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Comparison of excitation and emission ratiometric fluorescence methods for quantifying the membrane dipole potential

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Abstract

We are interested in developing fluorescence methods for quantifying lateral variations in the dipole potential across cell surfaces. Previous work in this laboratory showed that the ratio of fluorescence intensities of the voltage-sensitive dye di-8-ANEPPS using *excitation* wavelengths at 420 and 520 nm correlates well with measurements of the dipole potential. In the present work we evaluate the use of di-8-ANEPPS and an *emission* ratiometric method for measuring dipole potentials, as Bullen and Saggau (*Biophys. J.* 65 (1999) 2272–2287) have done to follow changes in the membrane potential in the presence of an externally applied field. Emission ratiometric methods have distinct advantages over excitation methods when applied to fluorescence microscopy because only a single wavelength is needed for excitation. We found that unlike the excitation ratio, the emission ratio does not correlate with the dipole potential of vesicles made from different lipids. A difference in the behaviour of the emission ratio in saturated compared to unsaturated lipid vesicles was noted. Furthermore, the emission ratio did not respond in the same way as the excitation ratio when cholesterol, 6-ketocholestanol, 7-ketocholesterol, and phloretin were added to dimyristoylphosphatidylcholine (DMPC) vesicles. We attribute the lack of correlation between the emission ratio and the dipole potential to simultaneous changes in membrane fluidity caused by changes in membrane composition, which do not occur when the electric field is externally applied as in the work of Bullen and Saggau. Di-8-ANEPPS can, thus, only be used via an *excitation* ratiometric method to quantify the dipole potential.

Keywords: Lipid vesicle; Voltage-sensitive styryl dye; Emission ratio; Excitation ratio; Cholesterol; Dipole potential

1. Introduction

The dipole potential, ψ_d, of a phospholipid membrane is an electrical potential that exists between the polar exterior of a membrane and its hydrocarbon interior [1–4]. This potential difference arises from the orientation of polar lipid residues and water dipoles near the surface of the membrane. For saturated phosphatidylcholine membranes like those investigated in this work, the dipole potential ranges from approximately 300 to 400 mV, which, because of the short distances over which it drops, results in electric field strengths in the range of 10^8–10^9 V/m [5]. In these membranes, the hydrocarbon-like interior is at a positive potential relative to the surface. For comparison, a typical membrane potential of 100 mV across the entire membrane of 4 nm thickness results in an electric field strength of 2.5×10^7 V/m [5]. Since it is known that field strengths of this magnitude can alter the conformation and orientation of membrane proteins, it is reasonable to assert that the much higher field strengths caused by the dipole potential could also significantly influence the conformation and orientation, and hence the activity, of membrane proteins. *Changes* in the dipole potential, then, could induce changes in protein activity and thus serve regulatory purposes.

Several studies suggest that the dipole potential does in fact influence membrane protein function. Work in this laboratory showed an increase in the molecular activity of the Na^+–K^+–ATPase ion pump [6]. Cladera and O'Shea studied the effects of the dipole potential on the insertion and folding of amphiphilic peptides in membranes [7] and Maggio reported on the modulation of phospholipase A_2 with the membrane dipole potential [8]. In addition to effects on membrane proteins, the

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dipole potential has also been shown to affect several other membrane processes such as the kinetics of redox reactions at membrane surfaces [9], skin permeability [10], membrane partitioning of anesthetics [11], and membrane fusion [12].

We are interested in developing fluorescence methods for quantifying lateral variations in the dipole potential across cell surfaces. To do this one must use an appropriate voltage-sensitive probe and carefully select the experimental parameters (e.g. excitation and emission wavelengths) so that the probe responds to the dipole potential and nothing else. Unfortunately, membrane components can have effects on a number of membrane properties. A prime example is cholesterol, which has been shown to increase the dipole potential of lipid membranes [13–16]. However, cholesterol also increases the lipid packing density [15,17–19] and decreases the membrane fluidity [15,20–22]. Therefore, if one wishes to detect variations in dipole potential caused by differences in the cholesterol content of a membrane, one must be sure that the fluorescent probe does not also respond to the simultaneous cholesterol-induced change in fluidity.

Work in this laboratory has shown that a fluorescence excitation ratio of the voltage-sensitive dye di-8-ANEPPS (see Fig. 1) correlates with other measurements of the dipole potential [6]. Specifically, it was shown that the ratio of fluorescence intensities using excitation wavelengths at 420 and 520 nm correlates well with the limited electrical measurements of the dipole potential available in the literature. Furthermore, in that study, it was shown that the excitation ratio is very sensitive to the presence of cholesterol and other dipole-potential modifying derivatives including 6-ketocholestenol, cholesten-3β-ol-7-one (7-ketocholesterol), coprostanol, and 4-cholesten-3-one. Other studies also indicate that di-8-ANEPPS incorporated in membranes is sensitive to phloretin [23], which is known to decrease the dipole potential [24,25]. The use of excitation ratios of di-8-ANEPPS to measure dipole potentials was first proposed by Gross, Bedlack, and Loew [26], later modified by Clarke and Kane [27] and recently used by Starke-Peterkovic et al. [6].

