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Orientational polarisability of lipid membrane surfaces

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Abstract

Here we present a fluorescence method based on the Stokes shift of the voltage-sensitive dye di-8-ANEPPS to quantify the orientational polarisability of lipid membrane surfaces, i.e. the polarisability due to molecular reorientation. Di-8-ANEPPS is already an established probe of membrane dipole potential. Its use, therefore, as a probe of both the dipole potential and orientational polarisability allows a direct comparison of these two properties in an identical region of the lipid bilayer. We applied the new technique on phosphatidylcholine vesicles to study the effects of different degrees of hydrocarbon saturation and of the incorporation of cholesterol and some of its oxidized derivatives. We found that lipids with unsaturated chains had a lower orientational polarisability than those with saturated chains. This could be explained by a reduction in membrane dipole potential as a result of a decrease in lipid packing density. Cholesterol derivatives were found to either increase or decrease the orientational polarisability depending on their molecular structure. The varying effects could be explained by antagonistic effects of the dipole potential and membrane order, which are both changed to varying degrees by the cholesterol derivatives and which lead to increases and decreases in orientational polarisability, respectively.

Keywords: Lipid vesicles; Voltage-sensitive styryl dyes; Fluorescence; Stokes shift; Cholesterol; Lippert plot; Solvent relaxation

1. Introduction

Lipid–protein interactions are known to be very important in determining the activity of membrane proteins [1]. This is particularly the case for ion pumps which must undergo significant conformational changes in order to allow ion translocation to occur. For example, the activity of the Na^+, K^+-ATPase (the first ion pump to be discovered) is critically dependent on its lipid environment. From kinetic measurements of the Na^+,K^+-ATPase reconstituted into lipid vesicles, Cornelius [2] showed that optimal steady state activity in the absence of cholesterol was achieved with a monounsaturated phosphatidylcholine chain length of 22. If 40% cholesterol was incorporated into the vesicles, the optimal chain length decreased to 18. Other authors have shown that cholesterol has a biphasic modulatory effect on Na^+,K^+-ATPase activity [3,4], with stimulation being observed at low cholesterol concentrations and inhibition at concentrations above the physiological level. The mechanism of such effects, however, remains unclear. Both specific lipid–protein binding and an indirect effect via lipid modulation of the membrane's physical properties (e.g. thickness, fluidity, curvature, charge, polarity) have been suggested [5].

Partial reactions of ion pumps involve the movement of ions through the membrane simultaneously with protein conformational changes, including the movement of charged and polar amino acid residues. It is feasible that charged and dipolar moieties in the surrounding lipids could reorient around the changed charge distributions of the enzyme's transition states or reacting enzyme intermediates. This reorientation could then accelerate or decelerate the enzyme's reactions. This would particularly be the case at the membrane–water interface, where charged and dipolar residues are located, i.e. the zwitterionic phosphocholine headgroup, carbonyl dipoles in the ester linkage between the phospholipid head groups and the fatty acid chains, water dipoles hydrating the membrane surface, and the hydroxyl group of cholesterol. Therefore, it is important to

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know not just the static charge distribution of the membrane interfacial region (reflected in the dipole potential), but also its polarisability, i.e. how well the surrounding lipid can respond to the changing charge distribution within the protein as it is carrying out its ion pumping. The aim of this paper is, thus, to develop a method for quantifying the orientational polarisability of the membrane interface.

In the field of fluorescence spectroscopy, the electrical environment of the headgroup region of lipid membranes has most commonly been studied using the probes PRODAN, PATMAN and LAURDAN [6–10]. In this paper we propose the use of a fluorescence method based on the Stokes shift of the voltage-sensitive styrylpyridinium probe, di-8-ANEPPS (see Fig. 1) to measure the orientational polarisability of the membrane surface. The major advantage of this probe over those listed above is that it is already a well-established probe for quantifying the dipole potential of lipid membranes [11–15]. Therefore, if it could also be used to quantify orientational polarisability, then in principle it would allow dipole potential and orientational polarisability trends to be directly compared because the probe would be reporting on the identical position within the membrane in both cases. Its exact orientation within a phosphatidylcholine bilayer has been determined by Lambacher and Fromherz [16] using fluorescence interferometry. The location within the membrane of dyes with very similar structures has been determined via fluorescence, NMR and resonance Raman spectroscopy [17–19]. The sulphonate group of the probe can be thought to act as a hydrophilic anchor, maintaining that portion of the molecule at the interface between the membrane and the adjacent aqueous phase. The molecule's two hydrocarbon chains anchor the other end of the molecule in the hydrophobic interior of the membrane, and the fluorophore is thought to extend from the level of the glycerol backbone towards the hydrocarbon interior, where its long axis forms an angle of approximately 38° to the membrane normal.

