Magnetically aligned membrane mimetics enabling comparable chiroptical and magnetic resonance spectroscopy studies

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Abstract

Lipidic bicelles have been widely used for the analysis of integral membrane proteins where their spontaneous alignment in an magnetic field has been exploited for oriented sample solid-state NMR studies. Many of their physical properties however make them well suited to the analysis of membrane proteins by circular dichroism (CD) and synchrotron radiation circular dichroism (SRCD). In this paper we have identified bicelle compositions that permit comparable studies of integral membrane proteins by solid-state NMR, CD, and SRCD; complementary methods that can provide insights into protein structure and the interactions with drugs and other small molecules. Furthermore we have been able to identify bicelle compositions that allow the magnetic alignment of the bicelles at fields routinely available in magnetic CD instruments. This potentially provides a route to the preparation of samples for oriented CD that mitigates many of the issues associated with the preparation of mechanically aligned samples, although we demonstrate that the dynamics present within the system can complicate the analysis of such spectra.

Introduction

Significant progress has been made in the structural analysis of membrane proteins with cryo-electron microscopy, X-ray crystallography and NMR spectroscopy making an important contribution to our understanding of this class of molecules. One of the major challenges remaining for the structural analysis of membrane proteins remains their isolation from, and if appropriate their reconstitution into, a lipid environment that is compatible with the analytical method employed. Recently however, there has been an increase in the diversity of membrane mimetics that can stabilise integral membrane proteins once they have been removed from the lipid bilayer [1-4]. This has led to the development of systems beyond the more traditional detergent micelles and lipid vesicles to include a range of discoidal membrane structures bounded by detergent, amphipathic proteins or polymers [5-8]. In contrast to detergent micelles these more closely mimic the structure of the native lipid bilayer with a lower degree of curvature and similar bilayer thickness, whilst permitting the incorporation of a greater diversity of lipids allowing the native membrane environment to be mimicked more accurately. In this paper we have focussed on one such class of membrane mimetics, namely bicelles which are frequently described as lipidic discs surrounded by a ring of detergent molecules, in an attempt to identify conditions that would allow comparable studies by circular dichroism (CD) and magnetic resonance-based spectroscopies. An attractive feature of bicellar structures is their ability to macroscopically align in a magnetic field that provides a platform in which to conduct oriented studies [9, 10], a property widely used in NMR but not exploited in general for CD spectroscopy. NMR studies of such macroscopically aligned samples give rise to spectra whose resonance frequencies reflect the orientation of sites within the protein. Similarly, CD spectra of oriented samples can provide valuable insights into the orientation of

secondary structural elements, such as α-helices, where differences in intensity are dependent on the orientation of the electronic transitions with respect to the incident light.

In contrast to X-ray crystallography and cryo-electron microscopy (cryo-EM), these techniques permit the structural analysis of integral membrane proteins under near physiological conditions (ambient temperatures), ensuring that the lipid environment retains a similar fluidic state similar to that in-vivo whilst potentially allowing functional studies of the protein to be conducted. These techniques also prove to be highly complementary. CD spectroscopy provides valuable insights into the global secondary structure both in terms of secondary structure content and average length and number of helices and strands of the protein from limited quantities of sample (typically micrograms) [11]. It is also amenable to high throughput screening of samples [12], whilst solid-state NMR methods can provide higher resolution and site specific structural data, albeit requiring larger quantities of sample (typically milligrams) and longer acquisition times.

In the selection of a membrane mimetic that would support both magnetic resonance and CD spectroscopy major considerations are the optical properties of the sample. As for any absorption optical spectroscopy the sample should be free of particles that can lead to scattering of the incident light which can compromise measurements distorting the spectral shapes. Secondly, clear membrane mimetics would have limited absorption in the far-UV region (185 to 250 nm) typically used for the analysis of sub milligrams of proteins thereby ensuring the signal is dominated by protein under study. In addition, CD spectroscopy places additional requirements as contributions from linear dichroism and birefringence also need to be minimised or accurately determined [13]. In contrast, magnetic resonance studies are typically limited by the concentration of protein present in the sample, usually requiring high

concentrations of isotopically labelled protein within the membrane (low lipid to protein ratios) and low levels of hydration to minimise the sample volume occupied by the aqueous phase.

Bicelles are widely used in NMR spectroscopy where their magnetic susceptibility is used to magnetically align the bilayer phase in the magnetic field. Alignment in the magnetic field depends on a number of properties including the ratio (q) of long chain lipid to detergent/short chain lipid, the degree of hydration, temperature and the strength of the magnetic field [14]. Once aligned however, the magnetically aligned bicelles can be used to obtain orientational restraints from proteins reconstituted into them [15-17]. Further control of the degree of alignment is possible by exploiting the anisotropy of magnetic susceptibility of paramagnetic trivalent lanthanide ions (Eu^{3+}, Er^{3+}. Tm^{3+} and Yb^{3+}) which interact directly with the lipid headgroups [18] or can be attached to the bilayer surface through the use of chelating lipids [19]. This allows the bilayer to be oriented either parallel or perpendicular to the applied field.

Under many conditions the physical properties of the bicelles are well suited to CD spectroscopy with little scattering across a broad range of conditions, and only moderate absorption in the range from 190 to 250 nm which is the spectral window widely used for the analysis of protein secondary structure. Furthermore, the magnetic alignment of bicelles in a magnetic field would provide an alternative to the preparation of aligned lipid bilayers on quartz plates that are typically employed for oriented CD (OCD) measurements but where variations in the thickness of bilayer deposition and poor optical properties make analysis challenging [13].

In this paper we undertake a study of bicelles to identify conditions which are compatible with both NMR and CD spectroscopic studies. These studies have identified conditions where magnetic alignment was obtained in a high magnetic field to support NMR studies of oriented

samples whilst simultaneously allowing an analysis of the secondary structure by CD spectroscopy. These have been demonstrated on a transmembrane peptide derived from the glycosyltransferase Fukutin-I [20, 21]. Subsequently EPR spectroscopy has been employed to identify conditions under which the bicelles will magnetically align in fields that are typically used for magnetic CD (~1.4 T). These results showed that under the low field conditions fluctuations of the bilayer/protein about the bilayer director occurring on a timescale of $<10^{-7}$ seconds which results in averaging of the anisotropic interactions apparent in the NMR spectrum results in a distribution of orientations when probed on the timescale of the CD experiment. This suggests that successful magnetically aligned oriented CD measurements may only be possible at higher magnetic fields.

Materials and Methods

Materials

Lipids (DMPC, DHPC, PC, DMPE, and DTPA (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid)) were purchased as lyophilised powders from Avanti Polar Lipids (Alabama, USA) and prepared as 10 mg mL^{-1} stocks in methanol. Unlabelled murine Fukutin TMD (Uniprot accession number Q8R507, residues 1-36) was synthesised by Peptide Protein Research Ltd (Fareham, UK) as a TFA salt at > 90 % purity with amino acid sequence:

MSRINKNVVLALLTLTSSAFLLFQLYYYKHYLSARN

For NMR studies, ^{15}N-leucine labelled Fukutin TMD was produced by Chinese Peptides (Hangzhou, China) as TFA salt supplied at > 98 % purity. The sequence was identical except for the removal of the N-terminal methionine (position 1). ^{15}N-leucine was incorporated for all

leucines with the exception of position 15. Both peptides were prepared at 1 mg mL^{-1} in methanol.

All other reagents (cholesterane spin label (CSL), D_2O) were sourced from Fisher Scientific (Loughborough, UK). Suprasil fused silica cuvettes used for CD measurements were manufactured by Hellma (Essex, UK). Cuvettes were cleaned by soaking in 70 % nitric acid for at least 1 hour, followed by repeated washing with H_2O and 70 % ethanol before drying at room temperature.

Bicelle Preparation

Stocks of lipids and Fukutin TMD were prepared in methanol. Appropriate aliquots of lipids and Fukutin TMD were mixed in glass vials and dried under a stream of nitrogen gas before being dried under high vacuum for at least four hours to remove any residual solvent. ddH_2O was added to the film to achieve the desired hydration (expressed as % w/v of dry lipid to ddH_2O), then subjected to six cycles of freezing in liquid nitrogen, thawing at 35 °C and vortexing for 30 seconds to ensure full hydration. Samples were prepared and used within 24 hours. Samples for CD were degassed to prevent cavitation during measurement in a 600 mmHg vacuum at 5 °C for 30 minutes. For samples with lanthanide, bicelles were prepared then the lanthanide solution was added and followed by further rounds of freeze/thawing to ensure homogeneity.