Bullen and Saggau [28] reported using an *emission* ratiometric method with di-8-ANEPPS to measure membrane potentials created via patch-clamp methods in neurons. It was shown that the probe responds rapidly to changes in the applied voltage and that the magnitude of the response was related to the magnitude of the applied voltage. Some adjustments to the data were required depending on the region of the neuron being probed in order to create a universal calibration curve relating the emission ratio of fluorescence above and below 570 nm to the applied electric field. Nevertheless, the study demonstrates that di-8-ANEPPS emission ratios, as well as excitation ratios, could, in principle, be used to track electric fields in membrane systems.

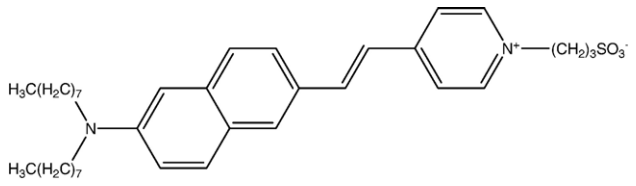

Fig. 1. Structure of di-8-ANEPPS.

Ultimately, it is desirable to develop a method for measuring dipole potentials that could be used with visualization methods such as fluorescence microscopy. Styrylpyridinium voltage-sensitive dyes are attractive candidates for such applications since they are designed to be sensitive to the electric field they are in. Additionally, they respond rapidly to changes in the local electric field suggesting that they could be used to monitor real-time changes upon various biologically significant perturbations to cells and their surrounding media.

Given our previous work using di-8-ANEPPS excitation ratios to measure dipole potentials and Bullen and Saggau's use of the same dye to probe membrane potentials via emission ratios, the work presented here is aimed at more fully characterizing the emission ratio response of di-8-ANEPPS, comparing it with the excitation method and discussing its potential application to the measurement and monitoring of dipole potentials in lipid vesicles and cells.

2. Materials and methods

All phospholipids used in this study except di-O-hexadecyl-sn-glycero-3-phoshocholine were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used as received. Di-O-hexadecyl-sn-glycero-3-phosphocholine, cholesterol, 6-ketocholestanol, cholesten-3β-ol-7-one, phloretin, ethylenediamine tetraacetic acid disodium salt (approx. 99%), and tris[hydroxymethyl]aminomethane (Tris, minimum 99.9%) were purchased from Sigma (Castle Hill, NSW, Australia) and used as received. Sodium chloride was from Merck (Darmstadt, Germany), 4-(2-(6-(dioctylamino)-2-naphthalenyl)ethyl-1-(3-sulphopropyl)-pyridinium inner salt (di-8-ANEPPS) was from Molecular Probes (Eugene, OR, USA), and concentrated hydrochloric acid was from Univar (Seven Hills, NSW, Australia). All were used as received. Purified water (18.2 MΩ) was obtained from a Millipore Direct-Q system.

2.1. Vesicle preparation—injection method

Two methods for making large unilamellar lipid vesicles were used in this study. In the injection method, 0.024 g of dimyristoylphosphatidylcholine (DMPC) was dissolved in 1.1 mL absolute ethanol. 1 mL of this solution was slowly and continuously injected into 10 mL of buffer at approximately 30 °C with stirring over a time span of 10 to 15 min. The buffer was 30 mM Tris, 150 mM NaCl, 1 mM EDTA, and adjusted to pH = 7.2 using hydrochloric acid. The resulting solution was then transferred to Spectra/Por dialysis tubing (MWCO 12-14,000, Spectrum Laboratory, Inc., Rancho Dominguez, CA, USA). The solution was dialyzed against 5 different 300 mL aliquots of buffer. Final lipid concentrations of 3 mM were confirmed using a Phospholipid C test kit from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The DMPC vesicles used in the temperature study described below were prepared via this method.

2.2. Vesicle preparation—extrusion method

The extrusion method of vesicle preparation was also used in this study. In this method, 0.024 g of DMPC and the required amount of cholesterol derivative were dissolved together in 1 mL of chloroform. The chloroform was then removed via overnight rotoevaporation to form a thin lipid film on the walls of a roundbottom flask. The material was resuspended in 10 mL of buffer (same composition as above) by allowing the material to rotate on the rotary evaporator (without vacuum) for 1 h. For some cholesterol compositions, some scraping was required to completely remove the material from the wall. The solution was vortexed for 30 to 60 s prior to extrusion in the next step. The solution was extruded through a 100 nm Nucleopore

polycarbonate membrane a minimum of eleven times using an Avanti Mini-Extruder (Alabaster, AL, USA). Throughout the resuspension and extrusion processes, temperatures were maintained above the known main transition temperatures for the different lipids used.