Upon excitation, styrylpyridinium dyes undergo a large electronic redistribution, with the positive charge that is generally localized on the pyridinium nitrogen in the ground state, moving towards the amino nitrogen in the excited state [20,21]. After excitation in a bulk solvent, the solvent dipoles surrounding the dye undergo significant reorientation in response to the altered electronic configuration of the dye. This lowers the energy of the dye's excited state below that of its initial Franck–Condon level and thus produces significant Stokes shifts, the magnitudes of which depend on the orientational polarisability of the solvent [22,23]. In the case of an anisotropic medium such as a membrane, however, the surrounding molecules (i.e. the lipids and associated water molecules) are not free to rotate equally in all directions. The degree of confinement can be quantified by the lipid orientational order parameter [24]. If the local membrane order is very high (which it can be, particularly at high percentages of cholesterol and high protein content), then the orientational polarisability could in principle be low even for a membrane with a high dipole potential. Therefore, measuring the Stokes shift in membranes, and thus their orientational polarisability, may shed light on the combined effects of dipole potential and membrane order.

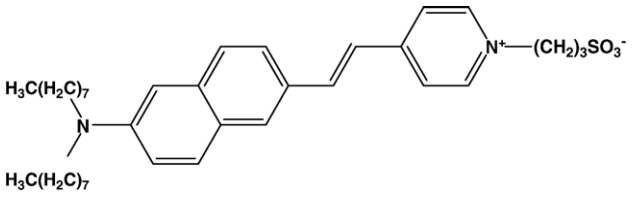

Fig. 1. Structure of di-8-ANEPPS.

A relationship between orientational polarisability and the Stokes shift was derived by Lippert in the 1950s [25,26]. It is described by the following equation:

$$10^2\left(\bar{\nu}_a^{sol} - \bar{\nu}_f^{sol}\right) = 10^2\left(\bar{\nu}_a^{gas} - \bar{\nu}_f^{gas}\right) + \frac{2}{4\pi\varepsilon_0 hca^3}(\Delta\mu)^2\Delta f \quad (1)$$

where $(\bar{\nu}_a^{sol}-\bar{\nu}_f^{sol})$ and $(\bar{\nu}_a^{gas}-\bar{\nu}_f^{gas})$ represent the Stokes shifts of the probe in cm^{-1} in solution and in the gas phase, respectively. The factor 10^2 has been introduced to convert the wavenumber from cm^{-1} to m^{-1}. h and c are Planck's constant and the speed of light in a vacuum, respectively. a represents the radius of the solvent cavity in which the fluorophore resides and $\Delta\mu$ is the change in dipole moment of the probe caused by excitation. For homogeneous isotropic solvents, Lippert, furthermore, utilized the reaction field theory of Onsager [27], which takes into account solvent–solvent interactions and their mutual polarisation, and which relates the orientational polarisability to the dielectric constant, ε, and the refractive index, n, of the solvent by:

$$\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad (2)$$

The first term on the right hand side of this equation, involving the dielectric constant, is the total polarisability (or low frequency polarisability) due to molecular reorientation of the solvent plus the mobility of the electrons within each solvent molecule. The second term, involving the refractive index, is the distortional polarisability (or high frequency polarisability) due to the mobility of the electrons alone. By subtracting the second term from the first, the result is the orientational polarisability of the surrounding medium, due to molecular reorientation alone. The Stokes shift of a fluorescent molecule is dependent on the orientational polarisability, but not the distortional polarisability, because the electronic redistribution of the surrounding solvent molecules is very fast and occurs virtually instantaneously upon excitation of the fluorophore.

If measurements of the Stokes shift of a fluorescent probe are made in a range of solvents of varying orientational polarisabilities, Eqs. (1) and (2) allow the estimation of the dipole moment change, $\Delta\mu$, of the molecule. This is the use which Lippert himself and many others since have made of his theory [28]. Here we propose a different application. The linear relationship between Δf and the Stokes shift given by Eq. (1) in principle allows the orientational polarisability of any probe environment to be estimated providing that its Stokes shift is

first calibrated against a range of solvents with known dielectric constants and refractive indices. Here we describe a calibration of di-8-ANEPPS for use as a probe of orientational polarisability and demonstrate its application on vesicles composed of various phosphatidylcholines in the presence and absence of cholesterol and some of its oxidized derivatives.

2. Materials and methods

All phospholipids used in this study except di-*O*-hexadecyl-*sn*-glycero-3-phosphocholine 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, coprostan-3-ol, cholest-4-en-3-one, ethylenediamine tetraacetic acid disodium salt (approximately 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. The purities and origins of the solvents used were as follows: chloroform (99.8%, Fluka, Castle Hill, NSW, Australia), dichloromethane (99.7%, Ajax Chemicals, Auburn, NSW, Australia), 1,2-dichlorobenzene (99%, Ajax Chemicals), hexanol (>95%, BDH, Poole, United Kingdom), pyridine (99%, Univar), 2-propanol (99.8%, Merck), acetone (>99%, Redox Chemicals, Minto, NSW, Australia), ethanol (99.8%, CSR Ltd., Yarraville, Australia), ethanolamine (≥99%, BDH), acetonitrile (99.9%, Lab-Scan Analytical Science, Bangkok, Thailand), methanol (99.8%, EM Science, Gibbstown, NJ, USA), ethylacetate (99.5%, Redox Chemicals) and triethylamine (≥99%, Riedel-de Haen, Castle Hill, NSW, Australia).

