

Quantitation of protein orientation in flow-oriented unilamellar liposomes by linear dichroism

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Abstract

The linear dichroism of the visible wavelength transitions of retinal have been used to analyse linear dichroism spectra to determine the orientation of aromatic and peptide structural motifs of Bacteriorhodopsin incorporated into unilamellar soy bean liposomes. The results are consistent with the available X-ray data. This proves that visible light absorbing chromophores can be used to analyse linear dichroism data to give the orientation of membrane proteins in membrane mimicking environments. The work has been extended by screening a wide range of hydrophobic molecules with high extinction coefficients in transitions above 300 nm to find molecules that could be used as independent probes of liposome orientation for experiments involving proteins incorporated into liposomes. Three probes were found to have potential for future work: bis-(1,3-dibutylbarbituric acid)pentamethine oxonol (DiBAC₄), retinol and rhodamine B. All three can be used to determine the orientation of the porphyrin of cytochrome *c*, the aromatic residues of gramicidin and the helices of both proteins. The orientation parameter, *S*, for the liposomes varied from batch to batch of unilamellar liposomes prepared by extruding through a 100 nm membrane. The value and variation in *S* was 0.030 ± 0.010 . Repeat experiments with the same batch of liposomes showed less variation. Film *LD* data were measured for DiBAC₄ and rhodamine B to determine the polarisations of their long wavelength transitions.

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1. Introduction

Processes taking place on or in lipid bilayer membranes are essential for biological systems, for example, for translocation of molecules into and out of cells and membrane-bound receptor activation. The understanding of inter-molecular interactions within the membrane, between the lipids themselves, between lipids and other species (proteins, drugs, and ions), and between membrane-bound molecules are all important. With the majority of future drug targets being anticipated to be membrane proteins,

knowing the locations and orientations of molecules bound to the membrane is an extremely important goal. However, to date no simple experimental method has been established to achieve this for membrane-bound proteins and little work has been done with small molecules. Ardhammar et al. [1] showed that liposomes (spherical bilayer systems) could be oriented by Couette flow and then linear dichroism (*LD*, the differential absorbance of light polarised parallel and perpendicular to an orientation direction) used to probe the orientation of small molecules in membranes. We extended this work by showing that the orientations of proteins both in (e.g. gramicidin) and on the surface of membranes (e.g. a thylakoid membrane protein, pre-PsbW, and cytochrome *c*) could be probed qualitatively using Couette flow linear dichroism [2,3]. Although *LD* only gives an

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average orientation, whereas other techniques such as X-ray or NMR can give specific information about the atoms in the structure, the LD method has the great advantage of being able to be readily performed on almost any system one might wish to study.

The reduced linear dichroism, LD^r , of a chromophore oriented in a liposome has been shown to be [1,3]:

$$LD^r = \frac{LD}{A} = \frac{A_{\parallel} - A_{\perp}}{A} = \frac{3S}{4}(1 - 3\cos^2\beta) \quad (1)$$

where LD is the measured LD signal, A is the isotropic absorbance of the same sample in a cell of the same path-length, A_{\parallel} is the absorbance of light polarised parallel to the orientation direction, S is the so-called orientation parameter ($= 1$ for full orientation and 0 for random orientation) and β is the angle between the chromophore's transition moment and the normal to the liposome surface (i.e. the average lipid direction) (Fig. 1). The quantitative interpretation of the LD spectra of membrane-bound proteins following, e.g., Ref. [4] has proved challenging due mainly to two factors: (i) the sloping baseline introduced by light scattering and (ii) the fact that we have not been able to tell the degree of orientation of the liposomes [1–3,5]. Ardhammar et al. used sucrose to match the refractive indices of the liposomes and the aqueous solution around the liposomes to reduce the light scattering. In our newly developed capillary Couette flow LD cell [6], whose light scattering is intrinsically lower than that of traditional Couette cells, we found this did not improve the signal to noise and at wavelengths below 210 nm the sucrose absorbance precluded measurements – thus rendering this approach inapplicable to proteins.

Our capillary LD cell is based on a rotating quartz capillary, sealed at one end, as the sample holder. A stationary quartz rod is inserted into the capillary to create the shear force and focusing lenses before and after the sample ensure the light is incident on the sample and then photomultiplier tube [6]. With this cell we have significantly reduced the scattering artefacts in a range of samples including fibrous proteins, liposomes and carbon nanotubes [7–10]. We have also found that further progress can sometimes be made following the methods of Nordén, Nordh, and Mikati [11] to correct for light scattering. Thus, while the issue of light scattering with liposome samples is still a problem, it is no longer dominant.

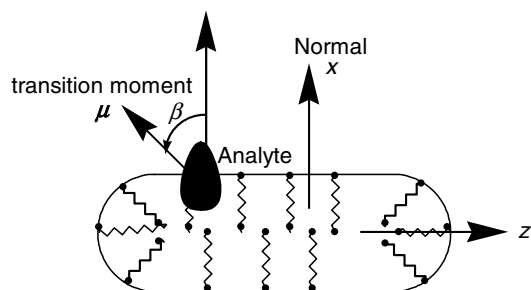


Fig. 1. Schematic illustration of an analyte in a shear-deformed liposome.

A variety of probe molecules and different techniques have been used to determine the lipid vesicle orientation factor. Ardhammar et al. used light scattering of flow-oriented liposomes to gauge the degree of orientation [1]. They also used $\text{Ru}(\text{phen})_2\text{dppzcpCOOCH}_3$ as a probe molecule to determine the S factor [12]. A further estimate of degree of orientation of the membrane was obtained by Brattwall et al. using penetratin [13]. Castanho and Lopes used Nystatin A_1 and Amphotericin B which belong to a polyene antibiotic family as probe molecules to study the orientational distribution function in a lipid bilayer film [14]. None of these approaches have been developed into a routine method for assessing the orientation of a protein molecule in cell-membrane mimicking environments.

The focus of the work reported in this paper is therefore to address the question of ‘what is the orientation parameter of the liposomes?’ The ideal would be an internal ‘standard that can be added to all samples to give a direct read-out of the orientation parameter, in much the same way as DNA bases can be used for determining the orientation on DNA of ligands, e.g. [4].