Phloretin was added to a solution of pure DMPC vesicles by first making an 88 mM stock solution in ethanol. Appropriate volumes of this solution and pure DMPC vesicle solution were mixed to achieve the desired mole percent of phloretin. The incorporation of phloretin into the vesicles is rapid, whereas cholesterol and its derivatives equilibrate more slowly. Thus, phloretin can be added directly to solution followed by rapid equilibration whereas to maximize cholesterol derivative incorporation into the membrane each derivative was mixed with lipid prior to vesicle formation, as described above.

2.3. Dye solution

A solution of dye was prepared by dissolving approximately 5 mg of di-8-ANEPPS in 9 mL of ethanol, which theoretically yields a concentration of 0.937 mM. The measured concentration was found to be 0.862±0.09 mM using an estimate of the molar absorptivity (37,000±4000 M^{-1} cm^{-1} at 498 nm in methanol) provided by Molecular Probes on the certificate of analysis. For fluorescence measurements, 5 μL of this ethanolic dye solution were added to 1 mL of vesicle solution. The ratio of the lipid to dye concentrations in these solutions is approximately 600. The solutions were allowed to remain at a temperature above the main phase transition for a minimum of 3 h for dye aggregates to dissociate and for dye monomers to insert themselves into the lipid membrane. No significant changes in fluorescence excitation or emission intensity are observed after 3 h. The effects of small volumes of ethanol added to these solutions on the emission and excitation spectra were checked in another study [27] and repeated in this study (up to 32 μL ethanol in 1 mL DMPC solution) and found to be negligible in both cases. Furthermore, essentially doubling the total dye concentration in the final solutions by adding 10 μL instead of 5 μL to a 1 mL aliquot of vesicle solution was found to have no effect on the measured emission and excitation ratios, verifying that the dye is dilute enough that dye–dye interactions within the membrane are insignificant.

2.4. Fluorescence measurements

Fluorescence measurements were performed on a Shimadzu RF-5301 PC spectrofluorophotometer. Quartz semi-micro cuvettes were used for all measurements. The emission wavelength was set at 670 nm for all excitation spectra with an RG645 cutoff filter (Schott, Mainz, Germany) in the emission path. Excitation wavelengths (in nm) and the corresponding filters used in the excitation path (shown in parentheses) were as follows 375 (WG320), 400 (WG320), 440 (GG400), 458 (GG420), 488 (GG455), 514 (GG495), 543 (GG495). These correspond to common laser lines and were chosen so that our work might be directly applicable to fluorescence microscopy. Temperatures were maintained using a circulating water bath connected to the sample holder.

Excitation spectra were corrected for the wavelength dependence of the excitation monochromator's transmission efficiency using rhodamine B as a quantum counter. The excitation ratios, R_{ex}, reported here are the ratio of emission intensity at 670 nm caused by excitation with 420 and 520 nm light. Emission spectra were uncorrected. The emission ratios, R_{em}, reported here are the ratios of emission intensity at 560 and 700 nm with the specified excitation wavelength (primarily 543 nm).

In all cases, the reported ratio is an average obtained from a minimum of five spectra recorded in succession. A single DMPC solution was carried through the temperature study described below. Measurements of the emission ratio at 30 °C were made at the start, middle, and end of the study and showed good internal agreement. Furthermore, they are in good agreement with ratios obtained many weeks later using a different vesicle preparation. For the work involving the cholesterol derivatives, each solution was prepared twice, with good internal agreement (typically under 10% difference and commonly under 5% difference in the measured ratios). Furthermore, good agreement with R_{ex} values previously reported by Starke-Peterkovic et al. was observed. [6,29].

3. Results

3.1. Temperature and excitation wavelength dependence of fluorescence emission ratios

Voltage sensitive dyes, while designed to have fluorescence characteristics that reflect their electrical environment, are also sensitive to other chemical or physical effects. In order to assign spectral changes of the dyes solely to electrical effects, care must, therefore, be taken to ensure that one is working under conditions where other effects are not contributing to the observed spectral changes. Changes in membrane fluidity are known to cause changes in the spectral characteristics of voltage-sensitive dyes [27]. To test for fluidity effects, we measured R_{em} of di-8-ANEPPS in DMPC vesicles as a function of temperature and excitation wavelength. The results are shown in Fig. 2.

Fig. 2 shows that R_{em} depends significantly on the excitation wavelength as well as on temperature. Larger ratios are observed for lower wavelength excitation, indicating a red shift in the fluorescence emission with increasing excitation wavelength. This shift is clearly shown in Fig. 3 with plots of the emission spectra recorded with 440 and 543 nm excitation wavelengths. For ease of comparison the spectra have been normalized to their maximum intensity (set as 100%). It is clear from Figs. 2 and 3 that the emission spectra, and hence R_{em}, vary significantly with excitation wavelength.