2.1. Vesicle preparation—extrusion method

Large unilamellar lipid vesicles were prepared via the extrusion method. In this method, 0.024 grams total of phosphatidylcholine plus cholesterol derivative were dissolved in 1 mL of chloroform. The chloroform was then removed via at least 2 h rotoevaporation at a slow rotation rate to form a thin lipid film on the walls of a roundbottom flask. Throughout the rotoevaporation the thermal bath was kept at a temperature above the main phase transition temperature of the phospholipid being used. The material was resuspended in 10 mL of buffer (preheated to a temperature above the main phase transition temperature of the phospholipid) by allowing the material to rotate at high rotation rate on the rotary evaporator (without vacuum) for 1 h. The buffer was 30 mM Tris, 150 mM NaCl, 1 mM EDTA, and adjusted to pH 7.2 using hydrochloric acid. 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/hydration and extrusion processes, temperatures were maintained above the known main transition temperatures for the different phospholipids used. After extrusion the final phosphatidylcholine concentrations were usually in the range 2–4 mM. These were determined using a Phospholipid C test kit from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Dye solutions

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 absorbance and fluorescence measurements, 30 μL of this ethanolic dye solution were added to 4 mL of vesicle solution. The ratio of the lipid to dye concentrations in these solutions is approximately 450–500. The solutions were vortexed for a few seconds and then allowed to remain at a temperature above the main phase transition overnight for dye aggregates to dissociate and for dye monomers to insert themselves into the lipid membrane. No significant changes in the maximum wavelength of fluorescence emission are observed after 3 h. The effects of small volumes of ethanol added to these solutions on the emission and excitation spectra were previously checked [12,29] and found to be negligible in both cases. Furthermore, essentially tripling the total dye concentration in the final solutions by adding 15 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 spectra, verifying that the dye is dilute enough that dye–dye interactions within the membrane are insignificant.

Dye solutions for the measurement of fluorescence and absorbance spectra in bulk solvents (excluding water) were prepared by adding a small quantity of solid dye to each solvent of interest. In the case of water, because of the dye's low solubility, 0.1 mL of the ethanolic solution described above was added to 5 mL of water, followed by a series of dilutions with water.

2.3. Absorbance measurements

Absorbance measurements were performed on a Shimadzu UV-2450 UV-visible spectrophotometer. Quartz semi-micro cuvettes were used for all measurements. Temperatures were maintained using a circulating water bath connected to the sample holder. For all spectra of di-8-ANEPPS in the presence of vesicles a reference cuvette containing vesicles at the same concentration was used for baseline subtraction in order to avoid any distortion of the absorbance spectrum via Rayleigh light scattering. Spectra were recorded a number of times to ensure reproducibility.

In the case of measurements of the maximum absorbance wavelength, λ_{Abs}^{max}, in bulk solvents, multiple dilutions were carried out to ensure that dye–dye interactions were not influencing the measured value.

2.4. Fluorescence measurements

Fluorescence measurements were performed on a Shimadzu RF-5301 PC spectrofluorophotometer. Quartz semi-micro cuvettes were used for all measurements. Temperatures were maintained using a circulating water bath connected to the sample holder. Emission spectra were uncorrected. All emission spectra were recorded over a range of excitation wavelengths in order to detect any excitation-dependent shifts in the position of the fluorescence emission maximum. The excitation wavelength had no effect on the wavelength of maximum emission of the dye in bulk solvents but did have an effect in membranes.

In the case of bulk solvents, dilutions of the dye were carried out as for the absorbance measurements to ensure that dye–dye interactions were not affecting the measured values of the wavelength of maximum emission, λ_{Em}^{max}. For the measurements in bulk solvents (excluding water) the excitation wavelength was set at 500 nm with a GG455 cutoff filter (Schott, Mainz, Germany) in the excitation path. In the case of dye in water, the excitation wavelength was 450 nm with a GG400 cutoff filter in the excitation path. For the measurements with lipids a GG420 cutoff filter was used in the excitation path.

3. Results

3.1. Solvent dependence of the Stokes shift

The maximum absorbance wavelength, λ_{Abs}^{max}, and the maximum fluorescence emission wavelength, λ_{Em}^{max}, of di-8-ANEPPS in a range of solvents of varying orientational polarisability were measured and the Stokes shifts of the dye in each solvent were calculated. The results are tabulated in Table 1. The orientational polarisability of each solvent was calculated according to Eq. (2) using tabulated values of their dielectric constants and refractive indices [30,31]. A linear fit of the Lippert equation (Eq. (1)) to the data (see Fig. 2) leads to the

Table 1
Stokes shift and wavelengths of absorbance, λ_{Abs}^{max}, and fluorescence emission, λ_{Em}^{max}, maxima of di-8-ANEPPS in solvents of varying orientational polarisability, Δf

Solvent	Δf	λ_{Abs}^{max} (nm)	λ_{Em}^{max} (nm)	Stokes shift (cm^{-1})
Chloroform	0.149	548	660	3116
Dichloromethane	0.218	532	680	4081
Hexanol	0.245	504	668	4858
2-Propanol	0.274	499	681	5372
Acetone	0.285	488	701	6237
Ethanol	0.289	500	694	5601
Ethanolamine	0.267	499	698	5691
Acetonitrile	0.308	489	710	6370
Methanol	0.309	498	704	5884
Water	0.320	456	653	6618

following equation of best fit: Stokes shift (cm^{-1})=19503 (±2000) Δf+207 (±542), with a correlation coefficient, r, of 0.922. Rearranging yields the following equation:

$$\Delta f = \frac{\text{Stokes shift } (cm^{-1}) - 207(\pm 542)}{19503(\pm 2000)} \quad (3)$$