Conventional CD Measurements

Conventional CD spectra were acquired on a nitrogen-flushed Chirascan Plus spectropolarimeter (Applied Photophysics, Leatherhead, UK). Far-UV spectra were obtained from 260 - 185 nm. A bandwidth of 1 nm, increment of 1 nm and integration time of 1 s were used throughout. As standard a minimum of four spectra were recorded and averaged for each

sample and appropriate baseline subtracted (either pure-lipid baseline for protein spectra, or buffer baseline for lipid spectra). Sample temperature was controlled using a Peltier cell holder (Quantum Northwest, Washington, USA). For temperature ramped experiments the start and end temperatures were 5 and 40 °C, with measurements at 5 °C intervals and a stepped ramp with a minimum 10 minute incubation at each point. All measurements were recorded using 0.1 mm pathlength fused silica demountable cell (Hellma, Essex, UK). Unless otherwise stated samples containing the protein, Fukutin, were measured at a L/P ratio of 1100:1.

Synchrotron Radiation CD (SRCD) Measurements

SRCD measurements were recorded at B23 beamline, Diamond Light Source, UK, with access provided through awarded beamtimes (SM12844, SM14084 and SM16501). Measurements were recorded using B23 module B station with a highly modified DSM 20 spectropolarimeter (Olis Inc, Georgia, USA) [22] with a removable 1.4 T permanent magnet for Magnetic CD (MCD) measurements. The field lines of the magnet run collinear to the direction of light propagation. All measurements were recorded without magnetic field and with the field in both S-N ('forward') and N-S ('reverse') orientations with respect to the incident light beam propagation. Far-UV measurements from 250 – 185 nm were carried out using slit width of 1 nm corresponding to about 1 nm bandwidth, 1 nm step increment and 1 s integration time unless otherwise stated. A 0.1 mm demountable fused silica cuvette (Hellma, Essex, UK) was used throughout. The spectrometer was calibrated using the 1S (+) 10-camphorsulphonic acid method.

To enable temperature control of samples in the magnetic sample holder, a stream of nitrogen gas was directed onto the face of the sample cuvette. The temperature of the nitrogen gas was regulated using a variable temperature controller (FTS Systems and Kinetics Air-Jet) and the

temperature of the sample measured using a fine tipped thermocouple (RS Components, UK) attached to the reverse of the cuvette (the configuration of the sample chamber on the beamline is shown in Supplementary Figure 1).

SRCD data was processed with CDApps [23] and MATLAB (MathWorks, Inc.) using custom written scripts. To yield the MCD or CD signal from the data acquired with the magnet in both forward and reverse orientations, spectra were calculated according to:

$$\begin{aligned} MCD &= (CD_{N\text{-}S} - CD_{S\text{-}N}) / 2 \\ CD &= (CD_{N\text{-}S} + CD_{S\text{-}N}) / 2 \end{aligned} \qquad (0.1)$$

To yield the OCD spectra and eliminate any MCD contributions, the spectra for forward and reverse magnet positions are summed and divided by two. Data was converted from ellipticity reported in millidegrees (mdeg) to $\Delta\varepsilon = (\varepsilon_L - \varepsilon_R)$ where ε_L and ε_R are the excinction coefficients of left and right circularly polarised light. Pure lipid spectra, for example, or protein spectra with large distortions were reported in millidegrees (mdeg).

Phosphorours-31 Solid-State NMR Measurements

All measurements were acquired using an Agilent DD2 600 MHz spectrometer equipped with a 3.2 mm magic angle spinning probe. For ^{31}P spectra samples were measured without spinning using a Hahn echo pulse sequence [24] at a frequency of 263 MHz with high power proton decoupling, typically with a 90° pulse lengths of 4 μs, echo delay of 50 μs, acquisition time of 50 ms and 2.5 s recycle delay. For temperature ramped experiments there was a 5 minute delay between temperatures to allow the sample temperature to equilibrate. The temperature was checked intermittently using ethylene glycol according to the method outlined in [25]. Data was processed by shifting to the top of fid, zero filling to 8192 points and applying 100 Hz exponential line broadening before Fourier transform.

EPR Spectroscopy

Bicelles for EPR studies were doped with 0.5 mol % cholestane spin label (CSL) with respect to DMPC as a reporter for orientation which was added to the lipids in solvent before drying. At this low concentration it is unlikely that the CSL has any influence on the bicelle morphology or phase, and in the literature similar concentrations of cholesterol and CSL do not impact the phase transition of PC lipids [26]. 20 µL samples were drawn into 1 mm diameter fused silica capillary tubes and heat sealed.

EPR experiments were conducted using an EMXmicro X-band continuous wave spectrometer (Bruker) with temperature control of the sample cavity using a flow of heated nitrogen gas. The microwave frequency was set to 9.85 GHz. The temperature was monitored using a thermocouple placed inside the capillary tube before measurement. To align the bicelles, the magnetic field was set at 0.58 T whilst the temperature was raised gradually from 25 to 45 °C over 15 minutes, and then incubated for an additional 20 minutes with the 0.58 T field. Presented spectra are the average of five scans recorded from 0.34 – 0.36 G with a 30 s scan length.

Results and Discussion

Analysis of phase behaviour and alignment in high (14.1T) magnetic fields by ^{31}P NMR

To probe the temperature and its role in bicelle phase behaviour we employed ^{31}P NMR. Initially, the optimum *q* ratio was determined by acquiring spectra from 0 to 60 °C in 2 °C intervals. The *q* ratios investigated were 2.5, 3 and 3.5, which were selected since it has been reported that above *q* of 4 samples begin to become translucent [27] and much below 2.5 yields isotropic bicelles which do not align in a magnetic field. The aim of these experiments was to determine which *q* ratio gave the highest degree of alignment, and the broadest temperature

window of alignment to avoid unnecessary sample heating and also to give maximum flexibility during future measurements. The ^{31}P spectra for bicelles at these three q ratios at 30 % w/v (dry wight of lipid to volume of solution) are shown in Figure 1, and the DMPC resonance positions extracted and plotted as a function of temperature (Figure 2) determined as either the most upfield peak or peak with the greatest intensity. In both figures the temperature dependence of the samples is clearly visible, with an initial isotropic phase characterised by a single resonance followed by the aligned bicellar phase dominated by two resonances for DMPC and DHPC respectively, and then the fluid bilayer/mixed micelle phase at higher temperatures. At all q ratios the isotropic chemical shift below the T_m was ~ −1 ppm, due to a rapidly tumbling micellar phase. When handling the samples at these temperatures they were found to be fully transparent and highly fluid. The full width at half maximum (FWHM) of the isotropic peaks subtly increased from 2.1 up to 2.3 and 2.4 ppm (at 0 °C) for q 2.5 to 3.5, reflecting the slower motions of the mixed micelles with increasing concentrations of DMPC.

Above the T_m, at 32, 28 and 16 °C for q = 2.5, 3.0 and 3.5 respectively, there was a transition to the bicellar phase (<5 minutes) which rapidly oriented perpendicularly to the magnetic field as indicated by the upfield resonances at about -5.8, -8.8 and -12.7 ppm for q 2.5, 3 and 3.5. The position of the peak was indicative of the both orientation and the degree of order in the bilayer, which is characterised with respect to DMPC multilayers by the parameter S_{bic}[28] that is also refered to in the literature as bicelle wobble, S_{bw} [14]) :

$$S_{bic} = \frac{\delta_{obs} - \delta_{iso}}{\delta_{\perp} - \delta_{iso}}$$

The slightly downfield peak of reduced intensity is assigned to the DHPC which is thought to form the rim of the bicelles. The peak position varies slightly with *q* ratio and temperature from ~3 ppm to ~7 ppm, similar to findings in the literature [27]. There is a large difference in the temperature range of orientation and the position of the 90° edge between the three *q* ratios, with the *q* of 3.5 fully aligning at the lowest temperature, around 24 °C, and having the most upfield DMPC resonance, indicating the smallest distribution around the bilayer director. This is confirmed by S_{bic} values at 36 °C of 0.31, 0.51 and 0.76 for *q* of 2.5, 3.0 and 3.5 respectively. These values were calculated from the 90° edge of ^{31}P powder spectrum of pure DMPC at -16.3 ppm (Supplementary Figure 2). In the bicellar phase, samples became much more viscous for all of the *q* ratios tested here and they retained their transparency. Above the critical temperature T_v, which has been assigned to morphologies of mixed micelles and fluid DMPC bilayers with incorporated DHPC molecules [27], there is a re-emergence of the isotropic peak in addition to an upfield peak close to the DMPC 90° edge. The T_v for the *q* ratios 2.5, 3 and 3.5 are 48, 44 and 40 °C. At *q* 2.5 and 3.0 there is a clear predominance of an isotropic peak, whereas at 3.5 the most intense peak at -15.3 ppm originates from a fluid bilayer phase, indicated by the S_{bic} of 0.96.

Samples with $q = 3.5$ were utilised because of their highest degree of order and alignment, were visually transparent in the bicellar phase, and importantly showed the lowest T_m, which prevented unnecessary heating of proteins embedded in the bicelles.