Fig. 2 also shows that R_{em} is quite sensitive to temperature, especially for shorter wavelength excitation. In all cases the dye is sensitive to the gel-to-liquid crystal phase change that occurs around 23 °C for DMPC [30,31]. But only with excitation at 543 nm is the dye insensitive to the fluidity (i.e. temperature effects) of its environment once the transition temperature has been exceeded. All other excitation wavelengths are still responsive to temperature-induced changes in the emission spectra of the dye. These changes likely arise, at least in part, from changes in the dye's immediate chemical environment (lipid packing density, water associated with the head groups, rigidity of the environment, dye alignment within the membrane, etc.) which alter the rate and degree of the dye's excited state relaxation prior to emission.

Given these results, it is clear that for our purposes of evaluating di-8-ANEPPS emission ratios as a probe of membrane dipole potentials, the only conditions under which R_{em} values might solely reflect electrical effects involve using 543 nm excitation with temperatures above the phase transition of the vesicles of interest.

3.2. Correlation of R_{ex} and ψ_d in different lipid vesicles

As stated in Introduction, this laboratory has already established a method for determining the dipole potential using di-8-ANEPPS and an excitation ratio [6]. This was accomplished by correlating R_{ex} with published literature values for dipole potentials in vesicles of different lipids. Specifically, dilauroylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC),

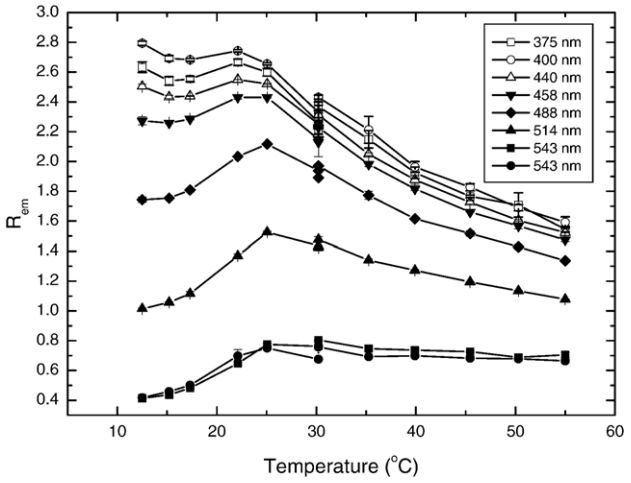

Fig. 2. R_{em} as a function of temperature at multiple emission wavelengths. R_{em} is defined as the fluorescence intensity of di-8-ANEPPS at 560 nm divided by that at 700 nm arising from excitation at the specified wavelengths. Conditions: 2.7 mM DMPC, 4.3 μM di-8-ANEPPS, excitation wavelengths (and filters) all in nm: □ 375 (WG320); ○ 400 (WG320); Δ 440 (GG400); ▼ 458 (GG420); ♦ 488 (GG455); ▲ 514 (GG495); ■ and ● 543 (GG495), excitation and emission bandwidths=3 nm. Error bars were calculated from three replicate emission scans recorded on the same solution and are generally smaller than the symbols.

dioleylphosphatidylcholine (DOPC), stearoyl-oleoylphosphatidylcholine (SOPC), and di-O-hexadecyl-sn-glycero-3-phosphocholine (di-O-C16) were investigated in that study and have been used in the present work.

In recording the R_{em} values, it was convenient to rerecord R_{ex} values at the same time using the same conditions as before [6]. The R_{ex} values obtained were consistent with those previously published. For comparison with the R_{em} values (see below) the correlation between R_{ex} and the dipole potential, ψ_d, from reference 6 is reproduced here (see Fig. 4).

3.3. Correlation of R_{em} and ψ_d in different lipid vesicles

The purpose of this work is to explore the possible use of an emission ratiometric method for determining ψ_d in the same way as was done using the excitation ratio. In Table 1 we present our average measured R_{em} values and uncertainties (from 5 replicate scans) and the values of ψ_d that were collected from the literature and used to establish the correlation between R_{ex} and ψ_d in previous work [6]. A plot of R_{em} against ψ_d is presented in Fig. 5. It is clear from visual inspection that there is no correlation between the emission ratio recorded at 543 nm excitation and ψ_d.

While no correlation was found, there is an interesting difference to be noted in Fig. 5, namely, that the two systems containing at least one unsaturated chain (DOPC and SOPC) both have R_{em} values that are larger than the systems with the saturated chains, i.e. the unsaturated lipids yield blue-shifted emission spectra relative to the saturated lipids. It is also interesting to note that whereas the excitation ratio clearly differentiated the lipid with the ether linkage (di-O-C16) from the acyl lipids, the emission ratio, R_{em}, for di-O-C16 falls squarely within the range of R_{em} established by the acyl lipids. Thus, it seems that while we tested for and used an excitation wavelength that seemed insensitive to fluidity effects, there are other chemical/environmental effects at play in the emission behaviour of di-8-ANEPPS that are not operative in its excitation behaviour.