Eq. (3) now allows the orientational polarisability of a membrane surface to be estimated from measurements of the Stokes shift of the probe in that membrane. It should be noted that the large uncertainties in Eq. (3) do not originate from uncertainties in the measured wavelengths, but rather reflect the fact that the model upon which it is based is general and represents a simplified view of solvent–dye interactions, treating every solvent molecule as a dipole and the fluorophore as being located within a spherical cavity. The model does not take into account differences in solvent molecular structure or any specific intermolecular interactions such as hydrogen bonding. It also does not take into account dipole-induced dipole interactions between the solvent and the fluorophore and vice-versa. Nevertheless, the fact that a relatively good correlation is observed between the Stokes shift and Δf (see Fig. 2) implies that for the solvents on which Fig. 2 is based, the general model of Lippert is a good approximation and there is no evidence for any specific solvent interactions. We did, however, observe that the solvents triethylamine and ethylacetate gave Stokes shifts which lay significantly off the line, possibly due to specific interactions with the dye in the excited state, and these were omitted from the linear regression analysis. The solvents 1,2-dichlorobenzene and pyridine also gave Stokes shifts which were higher than expected based on the line shown in Fig. 2. This could possibly be explained by dipole-induced dipole interactions between the fluorophore and the solvent. Both of these aromatic solvents have relatively mobile π-electrons, which results in a relatively high electronic polarisability (and refractive index) and hence a relatively strong dipole-induced dipole interaction. These two solvents were, therefore, also omitted from the linear regression analysis.

There is an additional reason for not including the aromatic solvents. In doing so, the solvent set used to construct the Lippert correlation is comprised solely of aliphatic compounds with permanent dipoles, many of which are also capable of hydrogen bonding. Thus, the solvent characteristics likely represent the type of environment that the dye experiences within the hydrated headgroup region of a lipid membrane. Furthermore, while the Lippert plot does not *explicitly* account for specific intermolecular interactions such as hydrogen bonding, the inclusion of several hydrogen bonding solvents within the training set may *implicitly* incorporate such effects, if operative, into the best-fit equation of the data. Thus, any effect of hydrogen bonding on the Stokes shift of the dye in the vesicles will be compensated for, at least in part, by the same effects present in the solvent set.

Based on the errors in the parameters in Eq. (3), the relative errors in the Δf values are all within the range ±11–12%. The precision of the trends in Δf described later between different lipid compositions and different cholesterol derivatives is, however, much higher, because the relative error in the Stokes shifts, which determine totally the difference between any pair of measurements, is only approximately ±1%.

It is important to point out that to calculate the true orientational polarisability of an environment using Eq. (3), the Stokes shift should correspond to that of the fully relaxed excited state. Strictly speaking this can only be determined from time-resolved fluorescence emission measurements because of the possibility that fluorescence emission could occur prior to the completion of solvent relaxation. Here we use the steady-state fluorescence emission spectrum as an approximation of the fully relaxed emission spectrum. This approximation is only valid under conditions where solvent relaxation goes virtually to completion during the fluorescence lifetime of the probe. If this is not the case, use of the steady-state Stokes shift in Eq. (3) will lead to an underestimation of the orientational polarisability.

3.2. Effect of lipid composition on orientational polarisability

The λ_{Abs}^{max} and λ_{Em}^{max} values of di-8-ANEPPS bound to lipid vesicles of different lipid compositions were measured and the Stokes shifts of the dye in each different lipid environment were calculated. The results are tabulated in Table 2. In some cases it was found that λ_{Em}^{max} and the Stokes shift were dependent on the wavelength of excitation. For this reason we report a range of

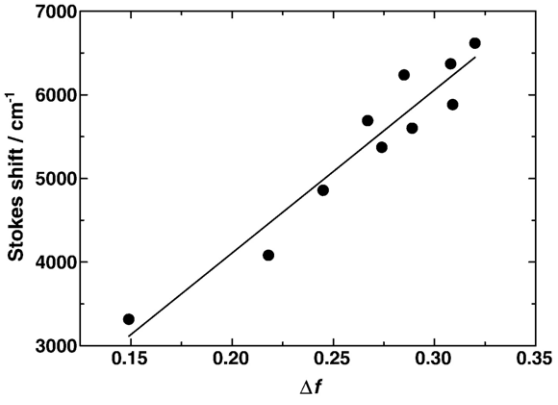

Fig. 2. Dependence of the Stokes shift, $\bar{\nu}_a^{sol}-\nu_f^{sol}$, of di-8-ANEPPS as a function of the solvent orientational polarisability, Δf (see Eq. (2)). The solvents used and the measured Stokes shifts are given in Table 1.