Having selected the *q* ratio of 3.5 we then aimed to optimise the weight percent of lipid. It is desirable for optical studies to reduce the quantity of lipid as much as possible since the lipid carbonyl groups absorb in the higher wavelength of far-UV region, although it is also be optimal to ensure the L/P ratio is large to prevent absorption flattening effects. This can give

rise to wavelength-dependant distortions of the spectra due to inhomogeniously distributed chromophores across the sample, resulting in localised changes in concentration [29]. Conversely, for NMR studies which are relatively insensitive to these issues, it is desirable to maximise the protein content of the sample and therefore also maximise the lipid content. In solid-state NMR measurements 20-30 % w/v are the most commonly used weight percentages, with ~50 % w/v the maximum which still aligns [30]. These levels of hydration have sufficient viscosity to allow and retain bicelle alignment, which is presumably stabilised by steric effects. Lower levels of hydration have high viscosity which reduces bicelle motion and prevents orientation [30], but when the level of hydration is too high the viscosity is such that the bicelles cannot stably align [31]. For these experiments samples of DMPC/DHPC with a q of 3.5 were prepared at 5, 10, 20 and 30 % w/v and measured using ^{31}P NMR over 0 to 60 °C (Figure 3 and Figure 4).

It appears that at 5 % w/v there is an aligned component above ~26 °C, as well as free micellar component indicated by the isotropic peak at all temperatures. The DMPC peak in the 5 % w/v sample is approximately 2 ppm upfield of the equivalent peaks for 10, 20 and 30 % w/v (-13.2 ppm compared to ~ -11.4 ppm). It is likely that there is a combination of bicellar and lamellar phases contributing to the spectra here, indicated by the broadened DMPC peak relative to the higher weight percent (FWHM at 40 °C is 1.15 ppm for 5 % w/v and 0.9 ppm for 10 % w/v). It is possible in this case that the peak occurs as a result of a combination of the 90° edge of a DMPC powder pattern and the downfield peak from the less ordered DMPC in bicellar phase. More dilute samples are known to be less amenable to alignment, with samples having q of 3 at less than 3 % w/v failing to align [32] and SANS studies indicate the presence of multilamellar vesicles rather than bicelles at 5 % w/v [33]. At 10, 20 and 30 % w/v there is a good degree of alignment from 28 to 42 °C. The S_{bic} parameter for 10, 20 and 30 % at 34 °C

were 0.69, 0.68 and 0.68 respectively, indicating that although there is a greater degree of motion in the bicelles than multilamellar vesicles there is little difference in phase between the weight percentages. In contrast, the S_{bic} of the 5 % w/v samples is 0.8, indicating a greater degree of rigidity, similarly to a lamellar phase sample.

Electron Paramagnetic Resonance analysis of phase behaviour and alignment of bicelles in low (0.6T) magnetic fields.

The alignment of lipid bilayers was determined using EPR spectroscopy. X-band EPR spectrometers operate with a maximum field of ~0.6 T and so it is likely that if bicelles can be aligned in these conditions, they could also be aligned for MCD measurements at 1.4 T. However, even at 20 % w/v the standard DMPC/DHPC bicelles failed to align at these low magnetic fields [34] and it was necessary to determine whether the lipid composition could be refined such that alignment could be achieved at reduced field strength.

Lorigan and coworkers have extensively studied the alignment of bicelles using X-band EPR, and have developed lipid compositions and protocols for alignment of bicelles in the 0.6 T field [34-39]. To achieve alignment at these low fields, it is necessary to gradually increase the sample temperature from the isotropic phase into the bicellar phase, and then to allow a further period of equilibration [37, 39]. Also a number of modifications to the basic DMPC/DHPC mixture were required such as the introduction of lanthanide ions to alter the natural $\Delta\chi$ of the lipid bilayer and control of the direction of alignment, the addition of PEGylated lipids to improve stability, and cholesterol to promote ordering. This mixture required the use of 10 mol % (with respect to DMPC) lanthanide chloride salt, which is challenging for three reasons. Firstly the use of chloride salts at this concentration weres prohibitive for CD measurements, even using SRCD, as chloride absorbs strongly in the far-UV region. Secondly

one of the aims of this work was to allow direct comparison between CD and NMR, and lanthanides at such high concentrations were likely to have deleterious effects on the NMR spectra. Finally, lanthanides can bind to proteins (especially acidic residues) which is likely to affect their folding [19]. We mediated these challenges using an alternative lipid mixture, based on a method established by Prosser et al. [19] to 'flip' bicelles for NMR studies through the use of DMPE-DPTA to chelate the lanthanide and effectively anchor it to the membrane surface. Added at 1:150 with DMPC, and a 1:1 ratio between the chelator and lanthanide, this substancially reduced the concentration required and sequestered the lanthanide away from proteins and phosphate groups due to its much higher affinity. The lanthanides selected were dysprosium and thulium. Dysprosium resulted in one degree of ordering such that the bilayers were aligned perpendicular to magnetic field and were distributed about the membrane normal to produce a cylindrical distribution. With thulium there was an additional degree of ordering, such that the normal was aligned parallel with the field. Similar to NMR, for interpretation of OCD data, the bicelles aligned parallel with the field were preferable as they give rise to a uniaxial distribution about the membrane normal. A visual representation is given in Figure 5, where *n* is the bilayer normal and *h* is the helical long axis. In these studies, both dysprosium and thulium have been used to provide a comparison between parallel and perpendicular alignment.

The alignment of DMPC/DHPC/DMPE-DTPA bicelles tagged with lanthanides and doped with CSL at low field was studied using EPR at 25 and 45 °C (Figure 6). In the measurements the temperature was set to 25 °C then raised at approximately 1 °C/minute to 45 °C, followed by a 15 minute incubation at 45 °C, in a field set at 0.58 T. It has been demonstrated that the temperature of 25 °C to enable a complete alignment was not above the T_m [39]. The field was then reduced to allow acquisition of spectra.

The EPR spectra acquired at 25 °C showed the typical CSL powder spectrum in which the A_{xx} and A_{yy} tensors averaged due to rapid rotation about the CSL long axis [40], with no significant differences observed with the two lanthanides. For the spectra at 45 °C there was a clear difference with changes of the splitting that were significant. The hyperfine splitting (measured between m = +1 and m = 0 peaks) was observed to be 12 G for thulium that is indicative of parallel alignment and 17 G for dysprosium indicative of perpendicular alignment. These were slightly different than those from the literature (17.5-19 G and 8-10 G for dysprosium and thulium respectively [34], where 19 G and 6 G were the rigid limits [41]), indicating slightly poorer alignment of the samples with thulium. One consideration is that the values quoted in the literature were for CSL in cholesterol-rich bilayers which are likely to alter slightly the values due to changes in the bilayer phase and therefore the spin label order. It is also clear from the thulium spectra that there was an underlying secondary component, a powder contribution, illustrated by the shoulders at outer side of the +1 and -1 lines. Although there may be a small disordered component in the sample, since the degree of alignment is dependent on the square of the field strength it is foreseeable that there will be an increase in the orientation in the MCD field. There does not appear to be a powder signal in the dysprosium spectra, presumably since the dysprosium serves to enhance the natural $\Delta\chi$ rather than oppose it, and has a greater absolute $\Delta\chi$, although it is also worth noting that the powder spectrum has similar splittings and so powder contributions may be less obvious than in the thulium spectra. Whilst it was tempting to increase the concentration of thulium to enhance the degree of alignment, this was avoided due to concerns regarding the amount of UV absorbing chloride ions present in the samples that would have reduced too much the spectral range available for CD measurements. (Fluoride and nitrate salts of thulium were obtained but were found to be mostly insoluble at the required concentrations.) Although in the literature cholesterol has been

used to improve the degree of alignment, this could add a further complication in these optical studies [42]. The samples studied by EPR at low field (~0.6T) were also analysed by ^{31}P NMR at 14.1 T. Data were acquired for bicelles in the presence and absence of Fukutin TMD when doped with both thulium and dysprosium as well as ytterbium. Ytterbium was also used as it possesses a smaller $\Delta\chi$ than thulium but is a weaker relaxation agent, thereby facilitating NMR measurements. A full analysis of the spectra and the corresponding changes in chemical shift as a function of temperature can be found in Supplementary Figures 3 and 4. As expected, and in agreement with EPR studies the ^{31}P spectra showed that the absence of lanthanide or in the presence of dysprosium the bicelles aligned with their normal perpendicular to the field, whilst the addition of thulium or ytterbium with positive $\Delta\chi$ resulting in the bicelle normal aligning parallel to the field.

CD analysis of Fukutin-I in bicelles in the absence of a magnetic field.