In our work, we have considered a range of liposome preparation methods and a series of probe molecules, aiming to find one to quantify the liposome orientation and consequently the protein orientation in the liposomes: if a probe molecule of known transition polarisation is inserted into liposomes, using Eq. (1) its LD could be used to estimate the S factor of the shear-deformed liposomes, and consequently the angle β between the protein's (or any other analyte's transition moments) and the lipids could be determined. Such a probe molecule must have a number of characteristics: it should bind to the membrane (rather than stay free in the aqueous solution); it should absorb outside the protein's absorption region (and very little within it); it should have a high extinction coefficient; it should not interact with the protein; and it should have the least possible effect on the protein's LD signal.

2. Materials and methods

2.1. Materials

All reagents, including soy bean lipid ($L\text{-}\alpha\text{-Lecithin}$, Type IV-s: from soybean approx. 40% (TLC)), Gramicidin D (from *Bacillus brevis*) and bacteriorhodopsin (from *Halo-bacterium salinarum* strain S9, lyophilised powder) were obtained from Sigma Chemical Company and were used as received except for bis-(1,3-dibutylbarbituric acid)pentamethine oxonol (DiBAC₄) which was purchased from Molecular Probes; undecylprodiginine which was kindly provided by Dr. G.L. Challis and Dr. Christophe Corre, University of Warwick; methanol and ethanol (analytical grade) which were obtained from BDH laboratory supplies; and chloroform which was purchased from Fisher Scientific. Polycarbonate membranes of 100 nm pore size were purchased from Avestin Inc.

2.2. Equipment

UV–Visible absorbance spectra were recorded using a Jasco V-550 or Cary IE spectrophotometer and circular dichroism (CD) with a Jasco J-715 spectropolarimeter using a 1 mm pathlength quartz cell. Flow linear dichroism spectra were recorded using a Jasco J-715 circular dichroism spectropolarimeter, which was adapted for flow *LD* measurements, and a 0.5 mm pathlength quartz capillary *LD* cell [6]. (Note the factor of two the difference in absorbance and *LD* pathlengths requires in any calculation of LD^r .) The *LD* cell is a cylindrical capillary flow cell, consisting of two coaxial cylinders, one of which is a capillary and the other is a quartz rod. There is a 0.25 mm annular gap between the rotating capillary and the stationary rod where the solution of interest is subjected to a constant flow, and hence viscous drag, by rotating the capillary at a speed large enough to cause significant orientation but not a turbulent flow (in our case 3000 rpm).

Absorbance and CD baselines were collected in the same cuvette using a liposome preparation identical to that of the corresponding sample but without the analytes. For *LD*, the best baseline to subtract from a recorded spectrum (as judged by the least scattering baseline in the final spectrum) was usually that of the sample in the same cell but without rotation. Alternatively, a spectrum of a rotating liposome solution was used as the baseline.

2.3. Liposome preparation

In this work liposomes were prepared by three different methods: the vortex method, the liquid-nitrogen method and the sonication method.

1. *Vortex method*: Aqueous buffer solution (e.g. 4 mL of 5 mM phosphate buffer, pH 7) was added to L- α -phospholipidylcholine lipids (e.g. 10 mg) to obtain a lipid concentration of 2.5 mg/mL. The sample was vortexed to obtain multilamellar liposomes and then extruded 11 times using a Liposofast Basic Extruder (Glen Creston Ltd.) with a polycarbonate membrane (100 nm pore size) to obtain unilamellar liposomes [15].
2. *Nitrogen method*: Chloroform (2 mL) or chloroform and methanol (2 mL and 1 mL, respectively) were added to L- α -phospholipidylcholine lipids (2.5 mg) and left overnight under a stream of nitrogen. The resulting dry lipid film was vortexed with phosphate buffer solution (1 mL, 5 mM, pH 7) to obtain multilamellar liposomes, then extruded 11 times using a Liposofast Basic Extruder with a polycarbonate membrane (100 nm pore size) to obtain unilamellar liposomes, and finally frozen using liquid nitrogen (-196°C) and thawed under running tap water (4 times).
3. *Sonication method*: L- α -Phospholipidylcholine lipids (2.5 mg) were placed in a vial with a mixture of organic solvents and was sonicated for 10 s at ambient tempera-

ture. The sample was left overnight under a stream of nitrogen. The resulting dry lipid film was vortexed with 1 mL of phosphate buffer (5 mM, pH 7).

2.4. Analyte introduction

The probe molecules and proteins were sometimes introduced to liposome solutions by adding dropwise an aqueous buffered liposome solution (4°C) to a concentrated solution of the analyte (also at 4°C) dissolved in an appropriate (water miscible) solvent. For samples containing probes and proteins, they were usually added in this order. Cytochrome *c* was added as a solid. Alternatively, the nitrogen method was used, with the analyte being added to the organic solution of lipid. If required, samples were subsequently diluted by adding either buffer or aqueous solutions of unilamellar liposomes. In most cases, samples were left overnight to equilibrate.

2.5. Film *LD* sample preparation

Film *LD* samples were prepared [4] by dissolving (heating to near boiling) low molecular weight polyvinyl alcohol (PVA) (0.48 g) in water (4.8 mL). For the sample films the viscous solution was allowed to cool before a saturated methanolic solution of the probe (0.2 mL) at an appropriate concentration (e.g. 0.1 mM for rhodamine B) was added. The mixture was cast onto a glass plate and allowed to dry in the dark over a period of two days. The dry film was removed from the plates with the aid of a scalpel and stretched in a mechanical film stretcher by a factor of two under heat from a hair dryer ($\sim 60^\circ\text{C}$). *LD* and normal absorption were recorded where the wavelength range and data interval of the two instruments were set to correspond. A control film was prepared by adding 0.2 mL of methanol in the place of probe solution.

3. Results and discussion

LD data for bacteriorhodopsin which has retinal as a convenient internal probe are described first followed by the results for a range of potential small molecule probes for determining the orientation of liposomes in a Couette flow cell. Finally, some data from selected probes together with the model proteins gramicidin and cytochrome *c* are described.