Table 2
Orientational polarisability, Δf, of phosphatidylcholine (PC) membrane surfaces based on the Stokes shift of the probe di-8-ANEPPS

PC [a]	λ_{Abs}^{max} (nm)	λ_{Em}^{max} (nm)	Stokes shift (cm^{-1})	Δf
12:0	465	608–609	5086–5104	0.250–0.251
14:0	463	599–601	4895–4946	0.240–0.243
16:0	464	603–608	4969–5120	0.244–0.252
18:0	464	610	5148–5157	0.253–0.254
18:1 cΔ^9/18:0	465	601–602	4847–4880	0.238–0.240
18:1 cΔ^9	469	603	4722–4748	0.231–0.233
18:2 c$\Delta^{9,12}$	472	609–610	4771–4798	0.234–0.235
18:3 c$\Delta^{9,12,15}$	475	614	4763–4772	0.234
20:4 c$\Delta^{5,8,11,14}$	477	614	4675–4679	0.229
Dihexadecyl	469	612–614	4980–5024	0.245–0.247

[a] The ester phosphatidylcholines have been given shorthand designations in the table based on the Δ-system [32]. For example, 18:1 cΔ^9/18:0 signifies a mixed chain diacylphosphatidylcholine with one chain having 18 carbons and a *cis* double bond linking the 9th and 10th carbons (counting from the carbon of the ester linkage) of the chain, whereas the second chain has 18 carbons and no double bonds.

λ_{Em}^{max} and Stokes shift values in Table 2. These values were determined by exciting the probe 20 nm either side of its wavelength of maximum absorbance, λ_{Abs}^{max}. Such behaviour has also been observed with other probes and is sometimes explained by the excited state relaxation not going 100% to completion on the timescale of the fluorescence lifetime of the probe [33,34]. A number of time-resolved fluorescence studies have shown that dyes of this type do undergo an excited state reaction in lipid membrane environments (as well as in micelles and viscous solvents), which is apparent as a 'growing-in' phase, particularly on the red edge of their emission [35–38]. This reaction has been attributed to solvent relaxation occurring simultaneously with the formation of a fluorescent twisted intramolecular charge transfer state (TICT). In the case of the related styrylpyridinium probe RH421, the degree of twist was estimated to be 9° [36] and the time constant of the 'growing-in' phase in dimyristoylphosphatidylcholine vesicles was determined to be 100 ps [35], which is around 20 times faster than the fluorescence lifetime of the probe's central-red emission of 1.7–1.9 ns [37]. If di-8-ANEPPS has similar fluorescence behaviour, it is very unlikely that the excitation wavelength dependent Stokes shifts are due to incomplete excited state relaxation, because over a fluorescence lifetime of close to 2 ns it can easily be shown if one assumes first order kinetics that the excited state relaxation should be extremely close to 100% complete. This can also be seen as justification for using the steady-state Stokes shift as an approximation of the fully relaxed Stokes shift in the calculation of the orientational polarisability.

Another possible explanation of excitation wavelength dependent Stokes shift is different probe locations within the membrane, i.e. ground state heterogeneity. The time-resolved fluorescence decays of the related styrlpridinium probe RH421 were found to be very heterogeneous [37], with at least four exponential time functions being necessary to obtain an adequate fit to the data. This is a strong indication of ground state heterogeneity. Time-resolved studies using di-8-ANEPPS would be required in order to resolve these issues. However, based on the studies performed using related dyes it would seem likely that ground state heterogeneity is the major cause of the wavelength-dependent Stokes shifts observed here. A number of other fluorescent membrane probes have already been shown to distribute between different locations within lipid bilayers [39]. In the case of di-8-ANEPPS, the probe's hydrophilic

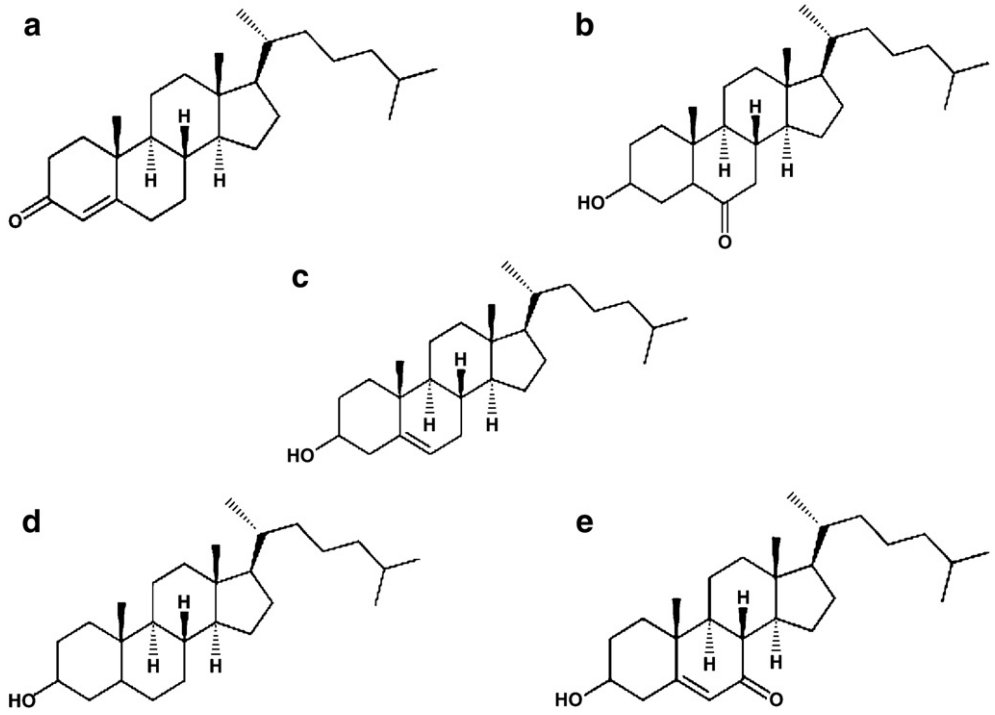

Fig. 3. Chemical structures of cholesterol and its derivatives. (a) 4-Cholesten-3-one, (b) 6-ketocholestanol, (c) cholesterol, (d) coprostanol, and (e) 5-cholesten-3β-ol-7-one.