Having characterised their phase behaviour of various lipid compositions and optimised conditions for alignment in a low magnetic field, the next step was to undertake CD measurements. These were initially performed using a conventional CD instrument without a magnetic field to understand the optical properties of the samples. Following the conditions used for EPR and NMR, the same DMPC/DHPC/DMPE-DTPA (3.5/1/0.023, 20 % w/v). composition was used A 0.1 mm pathlength was selected to minimise the amount of lipid in the beam and therefore its absorbance. The conformational behaviour of the lipid mixture after heating to 38 °C from 25 °C was monitored by acquiring consecutive CD spectra (Supplementary Figure 5). The first spectrum showed a large positive band below 190 nm, which increased rapidly within the first 10 scans to gradually reach a plateau after 25 scans. The positive band below 190 nm presumably reflects the gradual organisation of the lipids into

bicellar morphology that requires 45 minutes to equilibrate. The other positive band at about 216 nm, assigned to the lipid carbonyl chromophore, was affected to a lesser degree during equilibration.

CD spectra of DMPC/DHPC/DMPE-DTPA were acquired with and without Fukutin TMD in the absence of lanthanide, and with thulium or dysprosium (Figure 7). Due to the complex nature of the spectra, care must be taken when performing baseline corrections. In these measurements, lipid only spectra were corrected against a water blank, whilst Fukutin/lipid spectra were background corrected using the equivalent lipid only spectrym. For the pure lipid samples, from 5 to 25 °C the spectra were relatively similar, with a broad peak around 216 nm corresponding to the lipid carbonyl n-π^* transition. The large intensity of the lipid mixture spectra as a function of temperature in the 200-250 nm region was similar to that of the Fukutin TMD spectra. For the lipid mixture, above 25 °C there appears to be correlation between the appearance of a positive CD band below 200 nm and the phase transitions identified by ^{31}P NMR. It is important to notice that the bicellar phase of the lipid mixture DMPC/DHPC/DMPE-DTPA above 25 °C requires a lenghtly equilibration time to stabilise its morphology as revealed by CD spectroscopy (Supplementary Figure 5). The CD spectra of the lipid mixture with and without lanthanides in the presence and absence of Fukutin TMD reveal significant spectral differences, indicating that the incubation time to reach bicelle stability were also different. Due to the large contribution from the lipid signal at wavelengths below 200 nm and the time taken to stabilise we suggest that data should be interpreted with caution. Although this limits secondary structural deconvolution, for oriented studies it is less problematic since the band at ~208 nm is the prime indicator of helix alignment. This is consistent with the study of unoriented bicelles by CD spectroscopy conducted by Loudet et al. [43] where similar CD changes below 200 nm did not affect the author's conclusions.

The spectra of Fukutin TMD in the bicelles are characteristic of an α-helical protein, with negative bands at 210 and 223 nm respectively. In the micellar phase at 5 °C the CD spectra of Fukutin TMD with and without lanthanides were very similar, indicating similar foldings. With thulium there was an increase in the overall CD profile upon increasing the temperature suggesting an enhanced α-helix content.

NMR analysis of Fukutin-I TMD tilt in bicelles at high (14.1T) magnetic field.

Prior to the analysis of the tilt angle of Fukutin TMD by OCD, the orientation of the helical TMD was determined by well-established ^{15}N solid-state NMR methods. Spectra of $^{15}N_{leu}$ Fukutin TMD were acquired in bicelles of identical lipid composition to those studied by EPR, and ^{31}P and deuterium NMR (DMPC/DHPC/DMPE-DTPA). To enhance sensitivity though, the L/P ratio was decreased to 50:1 and the studies were increased from 20 % to 30 % w/v hydration, which is shown above to have little effect on the degree of alignment.

Prior to ^{15}N experiments studying the peptide, ^{31}P spectra were recorded to assess the degree of alignment in the sample. To obtain samples with the bicelle director aligned perpendicular and parallel to the magnetic field two samples were prepared, one in the absence of lanthanide (perpendicular orientation) and one with ytterbium (parallel orientation). The degree of alignment was assessed with ^{31}P NMR (Figure 8A and C). For comparison the ^{31}P spectra of the bicelles in the absence of protein are shown in in Supplementary Figure 3. The bicelles oriented perpendicular to the bilayer produce a DMPC peak at -11 ppm, as expected, but there is also a shoulder at -0.5 ppm from what may be an isotropic component, although this feature is absent in the corresponding deuterium spectra (Supplementary Figure 6). In the bicelles flipped with ytterbium there is a broad, shallow downfield contribution in the ^{31}P spectra, as well as a peak around -1.1 ppm which may result from an isotropic mixed micellar solution but

equally is fairly broad at 1.7 ppm (Figure 8C). It should also be noted that additional ytterbium was titrated into the sample to flip the bicelles completely, with a final ratio of ytterbium:DMPE-DTPA of approximately 1.5:1. It is highly likely therefore that some of the ytterbium is bound to the lipid phosphates. It has been noted previously that bicelles with protein often require additional lanthanides to orient [19]. From the ^{31}P spectra it is possible to determine the bicelle order parameter (S_{bic}) which characterise the size of the fluctuations around the bicelle normal, with values of 0.68 and 0.76 obtained for the perpendicular and parallel alignments. These values are in good agreement with those obtained from the corresponding ^{2}H data which exhibit better resolution due to the increased separation from the lanthanides (Supplementary Figure 6).

The ^{15}N spectra of Fukutin TMD labelled with ^{15}N-leucine at seven of eight leucine positions Fukutin in bicelles reveals a dominant peak centred around 114 ppm for perpendicular and 156 ppm for parallel aligned bicelles (Figure 8B and D respectively). These positions indicate a tilted transmembrane orientation. The leucines, which are distributed around the helix would be expected to give rise to resolved resonances, however due to the limited signal to noise obtained, they appear as a single envelope. Analysis of the lineshape obtained with the bicelle director parallel to the magnetic field indicates that the helix is tilted at approximately 26° with respect to the bilayer normal, when correcting for fluctuations about the bilayer normal as characterised by S_{bic}. This tilt angle is comparible to molecular dynamics simulations of monomeric Fukutin TMD in planar lipid bilayers composed of either DLPC or DPPC, whose fluid phase hydrophobic thicknesses are likely to be comparable to those of DMPC bicelles (21.7 Å for DLPC at 30 °C and 28.5 Å for DPPC at 50 °C)[21]. These are given as 39±11° and 30±9° for DLPC and DPPC respectively, indicating that the peptide compensates for hydrophobic mismatch by tilting to a greater or lesser angle. We note that the linewidth

obtained for the bicelles aligned perpendicular to the field is approximately twice that obtained for the parallel alignment, the opposite of what may be expected. This may arise from the presence of some unaligned peptide within the sample which gives rise to a weak signal at ~60 ppm in the sample with the bicelle director aligned parallel to the magnetic field.

SRCD studies of bicelles in the presence of a magnetic field.

SRCD spectra of DMPC/DHPC/DMPE-DTPA bicelles with and without Fukutin were acquired in the presence of lanthanide ions (Figure 9). Pure lipid bicelles were used as a baseline. Proteins containing aromatic amino acids can exhibit a magnetic CD (MCD) signal in the region of 300 to 250 nm, and thus in principle a weak MCD component may be expected from the peptide. To ensure that MCD made no contribution to the spectra, data were recorded in magnetic fields of opposite polarity and processed as described in the materials and methods resulting in a 'pure' CD signal. We note however, no detectable MCD signal was observed in the samples studied.

In all cases reported in Figure 9 the SRCD spectra were consistent with a dominant α-helix conformation. Surprisingly there were no significant changes between those with or without lanthanide nor in the presence of the magnetic field, suggesting there was no alignment under these conditions contradicting our observations made by EPR. The corresponding spectra from Figure 9 have been overlaid in Supplementary Figure 7. A slight change is observed in the intensity of the 190 nm band but as eluded earlier the large lipidic bilayer contributions in this region makes interpretation prone to error.

This result is somewhat surprising as EPR data collected under lower field conditions indicated that such preparations would align in the magnetic field, giving rise to oriented CD spectra. We believe that to understand the absence of an oriented signature in the CD spectrum it is

necessary to consider the timescale and amplitude of motions of the bicelles about the bicelle normal/director as the spectroscopic techniques employed are sensitive to significantly different timescales. NMR and EPR are sensitive to motions on the ~10^{-3} and 10^{-7} s respectively, much slower than that of CD at ~10^{-15} s. In NMR and EPR, fluctuations around the membrane director can be described as either static or dynamic mosaic spread dependent on whether the fluctuations occurs on a slower or faster timescale respectively [15, 28, 44]. In the case of a static mosaic spread this results in a spectrum containing a distribution of orientations, whilst a dynamic mosaic spread results in an averaging of the anisotropic interactions apparent in the spectrum. In the case of CD spectroscopy where the timescale is ~10^{-15} s, a timescale shorter than vibrations in bond length, any fluctuations about the bicelle director will manifest themselves as a static mosaic spread, and thus manifest in the CD spectrum as a summation of spectra arising from all orientations present.

