HEPES and the sulfonate group of the side chain (Figure S6). These results could prove the key to develop thermal sensors that could be

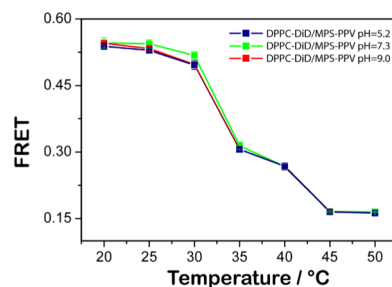


Figure 7. Thermal response of DPPC–DiD/MPS-PPV at pH values of 5.2, 7.3, and 9.0. Measurements were done in 10 mM HEPES buffer pH 7.3 and 150 mM NaCl and emission spectra were acquired upon excitation at 457 nm. The lines connecting the experimental points are for visual aid.

used in vitro and in vivo studies where experimental conditions are seldom well-controlled.

CONCLUSIONS

In this manuscript, we report on the thermal response of conjugated polyelectrolytes when embedded in a lipid membrane stained with an acceptor dye. The change in temperature restructured the polymer within the lipid membrane, resulting in a change in the measured FRET. The probe was also sensitive to the membrane fluidity with little effect from the external pH. This fluidity-dependent signal response might prove useful to assess lipid viscosity and estimate transition temperatures of lipid mixtures. In addition, lipid membranes have been formulated into microscopic structures such as silica beads and supported bilayers. The integration of our probes into such structures would allow probing local thermal changes at the meso- and macroscales.

MATERIALS AND METHODS

Materials. HEPES and poly[5-methoxy-2-(3-sulfopropoxy)-1,4-phenylenevinylene] potassium salt solution 0.25 wt % in H₂O (MPS-PPV) were purchased from Sigma-Aldrich. 1,1'-Dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate (DID) was purchased from Invitrogen. Cholesterol, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids.

Liposomes Preparation. DPPC powder was dissolved in chloroform to a final concentration of 10 mg/mL. The sample was then evaporated using a stream of nitrogen while rotating the vial to create a thin lipid film on its walls. The dried film was hydrated to a final lipid concentration of 21.5 mM with MPS-PPV (1.6 mM in monomer concentration) in 10 mM HEPES–150 mM NaCl, pH = 7.33. The resulting suspension was subsequently vortexed and sonicated at $T = 50.0\text{ }^{\circ}\text{C}$ for 10 min. The liposome solution was then extruded 15 times above the lipid-transition temperature through a 200 nm pore size polycarbonate membrane using a mini-extruder. For liposomes containing cholesterol, the procedure was the same except that different amounts of cholesterol were added to DPPC before the addition of chloroform to give 10 and 20 mole % cholesterol.

Steady-State Fluorescent Spectroscopy for Thermal Measurements. Steady-state fluorescence spectroscopy was carried out using a Thermo Fisher Lumina spectrometer equipped with a temperature controller unit (T3 Quantum Northwest). Fluorescent emission spectra were acquired upon excitation at 457 nm. Before each measurement, the solution was given time to stabilize for at least 5 min. To evaluate the thermal response, 50 μL of the liposome prepared solution was first diluted into 2 mL of 10 mM HEPES and 150 mM NaCl and then subjected to a gradual increase in temperature between 20.0 and 50.0 $^{\circ}\text{C}$ with a 5.0 $^{\circ}\text{C}$ increment. Relative FRET efficiency was calculated by first integrating the fluorescence signal between 490 and 640 nm, which is denoted as I_{Donor} . A second integration was calculated between 640 and 780 nm (I_{Acc}). The ratio $\left(\text{FRET} = \frac{I_{\text{Acc}}}{I_{\text{Acc}} + I_{\text{donor}}}\right)$ of the integrated signal was then calculated and plotted against the change in temperature.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.langmuir.9b03128>.

Liposomes characterization; the thermal response of DPPC–DiD; the thermal response of DOPC–DiD/MPS-PPV; and pH stability of MPS-PPV (PDF)

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Notes

The authors declare no competing financial interest.

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