3.4. Cholesterol effects on R_{ex} and R_{em}

To further characterize the dye's emission behaviour, we measured R_{em} in DMPC vesicles modified with varying amounts of cholesterol and 6-ketocholestanol, which are known to increase ψ_d [32,33], as well as with 7-ketocholesterol and phloretin, which are known to decrease ψ_d [15,34–37]. Starke-Peterkovic et al. previously measured R_{ex} as a function of mole percent cholesterol derivative [29]. Cholesterol and four derivatives were shown to produce more or less smooth curves of R_{ex} vs. mole percent additive from 0 to 60%, with all derivatives showing modest changes in R_{ex} up to approximately 25 mol%, followed by more rapid increases to roughly 45 mol%, and then levelling off or actually decreasing from 45 to 60 mol%.

In this work, we repeated the R_{ex} measurements and in addition performed R_{em} measurements for 0, 25 and 45 mol% cholesterol, 6-ketocholestenol, 7-ketocholesterol, and phloretin.

The excitation and emission ratios versus mole percent additive are shown in Figs. 6 and 7, respectively. The symbols for each additive are the same in both graphs. The average R_{em} value is shown in cases where two R-values were recorded. The ethanol added to the vesicle suspensions with the phloretin was found in control measurements to not significantly shift the R-values.

Comparing Figs. 6 and 7 it is obvious that once again the observed trends in R_{ex} and R_{em} do not agree and the two ratios are therefore responding to different properties of the vesicles. For example, 6-ketocholestanol induced the largest changes in R_{ex}, but cholesterol does so for R_{em}. In fact, R_{em} for 6-

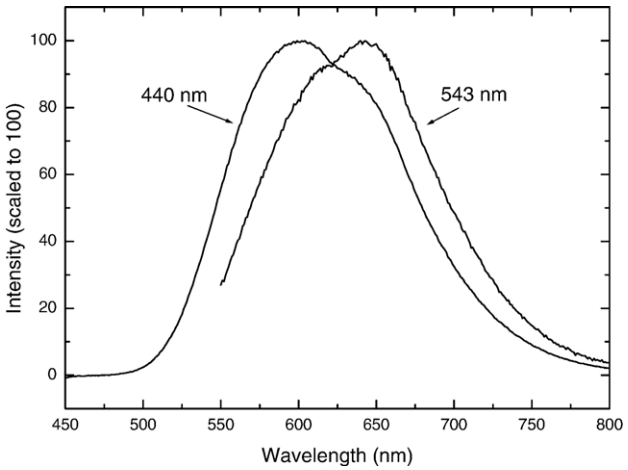

Fig. 3. Emission spectra of di-8-ANEPPS in DMPC vesicles with excitation λ_{ex}=440 nm and 543 nm at 30 °C scaled to a maximum intensity of 100. [di-8-ANEPPS]=4.3 μM, [DMPC]=2.7 mM, excitation and emission bandwidth=3 nm, excitation filters=GG400 and GG495 cut-off for λ_{ex}=440 nm and 543 nm, respectively.

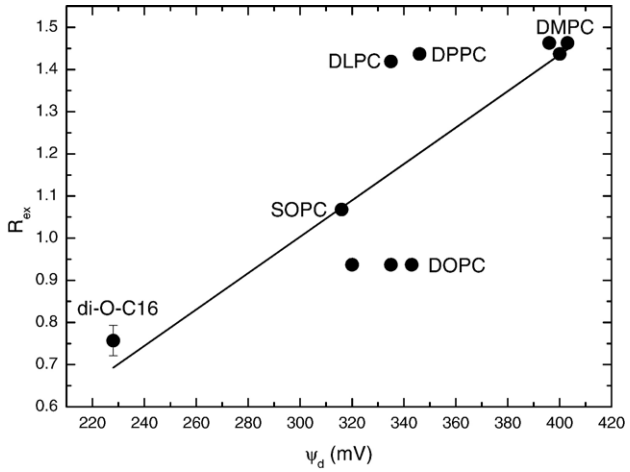

Fig. 4. Correlation of R_{ex} with ψ_d from Reference 6. R_{ex} is defined as the ratio of fluorescence intensity of di-8-ANEPPS at 670 nm arising from excitation at 420 nm divided by that arising from 520 nm. Emission filter=RG645 cut-off. The values of ψ_d were compiled from the literature (see Table 1 and ref. [6]). The line represents the best-fit through the data. Error bars are calculated from five replicate excitation scans recorded on the same solution. Reproducibility between two different solution preparations yielded an average percent difference of 10% in R_{ex}.