sulphonate group and its hydrocarbon chains act as anchors in the membrane–aqueous solution interface and the membrane hydrocarbon interior, respectively, and this should limit translational movement into or out of the bilayer, but rotational motion (within a cone with its axis perpendicular to the membrane surface) may still be possible. This could also lead to differences in Stokes shift, because there is likely to be a steep gradient in orientational polarisability going from the membrane interface into its interior. Time-resolved fluorescence anisotropy studies [37] have already been carried out on RH421 in dimyristoylphosphatidylcholine vesicles which would support such an idea. The rotational diffusion of the probe was described by a wobbling in a cone model [40] with a cone angle of 48–50° relative to the membrane normal.

From the results shown in Table 2 it can be seen that the orientational polarisabilities of the saturated phosphatidylcholine membranes are consistently greater than those of unsaturated phosphatidylcholines. It is not possible, however, from the data presented here to discern any clear trend in the effect of chain length on orientational polarisability.

3.3. Effect of cholesterol derivatives on orientational polarisability

The λ_{Abs}^{max} and λ_{Em}^{max} values of di-8-ANEPPS bound to dimyristoylphosphatidylcholine (DMPC) lipid vesicles containing different mole percentages of cholesterol and several of its oxidized derivatives (see Fig. 3) were also measured and the Stokes shifts of the dye in each different lipid environment were calculated. From the Stokes shifts the orientational polarisability was calculated according to Eq. (3). The orientational polarisability as a function of the mol% of each cholesterol derivative is shown in Fig. 4.

From the results shown in Fig. 4 it can be seen that the effects on the orientational polarisability varied significantly between the different cholesterol derivatives. Cholesterol, coprostanol and cholesten-3β-ol-7-one all cause a decrease in orientational polarisability compared to pure DMPC vesicles. 6-Ketocholestanol causes only a slight decrease up to 40 mol% and then a small increase. 4-Cholesten-3-one causes a small gradual increase over the concentration range studied. The reason for these differences in behaviour will be discussed later.

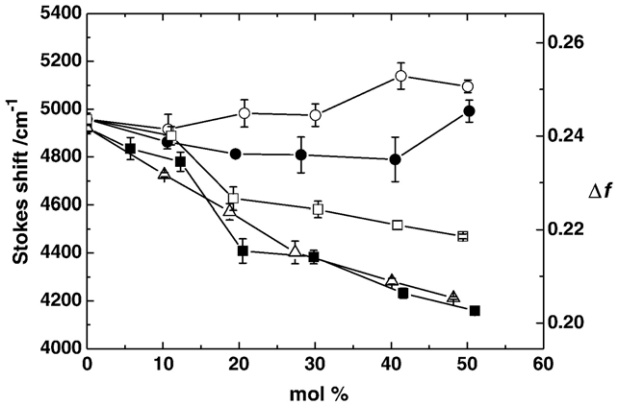

Fig. 4. Effect of cholesterol derivative concentration on the Stokes shift of di-8-ANEPPS and the orientational polarisability, Δ*f*. [di-8-ANEPPS]=6.4 μM, [DMPC]=3 mM, 30 °C. ●=6-ketocholestanol, ○=4-cholesten-3-one, ■=cholesterol, □=coprostanol, Δ=cholesten-3β-ol-7-one. Each individual point corresponds to a separate vesicle preparation at the given DMPC and cholesterol compositions. The bars on each point represent the range of the Stokes shifts measured by exciting 20 nm either side of the wavelength of the absorbance maximum.

4. Discussion

Here we have found that the fluorescent probe di-8-ANEPPS shows a linear dependence on the orientational polarisability of its environment, as predicted by the Lippert equation (Eq. (1)), and have calibrated it for use as a probe of the orientational polarisability of lipid membrane surfaces. Measurements using lipid vesicles composed of phosphatidylcholine of varying saturation demonstrated that the orientational polarisability is greater for saturated compared to unsaturated lipids. The highest orientational polarisability was found for distearoylphosphatidylcholine (PC 18:0) with a value of 0.254. The lowest value was found for diarachidonylphosphatidylcholine (PC 20:4) of 0.229. These values are comparable to the orientational polarisability of octanol (0.226), which would seem to support the wide use of octanol–water partition coefficients in modelling drug–membrane binding and permeability [41]. Now let us consider how the dependence of orientational polarisability on hydrocarbon chain saturation can be rationalised.