3.1. Bacteriorhodopsin orientation in liposomes

Bacteriorhodopsin (BR) is found in the purple membrane of Halobacteria that live in salt marshes [16,17], it is a 248 residue protein of mass 26,000 Da and includes a retinal chromophore (Fig. 2) covalently bound to a lysine. Each BR has 7 transmembrane helices, 3 of which in the crystal have their axis at $\sim 70^\circ$ to the lipids and the remaining 4 are parallel to the lipids [18–21]. The long axis of the

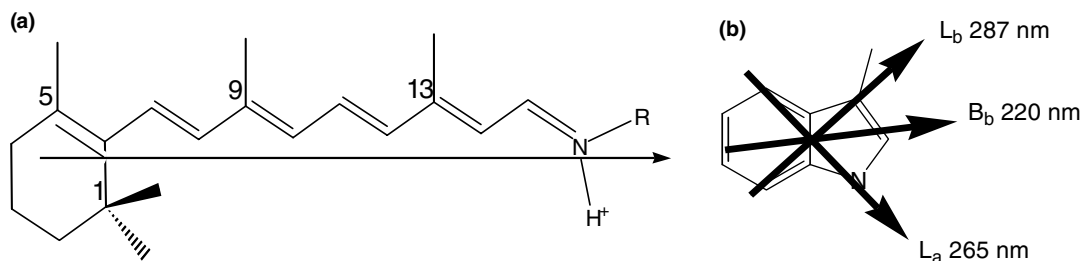


Fig. 2. (a) All-trans retinal converted to the Schiff base. The vector represents the transition dipole moment of the 570 nm transition of retinal [18]. (b) Tryptophan and its transition polarisations [23].

retinal lies at $\sim 69^\circ$ to the lipids in the crystal. An FTIR study on a dried suspension of BR in lipids gave an average angle of $90 \pm 20^\circ$ between the retinal plane and the plane of the purple membrane with the $C_9=C_{10}$ (Fig. 2) double bond of retinal being nearly parallel to the plane of the membrane (this makes it $\sim 30^\circ$ between the retinal long axis and the average lipid). BR is known to insert into liposomes in a single orientation [22].

The absorption, CD and LD spectra of bacteriorhodopsin in liposomes are given in Fig. 3. The absorption spectra (Fig. 3(a)) are consistent with those reported in the literature for the initial state of BR [24]. The absorption maximum at ~ 570 nm is due to a long axis polarised transition of the retinal chromophore. The broad peak in the near UV region (260–290 nm) is due to the transitions of the protein aromatic side chains phenylalanine, tyrosine and tryptophan and the peak observed in the far UV region (215 to ~ 230 nm) is due to the peptide $n \rightarrow \pi^*$ transition of the amide groups. The backbone CD spectrum of BR (Fig. 3(b)) is in accord with the CD spectrum for a highly α -helical protein as expected [25,26].

A peak at ~ 570 nm is observed in the LD spectrum of BR (Fig. 3). Taking the literature value of the orientation of the long axis of retinal with respect to the lipids of a lipid

bilayer (69°)⁹⁶ and the value for $LD'_{570 \text{ nm}}$ of 0.022 (from the Fig. 3(a) spectra, noting that the absorbance pathlength is twice that of the LD cell), it follows from Eq. (1) that $S \sim 0.048$. This value varies by about 5–10% for independently prepared samples with the same batch of liposomes and by up to 30% for different batches of the same average size liposomes.

The aromatic region (260–280 nm) of the LD spectrum is dominated by the indole chromophore of the tryptophan (W) residues [23]. Contributions from L_a (270 nm) and L_b (287 nm) (Fig. 3(a)) transitions are apparent, both showing positive LD. Using $S \sim 0.048$ and $LD'_{287 \text{ nm}} = 0.0076$ it follows from Eq. (1) that $\beta(L_a) \sim 60^\circ$. Similarly, $\beta(L_b) \sim 65^\circ$. Thus, the LD indicates that the average W is tilted so that the normal to the plane of the W is $\sim 40^\circ$ from the average lipid and the long and short axes are both at an angle of 60–65° to the lipids. This is consistent with the fact that the retinal is sandwiched by tryptophan residues in the X-ray structure [25].

The protein backbone LD spectrum shows a positive maximum at 220 nm ($n \rightarrow \pi^*$) and a negative maximum at ~ 213 nm ($\pi \rightarrow \pi^*$). Again using $S \sim 0.048$, it follows that the $n \rightarrow \pi^*$ transition (which is polarised perpendicular to the α -helix long axis) is at $\sim 58^\circ$ [3] ($LD'_{220 \text{ nm}} = 0.006$) from

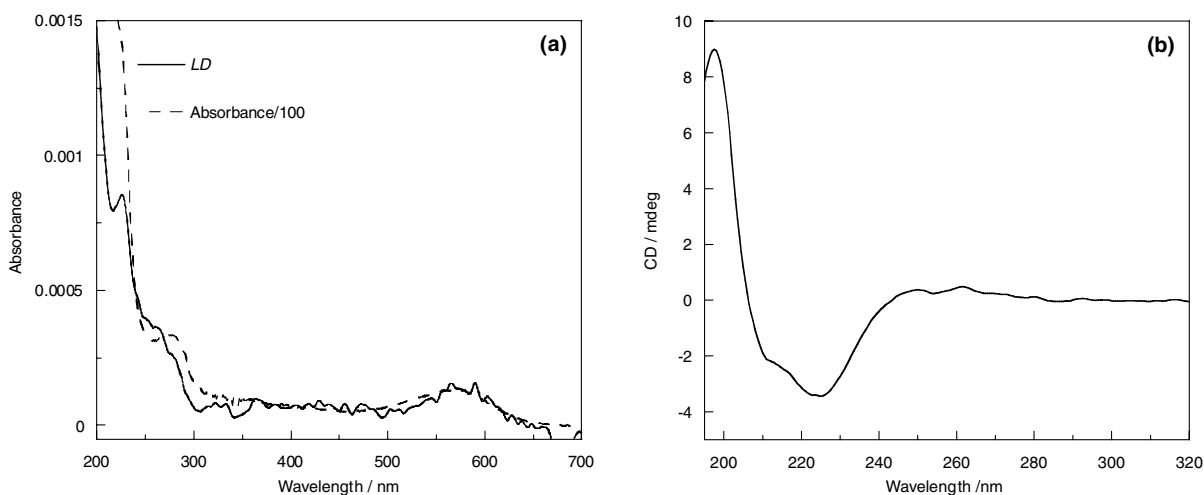


Fig. 3. Spectra of bacteriorhodopsin (0.2 mg/mL) added to a liposome solution (0.5 mg/mL): (a) Absorption (dashed line, 1 mm pathlength, baseline: liposome absorption spectrum) and LD (solid line, 0.5 mm pathlength, baseline: LD spectrum of sample without rotation spectrum); (b) CD (1 mm pathlength, baseline: liposome CD spectrum).

the average lipid direction. Thus, the average orientation of the transmembrane helices is $\sim 30^\circ$ from the membrane normal (Fig. 4). Our average value suggests that the protein is less rigidly held in a liposome than when dried or crystallised, which is entirely reasonable. Thus, we can conclude that a covalently bound internal probe of a membrane protein can be used semi-quantitatively to analyse the orientation of protein chromophores in liposomes.