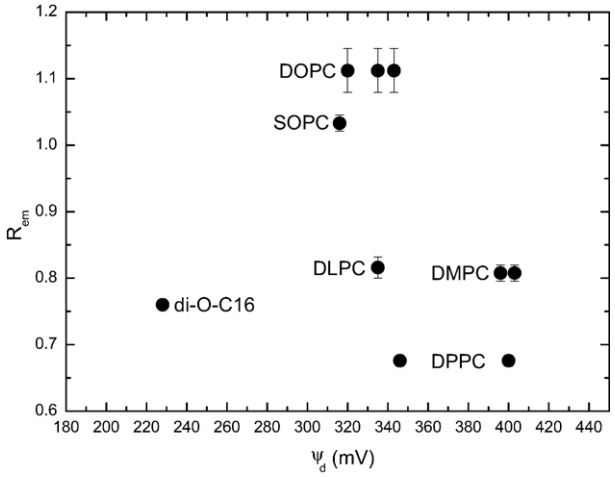

Fig. 5. Plot of R_{em} against ψ_d. R_{em} is defined as the fluorescence intensity of di-8-ANEPPS at 560 nm divided by that at 700 nm arising from excitation at 543 nm with a GG495 cut-off filter. Conditions: 3 mM phospholipids, 4.3 μM di-8-ANEPPS, excitation and emission bandwidths=3 nm. Error bars were calculated from five replicate emission scans recorded on the same solution.

ketocholestanol shows only a very mild increase even at 45 mol %. If it is accepted that R_{ex} provides a direct correlation with dipole potential as shown previously [6], then clearly R_{em} does not. Additional evidence for this is that the excitation ratio decreased upon addition of 7-ketocholesterol, whereas the emission ratio increases at 25 mol% and then decreases again at 45 mol%. It is also interesting to note that the change in R_{ex} from 25 to 45 mol% cholesterol is quite moderate, whereas a large change in R_{em} is observed.

4. Discussion

From our previous studies and those presented here, it is clear that the absorbance and fluorescence properties of di-8-ANEPPS are sensitive to changes in the local environment of the dye in lipid vesicles. From Figs. 4–7, it is also clear that the excitation ratio correlates with the known behaviour of the dipole potential, but the emission ratio does not. Thus, di-8-ANEPPS can be used as a ratiometric probe of dipole potential, but only if one uses an excitation ratio.

The fact that the emission ratio does not correlate well with the dipole potential (or with the excitation ratio) may be somewhat surprising given that Bullen and Saggau have described an emission ratiometric method using di-8-ANEPPS for the measurement of membrane potentials [28]. That work, however, used di-8-ANEPPS to probe the entire membrane potential in the presence of an externally applied electric field (via a voltage clamp). In contrast, in the present work the

Table 1
Comparison of fluorescence emission ration, R_{em}, of di-8-ANEPPS with electrical bilayer and monolayer measurements of the dipole potential, ψ_d, of various phosphatidylcholines

Lipid	R_{em}	T (°C)	ψ_d (mv)	Ref.
DLPC	0.816 (±0.016)	20	335	[40,41]
DMPC	0.808 (±0.012)	30	396	[41–43]
DMPC	0.808 (±0.012)	30	403	[41–43]
DPPC	0.676 (±0.008)	45	400	[41,44,45]
DPPC	0.676 (±0.008)	45	346	[46,47]
DOPC	1.112 (±0.033)	20	320	[40,41]
DOPC	1.112 (±0.033)	20	335	[40,41]
DOPC	1.112 (±0.033)	20	343	[47,48]
SOPC	1.033 (±0.012)	20	316	[47,48]
di-O-C16	0.760 (±0.007)	50	228	[46,47]

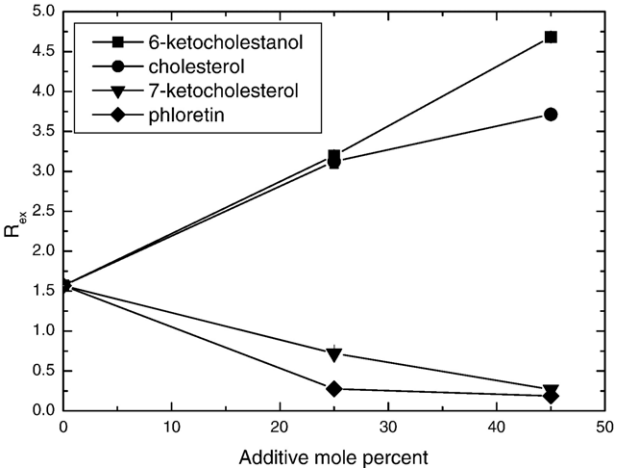

Fig. 6. R_{ex} as a function of additive mole percent in DMPC vesicles. R_{ex} is defined as the ratio of fluorescence intensity of di-8-ANEPPS at 670 nm arising from excitation at 420 nm divided by that arising from 520 nm. Emission filter=RG645 cut-off. [DMPC]=3.5 mM. [di-8-ANEPPS]=4.3 μM. Symbols: ■ 6-ketocholestanol, ● cholesterol, ▼ 7-ketocholesterol, and ◆ phloretin. Excitation and emission bandwidths=3 nm. Error bars were calculated from five replicate emission scans recorded on the same solution and are generally smaller than the symbols. Reproducibility between two different solution preparations yielded an average percent difference of 3.4% in R_{em}, with the lowest being 0.19% difference (pure DMPC) and the highest being 11.5% difference (45% 7-ketocholesterol).