The head group region of the lipid membrane contains dipolar groups which orient themselves to produce a dipole potential, ψ_d, positive in the membrane interior. The magnitude of the dipole potential is a reflection of the strength the dipole moment perpendicular to the membrane surface associated with each lipid molecule, $\mu_\perp$, and the density with which they are packed, $1/A$, where A is the area per lipid molecule in the surface.

Estimates of the value of ψ_d for the lipids studied here are already known from other studies using di-8-ANEPPS, which has previously been established as a probe of the dipole potential via excitation ratiometric measurements [11–14]. In Fig. 5 a plot of the orientational polarisabilities measured here against the corresponding values of the dipole potentials is shown. For the ester-linked phosphatidylcholines one observes the general trend that the orientational polarisability increases in parallel with the dipole potential. This makes sense in terms of the Debye equation [42], which describes the dependence of the orientational polarisability of an isotropic ideal gas of dipoles on the dipole moment of the gas molecule, μ, and the absolute temperature, T:

$$\Delta f = \frac{\rho N_A}{3M\varepsilon_0}\frac{\mu^2}{kT} \tag{4}$$

where ρ is the density of the gas, M is its molar mass, N_A is Avogadro's constant and k is Boltzmann's constant. In the case of an isotropic solvent in which the dipole moments of all solvent dipoles are influenced by the electric field of their

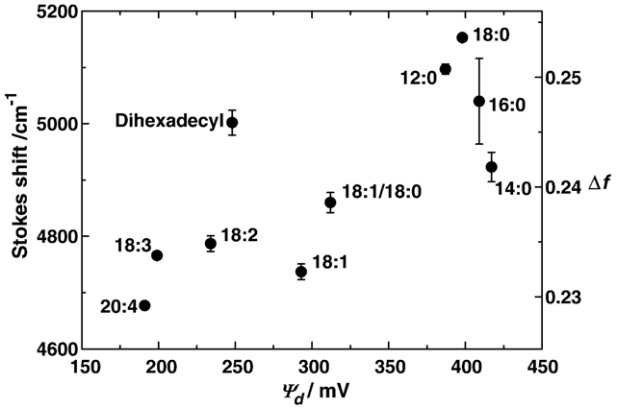

Fig. 5. Plot of the Stokes shift of di-8-ANEPPS and the orientational polarisabilities, Δf, of different phosphatidylcholine vesicle surfaces (from Table 2) versus their corresponding dipole potentials, ψ_d (from data in [13] and [14]). The bars on each point represent the range of the Stokes shifts measured by exciting 20 nm either side of the wavelength of the absorbance maximum.

neighbouring solvent molecules, a more complex theory due to Kirkwood [43] applies. However, for the qualitative purposes of this discussion, the Debye equation suffices. From Eq. (4) it can be seen that if the effective dipole moment of the membrane surface increases, then naturally the orientational polarisability, which comes about from the reorientation of the dipoles, must also increase. Therefore, an increase in orientational polarisability occurring in parallel with an increase in dipole potential can easily be rationalised in this manner. In fact the Debye equation (Eq. (4)) predicts a quadratic dependence of Δf on μ.

The result obtained for the ether lipid dihexadecylphosphatidylcholine is somewhat surprising. Although this lipid has quite a low dipole potential, its orientational polarisability is relatively high, comparable with that of the fully saturated ester-linked phosphatidylcholines. The reason for this is not clear at this stage. One interpretation could be that although the effective dipole moment of its surface is low, its dipolar groups might have greater freedom of movement than those of ester-linked phosphatidylcholines. The results of Hutterer et al. [44], who compared time-resolved fluorescence emission spectra of the probe PATMAN bound to ester- and ether-phosphatidylcholines, would support this idea. They concluded from their studies that ether linkages allow greater water penetration into the glycerol backbone region and faster solvent relaxation.

When the mole percentage of cholesterol or one of its derivatives was increased, it was found that the effect on the orientational polarisability varied significantly between the different cholesterol derivatives, some producing an increase whereas others (including cholesterol) caused a decrease (see Fig. 4). In a previous study we found that the effect of the same cholesterol derivatives on the membrane dipole potential also differed markedly [15]. These are reproduced here for comparison (see Fig. 6). Up to approximately 40 mol%, it was found that there was an increase in dipole potential for 6-ketocholestanol, 4-cholesten-3-one, cholesterol and coprostanol, but cholesten-3β-ol-7-one caused a decrease in dipole potential. These different effects of the cholesterol derivatives were explained based on quantum mechanical calculations [15] by their different molecular structures, which result in differences in their dipole moment components perpendicular to the membrane surface. The differences in their dipole moment are determined predominantly by the positions of their hydroxyl and carbonyl groups which vary in the different derivatives (see Fig. 3). Therefore, based on the strength of the dipole moment alone, one might have expected based on the Debye equation (Eq. (4)) that the effects of each of these derivatives on the orientational polarisability would follow the same pattern as that already observed for the dipole potential. Comparison of the results in Figs. 4 and 6 shows, however, that the effects are quite different. 5-Cholesten-3β-ol-7-one is the only cholesterol derivative which causes a decrease in both the orientational polarisability and the dipole potential with increasing mol%. 6-Ketocholestanol and 4-cholesten-3-one both cause a large increase in dipole potential, but only a small increase in orientational polarisability at high mol% values. Cholesterol and coprostanol cause an increase in dipole potential up to 40 mol%, but a decrease in the orientational polarisability. It must be remembered, however, that the Debye equation only applies for an isotropic gas. For an anisotropic medium such as a membrane, the orientational polarisability depends not only on the strength of the electric field arising from the dipoles but also on their degree of movement (not to be confused with their speed of movement), i.e. the angle through which they can move. Deuterium NMR [45] and fluorescence anisotropy [40,46] studies have firmly established that in the liquid crystalline state, cholesterol significantly increases the order of the phospholipid's hydrocarbon chains. It is also known from monolayer studies [47,48] that cholesterol has a condensing effect, i.e. increasing the density of phospholipid packing. It is, therefore, very likely that the cholesterol-induced increase in packing density would also restrict the degree of movement of the dipoles associated with the lipid headgroups, leading to a reduction in the orientational polarisability. The fact that experimentally, cholesterol decreases the membrane orientational polarisability while increasing the dipole potential,