It should also be noted that the results of the EPR experiments presented here are for pure lipid bicelles, and not those containing Fukutin. It may reasonably be expected that bicelles reconstituted with Fukutin are more likely to align when doped with lanthanides with positive diamagnetic anisotropy, since helical protein structures are well established to possess positive diamagnetic anisotropy. There is however no evidence indicating an improvement in alignment in the thulium doped samples by CD (Figure 9), and similarly no evidence of disruption of alignment in the samples doped with the negatively diamagnetic dysprosium. This suggests that the diamagnetic susceptibility of the Fukutin does not have a role in the alignment of bicelles under these conditions.

Despite the samples used for CD exhibiting identical compositions and the MCD field being higher than that employed for EPR studies, we acknowledge that we have no direct measure of

assessing the alignment in the CD instrument and it is conceivable that mechanical differences in sample geometry may influence alignment. We feel this is less likely however, as the dimensions of the EPR capillary and the CD cuvette are comparable.

An estimate of the distribution about the bicelle director at this field can be determined by measuring the order parameter S_{bic} from the EPR spectra recorded. Analysis of the splittings in Figure 6 results in a S_{bic} of 0.38 (for details of the calculations see Supplementary Material 8). If these fluctuations are modelled as fluctuations within a cone, this would lead to a distribution of bicelle directors over a range of angles up to 40°. Given that our estimates of the tilt of the Fukutin TMD is ~30°, it is clear to see that on the CD timescale a distribution of orientations is occupied that approximates closely to a powder distribution. Although the field applied for MCD measurements is higher than that used for EPR studies, comparable Q-band measurements (~1.2 T) have only increased the order parameter to 0.56 [45]. Clearly to utilise magnetically aligned oriented CD order parameters closer to those obtained from NMR are required, which necessitates significantly higher fields. We note that 8 T magnetic CD instruments are available, which approach fields used for NMR, but such studies were not possible within the scope of this work [46].

Conclusion

In this study we have established conditions for the preparation of bicelles which can be applied to both CD and magnetic resonance studies. These demonstrate the feasibility of using these complementary techniques to probe the structure of integral membrane proteins under near physiological conditions. Furthermore, we have identified bicelle preparations that permit their macroscopic alignment in the high magnetic fields used for NMR, as well lower fields typically employed in MCD measurements, preparations that would support analysis by oriented NMR

and CD/SRCD methods. This may have implications for studies of oriented membrane proteins by providing a more reproducible and straightforward approach than the preparation of mechanically aligned samples although careful consideration must be taken as to the dynamics that are present in these bicellar preparations.

Acknowledgements

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Figures

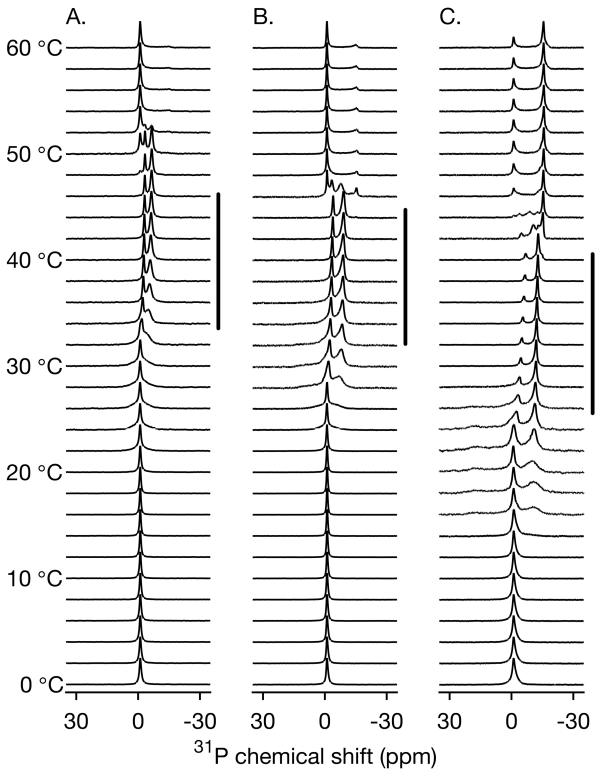

Figure 1. ^{31}P NMR spectra of DMPC/DHPC bicelles at 30 % w/v at q *ratios 2.5 (A.), 3.0 (B.) and 3.5 (C.) from 0 to 60 °C in 2 °C steps. Samples were incubated for five minutes at each temperature before measurement. The bars represent the regions that form magnetically aligned bicellar phases.*

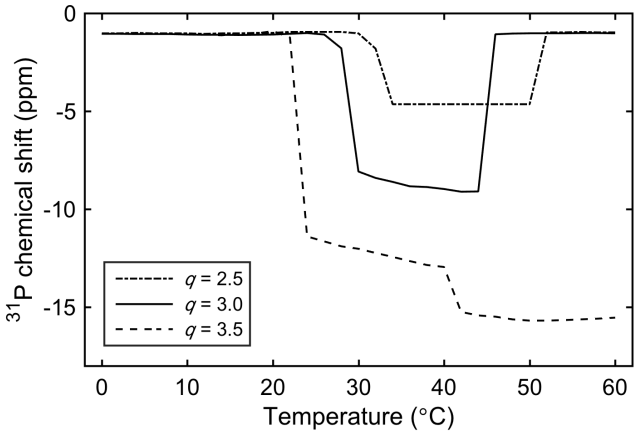

Figure 2. ^{31}*P chemical shifts of DMPC resonance for DMPC/DHPC bicelles at 30 % w/v with* q *ratios 2.5 (dash dot line), 3.0 (solid line) and 3.5 (dashed line) from 0 to 60 °C.*

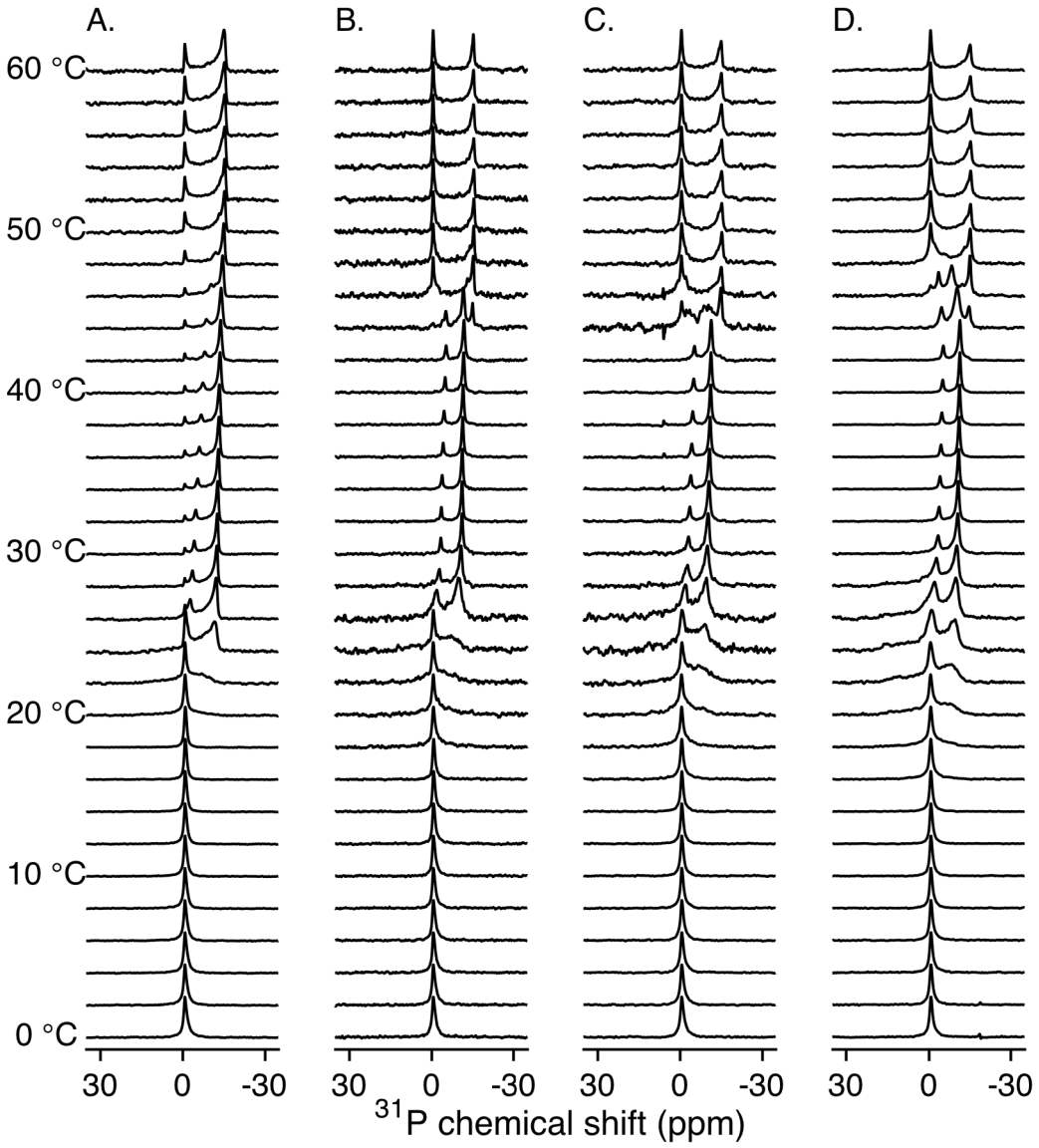

Figure 3. ^{31}*P NMR spectra of DMPC/DHPC bicelles at 5 (A.), 10 (B.), 20 (C.) and 30 (D.) % w/v from 0 to 60 °C in 2 °C steps. Spectra were acquired with 128 (20 and 30 % w/v) or 512 (5 and 10 % w/v) scans at each temperature.*