3.2. Independent probes to determine liposome orientation

Very few proteins have unique spectroscopically isolated internal probes of known orientation with respect to the rest of the protein, such as retinal in BR. Having proved the concept of using a chromophore with visible spectroscopy to probe protein motif orientation our aim was therefore to find one or more probe molecules that could be added to a liposome preparation to give an orientation read-out for other proteins. The holy grail is a molecule with no spectroscopic signature in the protein region, 100% membrane affinity (so there is no probe in the aqueous part of the solution contributing to the absorbance but not the LD), no precipitation from an aqueous liposome solution, and no perturbing effect on the membrane environment.

Potential probe molecules were screened for their ability to give an LD spectrum when added to an aqueous liposome solution following any of the preparation methods outlined above. From over 30 candidates, 7 (Fig. 5) were selected for further study on the basis of our ability to get them into solution and bound to liposomes: Amphotericin B (AmB), hemin, undecylprodigine, DiBAC₄, retinol, protoporphyrin and rhodamine B. AmB, a polyene antibiotic used in the medical treatment of fungal infections [27], was prepared by adding an aqueous liposome solution dropwise to a concentrated DMSO solution of AmB. Hemin, whose visible absorbance has two non-degenerate components of the Soret band at ~ 400 nm, was similarly prepared from a concentrated methanolic solution as was the red coloured antibiotic undecylprodigine (whose linear form was obtained from a cell extract using HPLC [28]). Bis-(1,3-dibutylbarbituric acid)pentamethine oxonol (DiBAC₄), which is used as a probe for ion channels and

carriers in lipid membranes, has a very high extinction coefficient ($160,000 \text{ mol dm}^{-3} \text{ cm}^{-1}$) in the visible [29]. It was simply added to liposome solutions from methanol. Retinol was dissolved in isopropanol. Protoporphyrin samples were prepared by the nitrogen/freeze-thaw method as it was not sufficiently soluble in a water miscible solvent. rhodamine B samples were also prepared in this manner as this method seemed to give better spectra.

The absorbance and LD of each of these probes when in solution with unilamellar soy bean liposomes are given in Fig. 6. AmB, hemin, undecylprodigine and protoporphyrin were eliminated from our search for the ideal probe for different reasons. AmB absorbance and LD signals in liposome solution are not proportional to its concentration in the solution. Gruszecki et al. have previously concluded that AmB adopts two different orientations when bound to a lipid bilayer [27] and we presumably are seeing a concentration dependence of the occupancy of the two binding modes. In any case it renders AmB unsuitable for use as a generic probe. However, AmB does have a sharp peak at 325 nm and a fairly flat spectrum between 200 and 300 nm. In addition it does co-incorporate into the membranes with proteins (data not shown). So there may be instances where it could be used. Hemin proved to absorb too much at 200 nm to be suitable and protoporphyrin has a complicated spectrum with significant intensity below 300 nm. Undecylprodigine was also eliminated because it stuck to the quartz of cuvettes and LD cell capillaries. The remaining 3 probes: DiBAC₄, retinol and rhodamine B have LD intensities proportional to their concentration (Fig. 7). Their LD' spectra are given in Fig. 6.

DiBAC₄, retinol and rhodamine B all have potential as liposome orientation probes. To determine how they are oriented on or in the liposomes one needs to know their own transition polarisations. Retinol is available from the literature: its long wavelength visible transition is along its long axis as illustrated in Fig. 2. For DiBAC₄ and rhodamine B it was necessary to measure film LD spectra (Fig. 8). For DiBAC₄ in a PVA film, $LD' = 0.48$ at 509 nm and 0.83 at 602 nm (Fig. 8). In a film, DiBAC₄ can be treated as a uniaxially oriented 'rodlike' molecule [4]. So

$$LD' = \frac{LD}{A} = \frac{3}{2}S(3 \cos^2 \alpha - 1) \quad (2)$$

where S is the orientation parameter and α the angle between the orientation direction and the transition moment of interest. We assume the maximum LD' is related to the orientation parameter according to

$$LD'_{\max} = 3S_z \quad (3)$$

As DiBAC₄ transition polarisations are almost determined by symmetry to be either long or short axis polarised, it is reasonable to conclude that the 602 nm transition lies along the long axis of DiBAC₄ as it has the largest LD' signal. This gives $S = 0.28$, or more strictly, $S \geq 0.28$ (allowing for the fact the molecule is not quite of D_{2h} symmetry). It

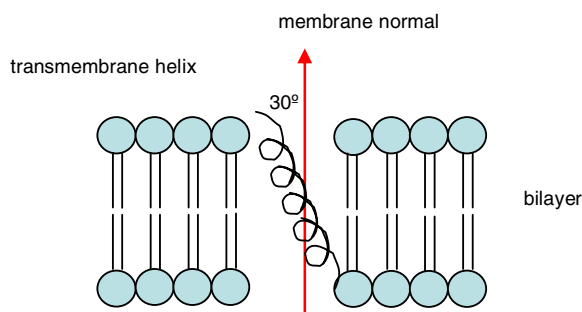


Fig. 4. Schematic illustration of the average orientation of the transmembrane helices of BR in the liposome bilayer deduced from the LD .