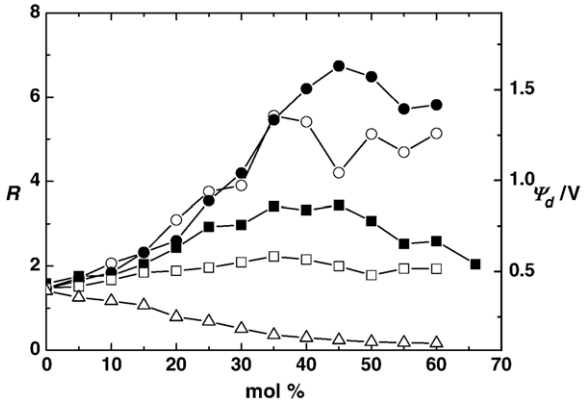

Fig. 6. Effect of cholesterol derivative concentration on the fluorescence excitation ratio, R, of di-8-ANEPPS and the dipole potential, ψ_d (reproduced from [15]). [di-8-ANEPPS]=5 μM, [DMPC]=3.6 mM, 30 °C. ●=6-ketocholestanol, ○=4-cholesten-3-one, ■=cholesterol, □=coprostanol, Δ=cholesten-3β-ol-7-one. Each individual point corresponds to a separate vesicle preparation at the given DMPC and cholesterol compositions.

implies that cholesterol's effect in restricting the degree of movement (i.e. increasing membrane order) dominates over its effect on the dipole strength. Supporting this interpretation of antagonistic effects of dipole strength and membrane order are the observations that the changes in orientational polarisability are the least for 6-ketocholestanol and 4-cholesten-3-one, which cause the greatest increase in dipole potential (see Fig. 6). For these two derivatives it would seem that the increase in dipole potential is compensated by a reduction in degree of movement, leading to little overall change in the orientational polarisability. In the case of cholesterol and coprostanol, it appears that a decrease in the degree of movement overcompensates for these derivatives' smaller effects on the dipole potential, resulting in a reduction in orientational polarisability. Finally, 5-cholesten-3β-ol-7-one causes a decrease in dipole potential [14], which itself would result in a decrease in orientational polarisability, as experimentally observed, and any restriction in the degree of movement that this derivative causes would simply enhance the effect.

It is interesting now to discuss the results presented here within the context of the activity of an ion pump. As is the case for chemical reactions in homogeneous solvents, where it is very difficult to attribute changes in reaction rates to a single solvent parameter (e.g. dielectric constant, dipole moment, refractive index, etc.) [49], it is hard to imagine that one individual physical parameter could explain all of the kinetic effects of changes in lipid composition on the kinetics of an ion pump. Ion pumps would no doubt be influenced by both the electrical properties of the membrane and by steric factors, e.g. lipid packing, order and membrane thickness. Orientational polarisability is an interesting parameter, however, firstly because, as discussed above, it incorporates in one parameter both electrical and steric effects, and secondly one can clearly understand, based on dipole reorientation and transition-state theory, how it might affect pump kinetics. The pumping of ions across a membrane against an electrochemical potential gradient relies critically on the formation of unstable enzyme intermediates which can only relax to a lower energy state if they release ions to the side of the membrane with the higher electrochemical potential for that ion [50,51]. If the surrounding lipid could stabilise such enzyme intermediates via dipole reorientation, this would obviate the necessity for ion transfer and the efficiency of ion pumping could be significantly reduced. An estimate of the possible degree of energy stabilisation which lipid reorientation could cause can be calculated based on the results presented here. In the case of a fully saturated phosphatidylcholine a typical Stokes shift of di-8-ANEPPS was found to be around 5000 cm^{-1} (see Table 2) which corresponds to an energy of 60 kJ mol^{-1}. In comparison, the free energy released by ATP hydrolysis via the Na^+,K^+-ATPase is approximately 56 kJ mol^{-1} [50], i.e. both are of a very similar magnitude. Therefore, unless high energy intermediates of ion pump proteins are shielded from the surrounding lipid by the protein matrix and/or the surrounding lipids have a particularly low orientational polarisability, it is entirely feasible that lipid reorientation could dissipate the entire free energy of ATP hydrolysis and totally inhibit ion pumping. Whether such a mechanism has physiological relevance awaits further experimental study.

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