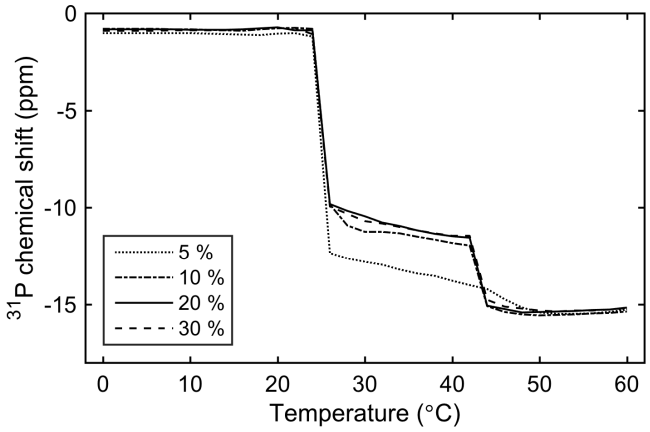

Figure 4. ^{31}P chemical shifts of DMPC in DMPC/DHPC bicelles at 5 (dotted line), 10 (dash-dot line), 20 (solid line) and 30 (dashed line) % w/v as a factor of temperature.

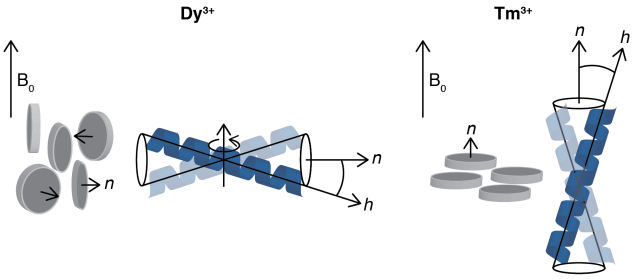

Figure 5. Representation of helix distribution in bicelles oriented either perpendicular (dysprosium) or parallel (thulium) for a protein inserted in the bicelle with long axis h. In MCD experiments the director of light propagation is collinear with the field director.

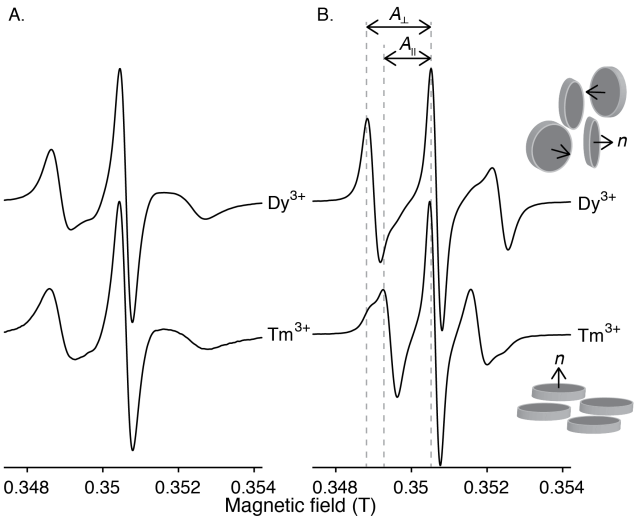

Figure 6. EPR spectra of CSL in DMPC/DHPC/DMPE-DTPA (3.5/1/0.023) bicelles at 20 % w/v with either dysprosium (top) or thulium (bottom). Spectra were acquired at 25 °C (A) or 45 °C (B) following temperature equilibration (as described in main text). Dashed lines indicate splitting for CSL aligned parallel to the field and dotted the for perpendicular. Spectra were acquired with a centre field of 0.3505 T, sweep width of 0.01 T, sweep time 30 s and frequency of 9.85 GHz. Temperature was confirmed using a fine thermocouple inserted in the sample.

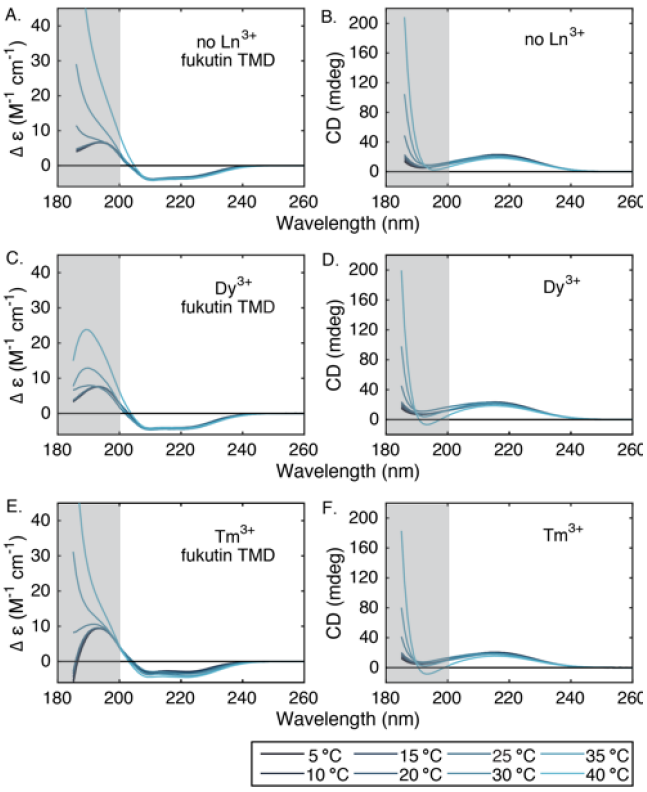

Figure 7. Conventional CD spectra of pure lipid bicelles (DMPC/DHPC/DMPE-DTPA, q 3.5, 20 % w/v) from 5 to 40 °C in 5 °C steps. Left column, spectra of Fukutin TMD in lipid bicelles; right column, equivalent pure lipid spectra. A. and B. are without lanthanide; each spectrum is an average of four scans. C. and D. are with dysprosium at 1:1 ratio with DMPE-DTPA; each spectrum is an average of two scans. E. and F. are with thulium at 1:1 ratio with DMPA-DTPA; each spectrum is an average of four scans. A 15 minute incubation was allowed at each temperature steps. All spectra were baseline corrected against H_2O and acquired with 1 s integration time and 1 mm slit width in a 0.1 mm pathlength fused silica demountable cuvette. The grey areas highlight the regions with the possible contribution from the lipids.

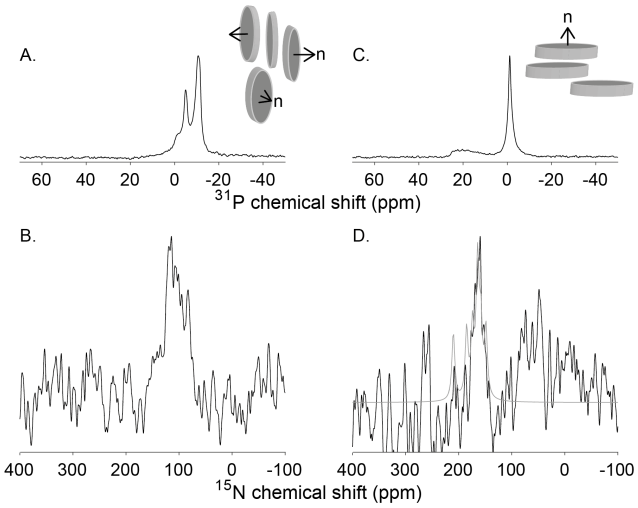

Figure 8. Spectra of bicelles containing $^{15}N_{leu}$ Fukutin TMD aligned either perpendicular (left) or parallel (right) to the magnetic field (14.1 T) at 40 °C. The lipid composition was DMPC/DHPC/DMPE-DTPA (3.5/1/0.023) at 30 % w/v with an L/P ratio of 50 in both samples. 20 mol % DMPC was exchanged for DMPC-d_{54}. For samples aligned perpendicular to B_0 no lanthanide was added, and to align bicelles parallel ytterbium was added at approximately 1.5:1 with DMPE-DTPA. To check alignment samples were measured using both ^{31}P (A and C) NMR. The protein orientation was measured using ^{15}N NMR (B and D), with 40000 and 12000 scans respectively. D. was fitted with a simulated ^{15}N spectra (grey line).