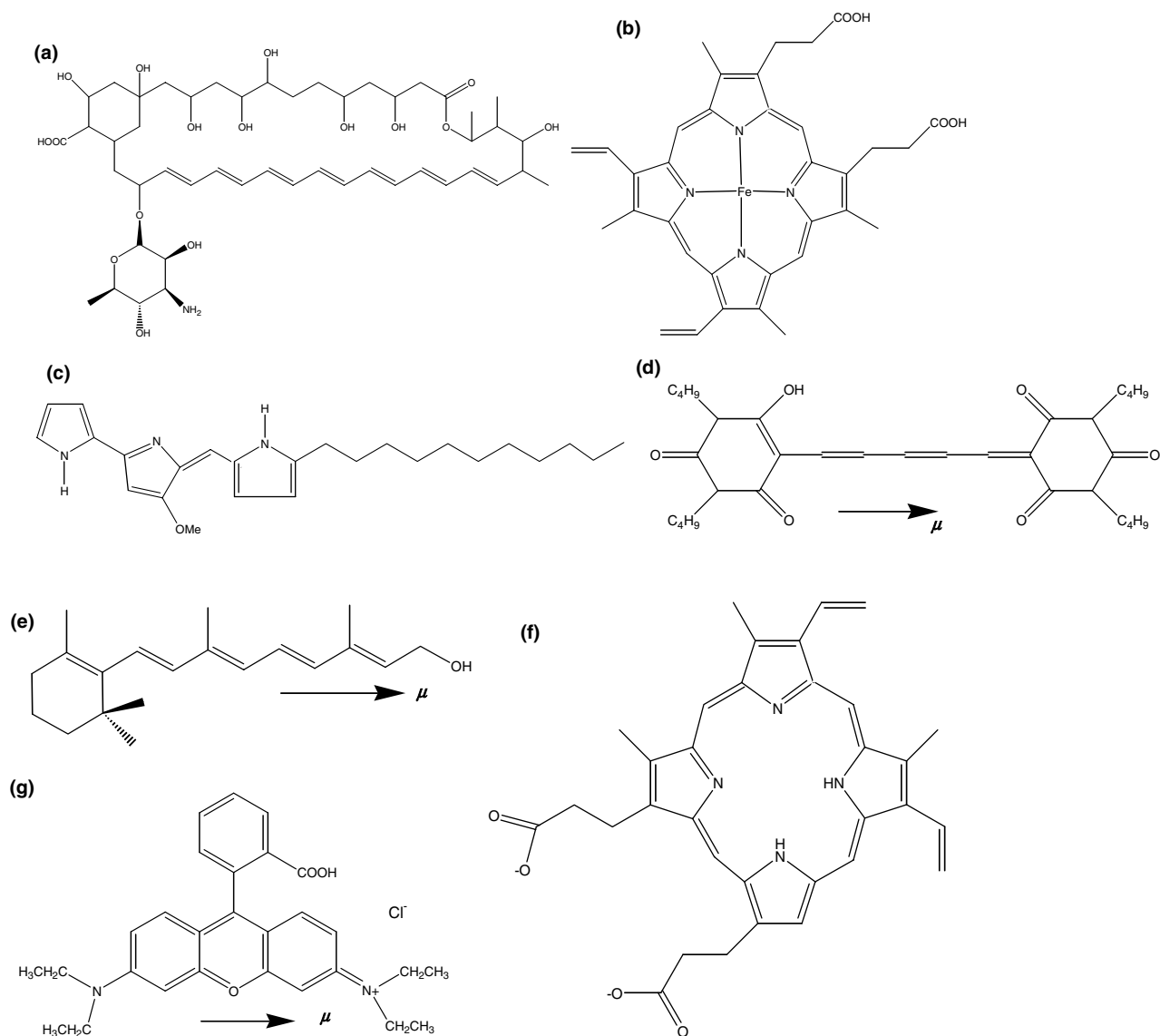


Fig. 5. (a) Amphotericin B (AmB), (b) hemin, (c) undecylprodiginine, (d) DiBAC₄, (e) retinol, (f) protoporphyrin and (g) rhodamine B. Arrows indicate the polarisations of the long wavelength transitions.

then follows that the 509 nm region of the spectrum is polarised $\geq 32^\circ$ from the long axis of DiBAC₄. Given the approximate symmetry of the molecule and also the region of the spectrum, this means that this is the result of overlapping transitions of different polarisations. The rhodamine B chromophore similarly has approximate C_{2v} symmetry so it is reasonable to conclude that the 580 nm component of its 550 nm band (largest LD') is along its long axis and the 540 nm component is perpendicular to this. (Although the film LD signal is not negative at 540 nm, if the actual component spectra are determined using Thulstrup's trial and error method it is apparent that rhodamine B's absorbance is short axis polarised at 540 nm [4]).

Using the transition polarisation information and the liposome LD spectra of Fig. 6, we can then determine the orientation of the probes in the liposomes and hence the liposome orientation parameters. DiBAC₄ loses the mixed

polarisation 509 nm band in the lipid environment, but the 602 nm band seems unaffected. The strong positive LD of this transition in the liposomes says it is well-oriented perpendicular to the average lipid, perhaps lying on the liposome surface with its alkyl chain inserting in. $LD'_{600\text{ nm}} \sim 0.019$, which from Eq. (1) gives: $S \sim 0.026$, assuming $\beta_1 = 90^\circ$. The rhodamine B in the liposome LD spectrum of Fig. 6 has a similar profile to that of the film data (Fig. 8) suggesting that the long axis of the probe is lying parallel to the surface and the short axis parallel to the lipids (cf. Eq. (1)). If this is the case, it then follows from the 580 nm LD' value (which is not overlaid with any other transition) of 0.025 that S for the liposomes ~ 0.032 . Retinol, assuming $\beta = 90^\circ$, yields $S = 0.020$. Allowing for any flexibility within the membrane and a less rigid orientation results in a decrease in β and an increase value for S . Repeat experiments with all three probes, lead