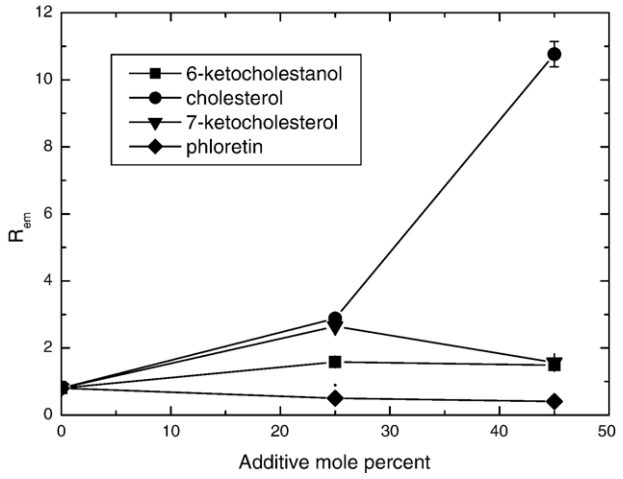

Fig. 7. R_{em} as a function of additive mole percent in DMPC vesicles. R_{em} is defined as the fluorescence intensity of di-8-ANEPPS at 560 nm divided by that at 700 nm arising from excitation at 543 nm with a GG495 cut-off filter. [DMPC]=3.5 mM. [di-8-ANEPPS]=4.3 μM Symbols: ■ 6-ketocholestanol, ● cholesterol, ▼ 7-ketocholesterol, and ♦ phloretin. Excitation and emission bandwidths=3 nm. Error bars are calculated from five replicate emission scans recorded on the same solution and are generally smaller than the symbols. Reproducibility between two different solution preparations yielded an average percent difference of 3.7% in R_{ex}, with the lowest being 0.74% different (pure DMPC) and the highest being 8.5% different (45% 6-ketocholestanol).

electric field being probed arises from the lipids and modifiers themselves. Therefore, although an emission ratiometric method using di-8-ANEPPS appears to work for the transmembrane potential measured in the presence of an applied field, it does not work when the electric field is produced solely by the lipid components of the membrane. One possible explanation is that this may be due to excited state relaxation mechanisms that can occur in the absence of an applied electric field which are not available to the dye and/or lipid components when an electric field is externally applied. However, a more likely explanation is possibly that an externally applied electric field at constant temperature and constant membrane composition has a negligible effect on membrane fluidity, so that any changes in R_{em} can in this case be confidently attributed to changes in the transmembrane potential alone. In contrast, if the lipid composition is varied, then changes in both the membrane dipole potential and the fluidity will occur, and in these circumstances only excitation ratiometric measurements allow the change in dipole potential alone to be extracted.

The fact that R_{ex} and R_{em} in our measurements are not correlated arises from the fact they are based on fundamentally different processes, namely the absorption and emission of photons, respectively. During the absorption process, the absorbing species undergoes a ground to excited state transition, with a concomitant change in the electron distribution within the molecule. The Franck–Condon principle asserts that the absorption process occurs so rapidly that the solvent nuclei clustered around the absorbing species do not have time to reorient around the new charge distribution of the excited state chromophore. Thus, the energy required for absorption is dictated solely by the difference in the ground and excited state energy of the absorbing species, with both states interacting with the same configuration of neighboring solvent molecules (i.e. there is no atomic motion, although the electrons in the solvent molecules can respond to the new local electric field induced by the excited state electron distribution of the chromophore).

In contrast, R_{em} is based on fluorescence emission. As in absorption, the energy of the emitted light also depends on the energy difference between the ground and excited states of the absorbing species. Unlike absorption, however, the time scale of emission is such that the dipoles of the neighboring solvent molecules can reorient to stabilize the excited state species. This is known as solvent relaxation and decreases the energy gap between the excited and ground states. The decrease in this energy gap manifests itself in a difference in the energy of the emitted light relative to that absorbed. This difference is referred to as the Stokes' shift. The degree to which the surroundings stabilizes the excited state is determined by the orientational polarizability of the medium, meaning the polarizability due to molecular reorientation alone, excluding electronic redistribution.

Another effect that can alter the energy of the emitted light in rigid environments such as membranes is the speed with which the excited state molecule can relax. If the solvent relaxation is slow due to the rigidity of the environment, then the probability of emission from a higher energy state increases, with a subsequent shift towards higher energy (lower wavelength) fluorescence. This is the origin of so-called fluidity effects.

Thus, R_{em} depends on (1) the energy gap between the ground and excited states, (2) solvent reorganization around the excited state, and (3) the rate of solvent reorganization (fluidity effects). Even if conditions are found such that R_{em} is independent of fluidity effects in a pure lipid vesicle, such as was done in this study, the effect of additives such as cholesterol and its derivatives on the energy gap and on the orientational polarizability (or in other words the degree of solvent reorganization) do not necessarily act in parallel. Thus, differences in the response of R_{ex}, which depends only on the energy gap, and R_{em}, which depends both on the energy gap and on the orientational polarizability, can arise. More specifically, additives affect the energy gap by altering the polarity of the probe's microenvironment within the membrane. In the case of di-8-ANEPPS, which resides near the membrane surface, this means modifying the dipole potential. But the additives also affect the freedom of motion within the membrane via changes in packing density or other structural effects (waters of hydration, associated ions, etc.) and hence alter the orientational polarizability and consequently the degree of solvent relaxation. Furthermore, there is no reason to believe that the changes in dipole potential and orientational polarizability induced by an additive are necessarily linked or correlated. Thus, R_{ex} and R_{em} are influenced by independent effects and need not necessarily display parallel responses to the various types and amounts of additives used in this study.