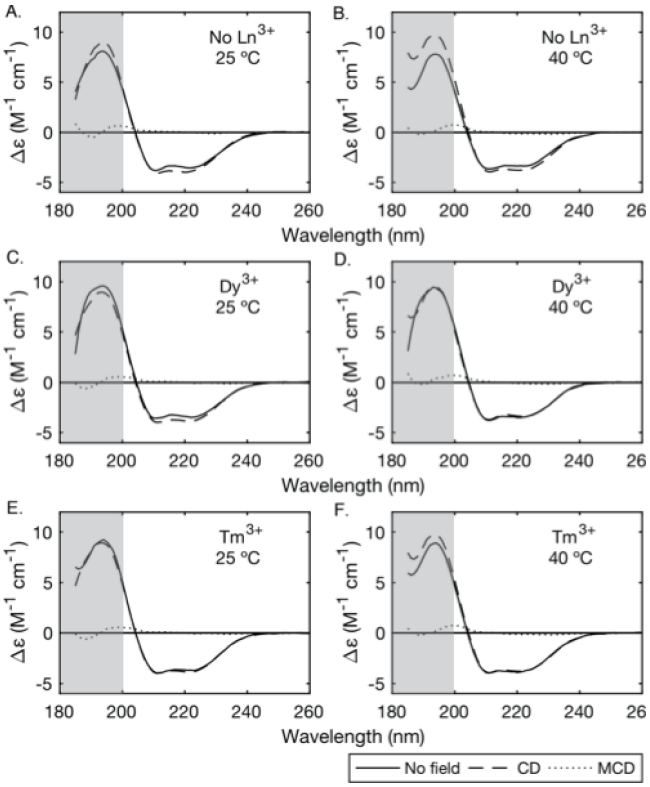

Figure 9. SRCD spectra of Fukutin TMD in DMPC/DHPC/DMPE-DTPA (3.5/1/0.023, 20 % w/v) in the presence and absence of a magnetic field.Samples were prepared either without lanthanide (A. and B.), with dysprosium (C. and D.) or thulium (E. or F.) at 1:1 molar ratio with DMPE-DTPA. A., C. and E. were at 25 °C whilst B., D. and F. were at 40 °C. Spectra were acquired without magnetic field (solid line) or with the 1.4 T field, and are presented as either CD (dashed line) or MCD (dotted line; see processing details in methods). The pathlength of the fused silica demountable cell was 0.1 mm, the slit width 0.5 mm and the integration time 1 s. The grey areas highlight the regions with the possible contribution from the lipids.

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Supplementary Material

Magnetically aligned membrane mimetics enabling comparable optical and magnetic resonance spectroscopy studies

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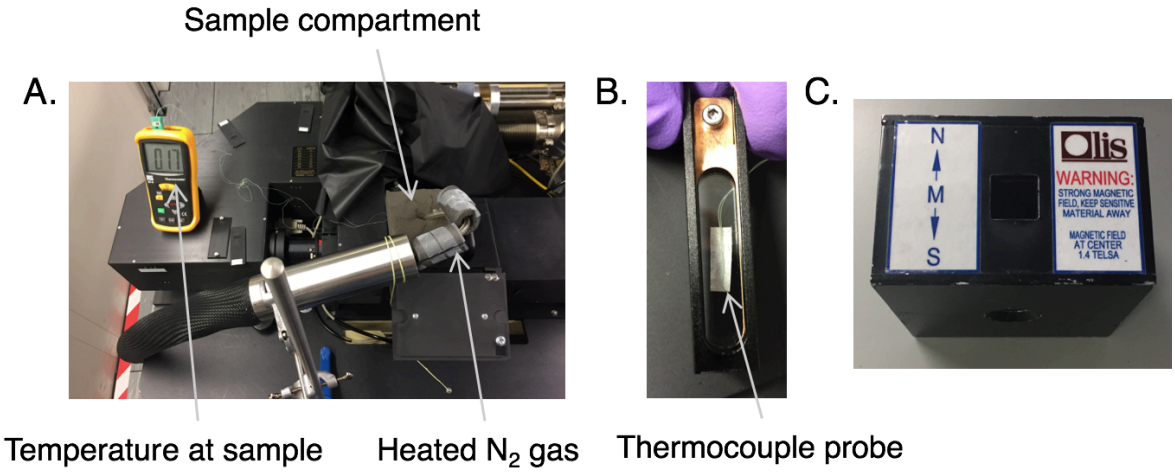
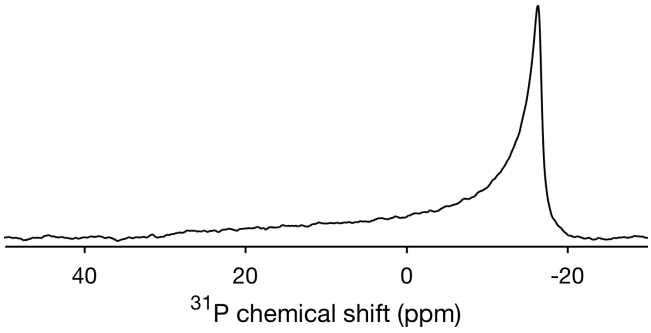
Supplementary Figure 1



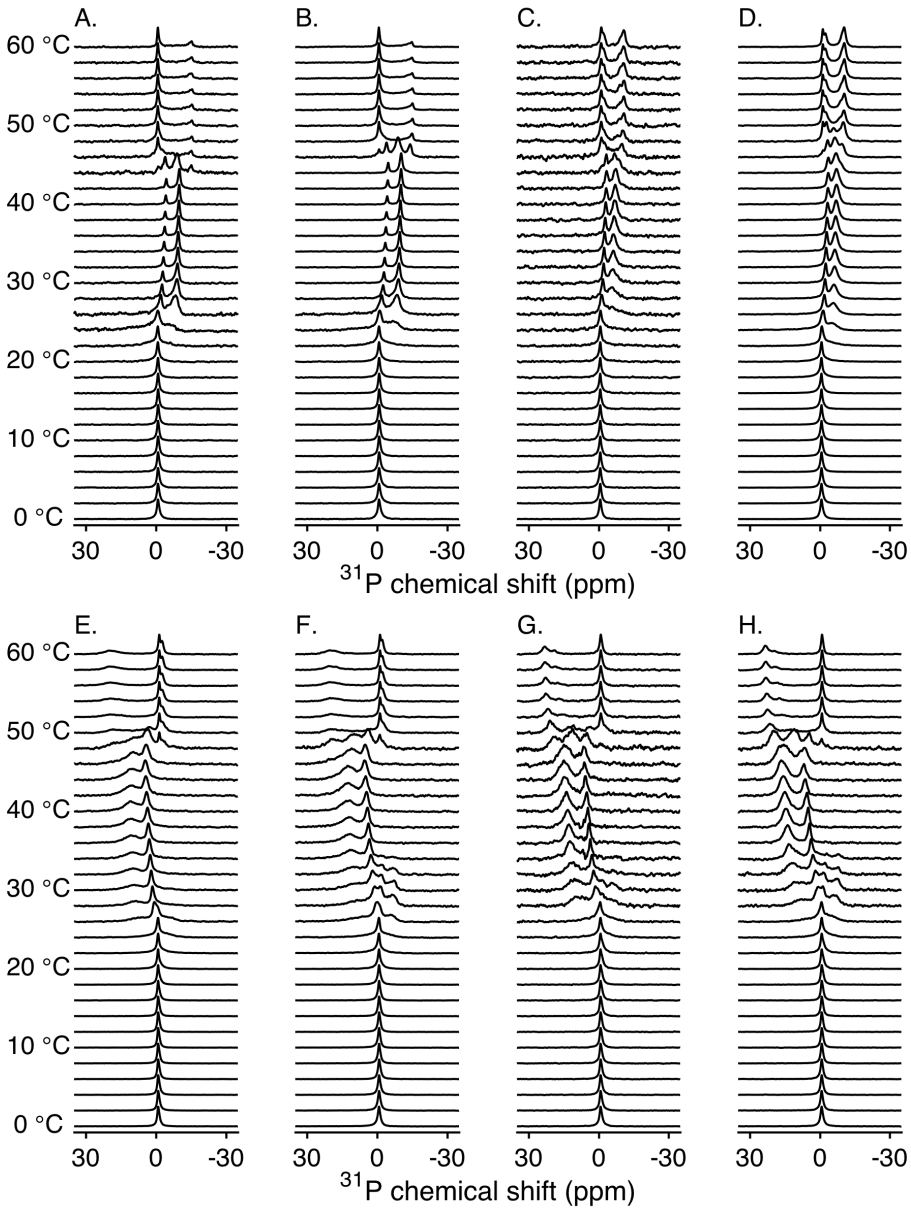
Supplementary Figure 1. Set up of magnetic CD instrument temperature control. A. *Sample compartment was supplied with heated nitrogen from a variable temperature controller (FTS Systems). The tube was passed through a foam support and rested between the magnet and reverse of the cuvette. B. A fine probed thermocouple was taped to the reverse of the cuvette to measure temperature on an external thermometer. C. 1.4 T magnetic sample holder used for magnetic CD studies. The magnetic field runs parallel to the light director. The magnet is removable and reversible to allow 'forward' and 'reverse' field directions.*