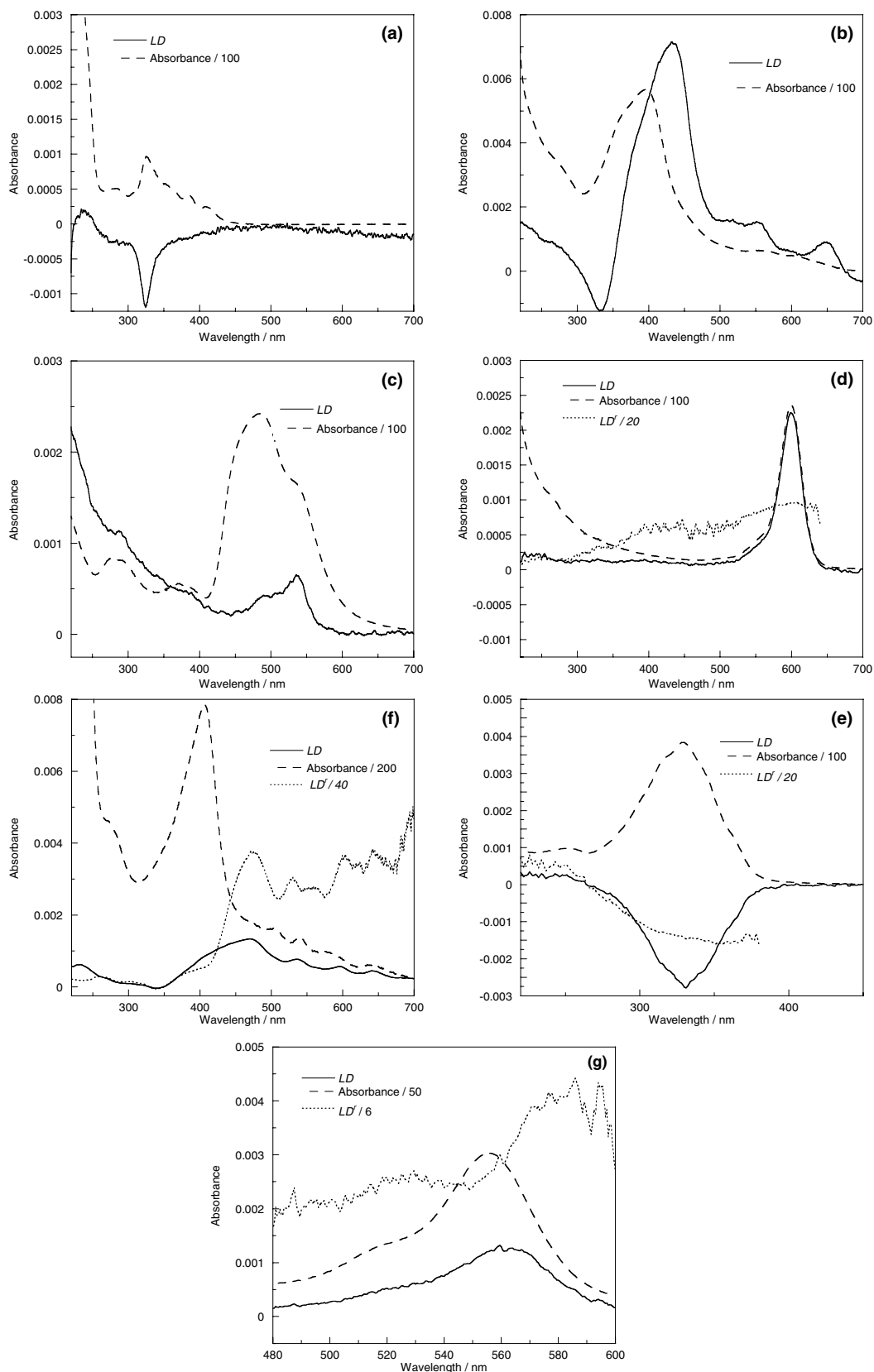


Fig. 6. Absorbance, LD and in some cases LD' (note LD cell pathlength is half that of the normal absorbance cell used) of: (a) AmB (0.02 mg/mL) in aqueous liposome solution (5 mg/mL); (b) hemin (0.5 mg/mL) in aqueous liposome solution (2.5 mg/mL); (c) undecylprodiginine (0.5 mg/mL) in aqueous liposome solution (1 mg/mL); (d) DiBAC₄ (0.01 mg/mL) in aqueous liposome solution (4 mg/mL); (e) retinol (0.05 mg/mL) in aqueous liposome solution (0.5 mg/mL); (f) protoporphyrin (0.1 mg/mL) in aqueous liposome solution (0.5 mg/mL); (a) rhodamine B (0.01 mg/mL) in aqueous liposome solution (0.5 mg/mL). A soybean lipid spectrum was used as blank for absorbance and sample without rotation used as blank for LD in each case.