Jin et al. have recently published similar findings and conclusions regarding the origin of emission shifts of dyes in lipid vesicles [38]. Specifically, they investigated the excitation and emission properties of di-4-ANEPPDHQ, a dye with a

chromophore that is structurally highly related to that of di-8-ANEPPS, in large unilamellar vesicles (LUVs) of DPPC and DOPC. They conclude that the emission shifts they observe upon the addition of cholesterol to the LUVs cannot be explained solely by changes in the dipole potential. They hypothesize that the increase in rigidity of the molecular environment of the dye induced by the cholesterol may also contribute to the emission spectrum shifts. Thus, our work is consistent with theirs in the observation that variations in R_{em} cannot be linked to changes in the dipole potential alone.

To extend this analysis in the light of the specific R_{ex} and R_{em} values observed in this study, it is known that unsaturated lipid vesicles are less closely packed (i.e. require greater surface area per monomer) than are saturated lipid vesicles. Therefore, the dipolar headgroups, which influence the Stokes' shift, are on average further away from the probe molecule in unsaturated compared to saturated lipid vesicles. Thus, the change in the electric field that is sensed by an unsaturated lipid upon the probe's absorption of energy is likely to be less than that sensed by a saturated lipid. This could result in less reorganization and higher energies (shorter wavelengths) of emitted light, ultimately increasing R_{em} for unsaturated lipid vesicles relative to saturated vesicles, which is what we observed in Fig. 5. Conversely, vesicles that have high dipole potentials (e.g. tightly packed saturated lipid vesicles) will lower the ground state energy of the dye relative to its excited state, causing higher energies and shorter wavelengths of absorption compared to less tightly packed unsaturated lipids with lower dipole potentials. This will result in larger R_{ex} values for saturated lipid vesicles compared to unsaturated vesicles as is observed in Fig. 4.

Thus, while the excitation and emission behaviour of di-8-ANEPPS can be rationalized on the basis of the different processes occurring, the differences in the processes also leads to the result that R_{em} does not respond solely to the dipole potential. Thus, it appears that emission ratios of di-8-ANEPPS, or any other probe that is sensitive to both orientational polarizability and dipole effects, cannot be used to measure dipole potentials in lipid vesicles. If one wishes to perform emission ratiometric measurements (which have several practical advantages over excitation ratiometric measurements for fluorescence microscopy) of the dipole potential, then other probes are required. In this regard the work of Klymchenko et al. should be mentioned [39]. They have studied two different 3-hydroxyflavone probes that undergo an excited state intermolecular proton transfer (ESIPT) reaction upon excitation that results in two emission bands resulting from the normal (N*) and tautomeric (T*) excited state species. The fluorescence emission of these dyes appears to have relatively weak dependence on the orientational polarizability, while the distribution between the N* and T* forms is very sensitive to the local electric field (i.e. dipole potential). Thus, R_{em} for the 3-hydroxyflavones, like R_{ex} for di-8-ANEPPS, is dominated by dipole effects, making it possible to use the dyes and R_{em} values to track changes in the dipole potential of lipid vesicles. The authors did not, however, correlate R_{em} with any electrical measurements of dipole potential, citing the lack of experimental methods for obtaining reliable values of the dipole potential. Instead, they showed that R_{em} of the 3-hydroxyflavones is linearly related to R_{ex} of di-8-ANEPPS in a series of egg yolk phosphatidylcholine vesicles in which the dipole potential was modified using phloretin and 6-ketocholestanol. Thus, if R_{ex} of di-8-ANEPPS is linearly related to the dipole potential as has been suggested, then so too is R_{em} for the 3-hydroxyflavones.

In summary, while *excitation* ratios of di-8-ANEPPS have been shown to correlate with lipid vesicle dipole potentials and with ratiometric measurements made with other probes that also likely respond to dipole potential, we have shown that the *emission* ratio of di-8-ANEPPS cannot be used for the purpose of measuring dipole potentials. This arises because of the complex nature of the fluorescence process and the sensitivity of di-8-ANEPPS to both dipole and orientational polarizability effects. These effects appear to lead to a discrimination between saturated and unsaturated lipid vesicles, which may in itself be useful, but does not lead to quantitative measurement of dipole potentials. For emission ratiometric measurements, other dyes are required. The 3-hydroxyflavones described by Klymchenko et al. [39] appear to be promising in this regard.

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