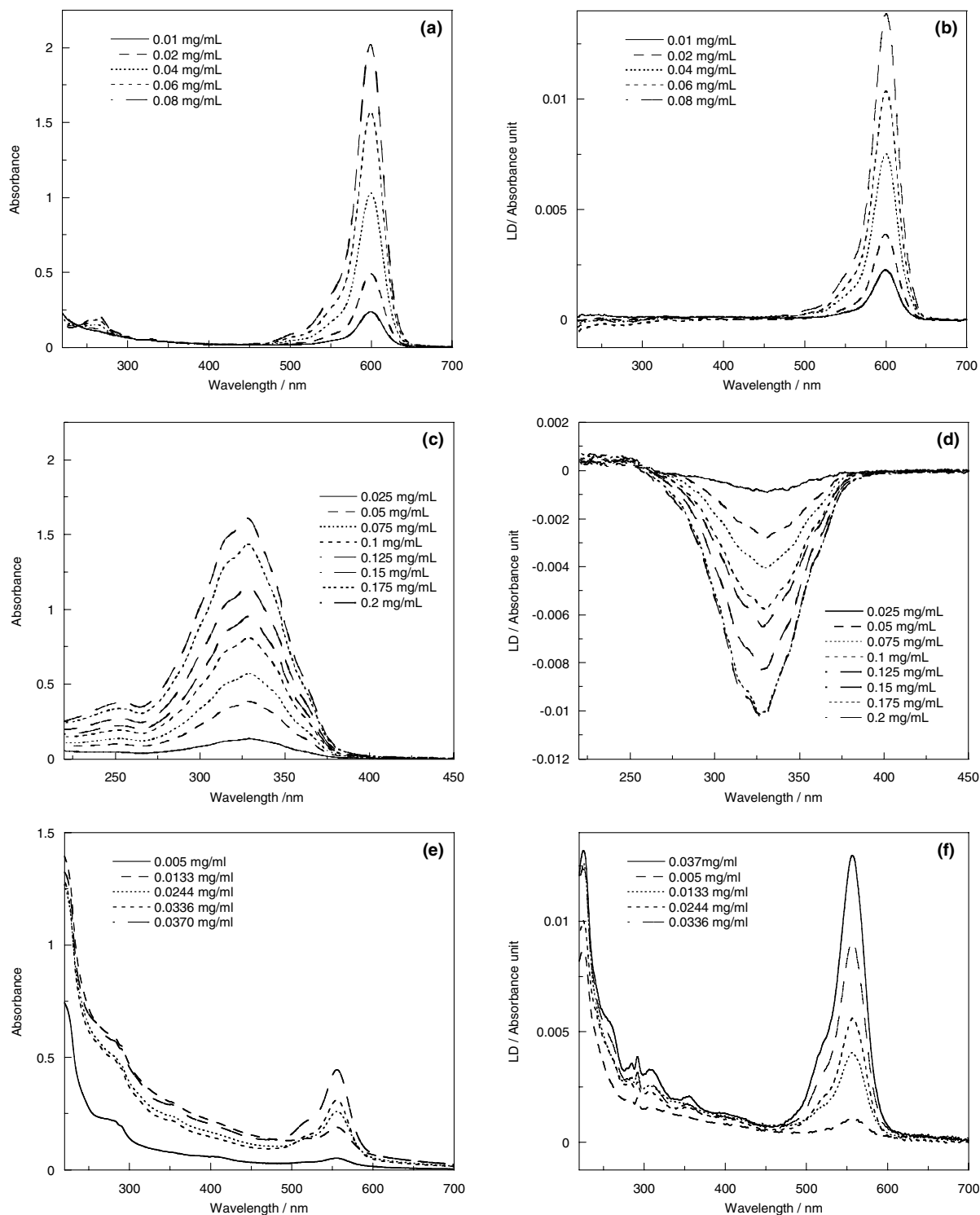


Fig. 7. Absorbance and LD spectra of different concentrations of selected probes (a), (b) DiBAC₄ (liposome concentration: 4 mg/mL); (c), (d) retinol (liposome concentration: 4.5 mg/mL); (e), (f) rhodamine B (liposome concentration: 2.5 mg/mL) in aqueous liposome solutions. Probe concentrations are indicated in the figures. Absorbance and LD spectra are base line corrected by subtracting liposome (4 mg/mL) absorbance and LD, respectively.

us to conclude that the liposomes are oriented in our experiments with an orientation parameter of $S \sim 0.030 \pm 0.010$. Independently prepared sample using the same batch of liposomes had a 5–10% variation in value of S . Different batches of liposomes resulted in the wider range.

3.3. Probe molecules and proteins

We have previously shown that both gramicidin and cytochrome *c* can be oriented in Couette flow and give LD spectra when in the presence of liposomes. So we were

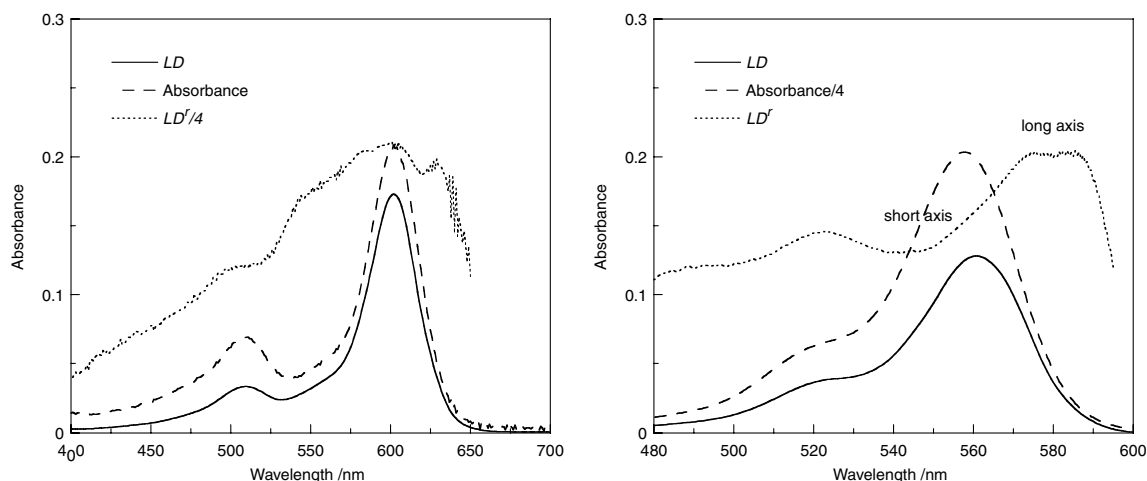


Fig. 8. LD , absorbance and LD' of: (a) $DiBAC_4$ and (b) rhodamine B in stretched PVA films.

interested to see whether the probe molecules could be used to make the geometric information deduced more quantitative. Gramicidin is a linear polypeptide with an alternating sequence of L- and D-aminoacids [30]: formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine. We have previously shown that using our preparation methods gramicidin forms head to tail dimers in liposomes [31,3]. Cytochrome *c*, by way of contrast, oriented in such a way as to be consistent with it being bound on the surface of the membranes [3]. So the two proteins are simple systems to let us address the question of whether the probe affects protein membrane binding.

3.4. $DiBAC_4$

Fig. 9(a)–(d) shows that both proteins and $DiBAC_4$ can be simultaneously oriented. The degree of protein orientation does not seem to be affected by $DiBAC_4$, however, intriguingly, the light scattering is reduced in its presence. This is not due to the reduction in the size of the liposomes which have an average length of 160 nm in each case (data not shown). It may represent a loss of a small population of very large liposomes, or perhaps it is due to the probes facilitating the solubilisation of the protein – particularly in the case of gramicidin. The data of Fig. 9(b) show the LD of the porphyrin group of cytochrome *c*. Eq. (1) then leads to a value of $\sim 50^\circ$ for the angle between the average lipid and the porphyrin plane which is consistent with our expectations based on a perpendicular attachment to the liposome surface.

In dealing with the protein backbone region it is important to ensure that the photomultiplier tube is responding correctly to the signal by diluting the sample by e.g. a factor of 2 and ensuring the signal halves in magnitude and the wavelengths of the maxima stay the same. This was done for the systems reported in this paper (data not shown) and it was confirmed that the samples obeyed the

Beer–Lambert Law. Geometry calculations can then be undertaken. For example, the S factor for gramicidin (~ 0.2 mg/mL) incorporated $DiBAC_4$ (0.01 mg/mL)/liposomes (~ 1.5 mg/mL) system is 0.021. It follows that the angle between the $n \rightarrow \pi^*$ transition (which is perpendicular to the axis of the helix) at 228 nm and the lipid normal is $\sim 70^\circ$ suggesting that the helices of gramicidin are on average tilted from the lipid normal.

3.5. Retinol

Retinol does not affect the shape of the CD spectra of either gramicidin or cytochrome *c*, but its absorbance is broad and sufficiently close to where the protein absorbs that the effect it has on reducing the sample scattering (as in the case of $DiBAC_4$) affects the protein's apparent LD spectra (e.g. Fig. 9(f)). Despite this, if one uses retinol as an internal probe to determine orientations for the cytochrome *c*, porphyrin or the gramicidin tryptophans, the results are the same as those determined with $DiBAC_4$ – which is encouraging.

3.6. Rhodamine B

The high extinction coefficient of rhodamine B makes it an attractive probe as it can be used at low concentrations. Its disadvantage is that the best data are collected using the nitrogen preparation method which is more laborious than simply adding lipids to a solution of the probe. Analysis of the orientations of the protein chromophores in the visible, near UV and far UV regions (data not shown) all indicate that it can be used as a probe.

4. Conclusions

The goal of this research was to show that LD could be used more quantitatively to determine the orientation of proteins in membrane mimicking systems. The proof of

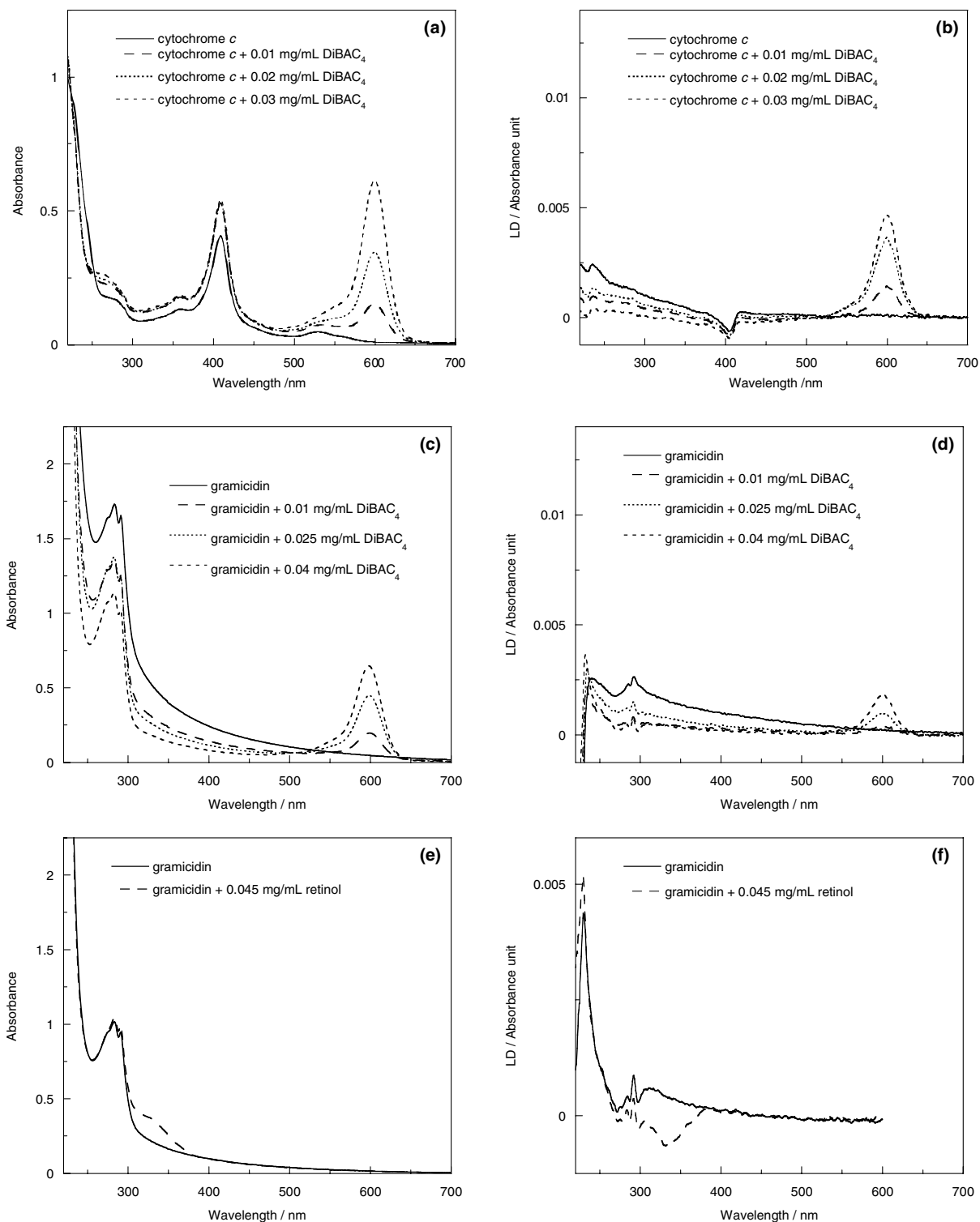


Fig. 9. (a) Absorbance and (b) *LD* of cytochrome *c* (0.6 mg/mL) in a DiBAC₄/lipid (5 mg/mL) system. (c) Absorbance and (d) *LD* of gramicidin (0.8 mg/mL) in a DiBAC₄/lipid (5 mg/mL) system. DiBAC₄ concentrations are indicated on the figures. (e) Absorbance and (f) *LD* of gramicidin (0.6 mg/mL) in a retinol/lipid (5 mg/mL) system. Retinol concentrations are indicated on the figures. Baselines were corrected by subtracting spectra of liposome solutions.

concept experiment was achieved with bacteriorhodopsin which has its own internal probe, retinal, covalently bound to it and for which X-ray data on relative orientations of chromophores are available. For the more generic problem of a protein without a covalently bound probe, a wide

range of hydrophobic molecules were screened to find some molecules that had high membrane affinity, little spectroscopy below 300 nm but also have a large absorbance above 300 nm which could be used to determine the orientation of the liposomes. Three probes were found to have potential

for future work: bis-(1,3-dibutylbarbituric acid)pentamethine oxonol (DiBAC₄), retinol and rhodamine B. Film *LD* data were measured for DiBAC₄ and rhodamine B to determine the polarisations of their long wavelength transitions. The orientation parameter, *S*, varied from batch to batch of liposomes but for unilamellar liposomes prepared by extruding through a 100 nm membrane, $S \sim 0.030 \pm 0.010$. Bacteriorhodopsin with its high protein load had a slightly higher value of *S*. The values show quite a high degree of orientation in flow of the originally spherical liposomes. The orientation parameter for a typical very long DNA sample is $\sim 0.03\text{--}0.1$ [32]. A single preparation of liposomes showed much less variation. When both probe molecules and proteins were added to liposomes it was generally possible to use the probes to determine the liposome orientation and hence to determine the protein orientation within the membranes. At the low wavelengths relevant for protein backbone motifs, it is important to ensure that the photomultiplier tube is responding properly by measuring on a series of samples of different concentration. If the wavelengths of *LD* maxima remain the same and intensities follow the Beer–Lambert Law then data can be used. The probes seemed to have the general effect of reducing light scattering.

So in conclusion a significant step forward has been taken towards being able to use flow linear dichroism as a quantitative structural tool for proteins in fully hydrated membrane environments such as liposomes.

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