Supplementary Figure 2



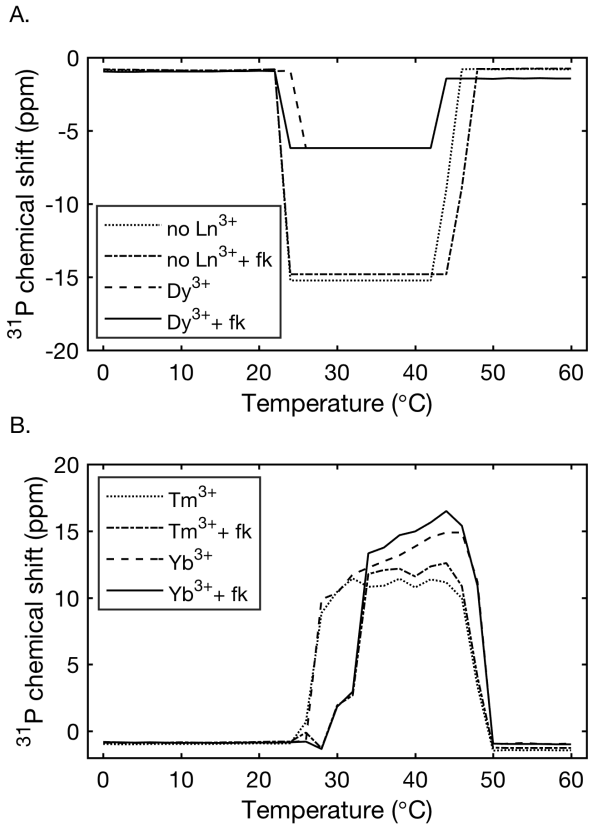
Supplementary Figure 2. ^{31}P NMR spectrum of DMPC multilamellar vesicles at 35 °C. Vesicles were prepared at 30 % w/v in ddH_2O. 1024 scans were acquired and processed with 50 Hz exponential line broadening prior to Fourier transform.

Supplementary Figure 3



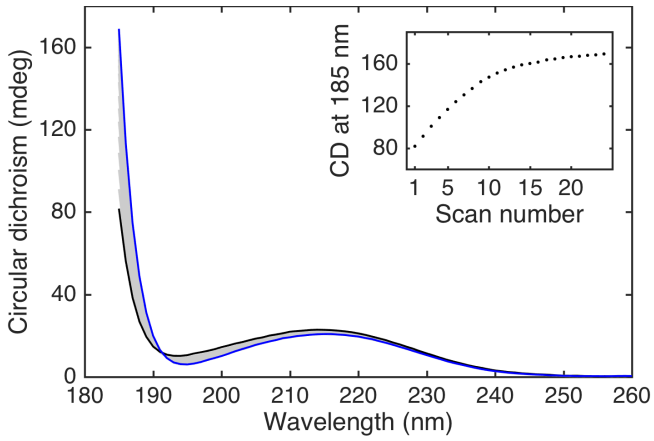
Supplementary Figure 3. ^{31}P NMR spectra of DMPC/DHPC/DMPE-DTPA bicelles (3.5/1/0.023) at 20 % w/v from 0 to 60 °C with and without fukutin TMD and lanthanide salts. Samples contained no lanthanides (A. and B.), dysprosium (C. and D.), thulium (E. and F.) or ytterbium (G. and H.) at 1:1 DMPE-DTPA:lanthanide. A., C., E. and G. were pure lipid, whilst fukutin TMD was present in B., D., F. and H. at 848 molar L/P. Either 256 or 512 scans were acquired for samples without or with lanthanides respectively.

Supplementary Figure 4



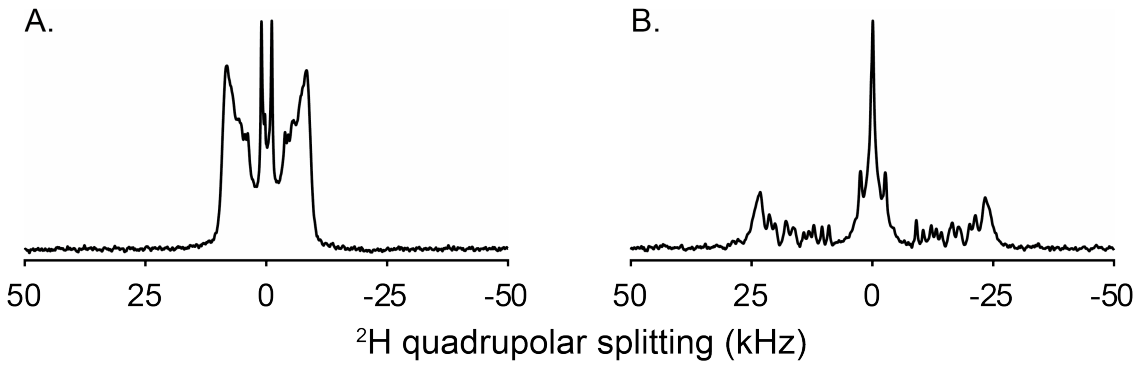
Supplementary Figure 4. ^{31}P chemical shifts of DMPC in DMPC/DHPC/DMPE-DTPA (3.5/1.0/0.023) bicelles with lanthanides thulium, dysprosium or ytterbium and with or without fukutin TMD (fk). A. Bicelles oriented perpendicular to the field prepared without lanthanide and without fukutin TMD (dotted line) and with fukutin TMD (dot-dash line), or with dysprosium without fukutin TMD (dashed line) and with fukutin TMD (solid line). B. Bicelles oriented parallel to the field prepared with thulium either without fukutin TMD (dotted line) or with fukutin TMD (dot-dash line); or with ytterbium either without fukutin TMD (dashed line) or with fukutin TMD (solid lines).

Supplementary Figure 5



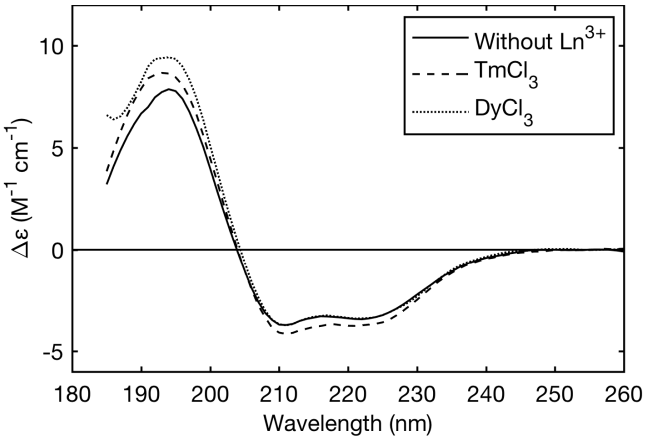
Supplementary Figure 5. Conventional CD spectra of DMPC/DHPC/DMPE-DTPA (3.5/1/0.023, 20 % w/v at 38 °C. A five-minute incubation was allowed after the temperature was raised from 25 °C to 38 °C, and each scan was approximately two minutes. Consecutive scans were acquired until the spectral intensity began to stabilise. First scan is coloured black and the last blue. Inset: plot of CD signal at 185 nm against number of scans. Spectra presented are baseline corrected against water. Samples were measured in a demountable 0.1 mm pathlength fused silica cuvette with 1 s integration time and 1 mm slit width that corresponds to about 1nm bandwidth.

Supplementary Figure 6



Supplementary Figure 6. Deuterium spectra of bicelles containing $^{15}N_{leu}$ fukutin TMD as per Figure 8 aligned either perpendicular (left) or parallel (right) to the magnetic field (14.1 T) at 40 °C. The lipid composition was DMPC/DHPC/DMPE-DTPA (3.5/1/0.023) at 30 % w/v with an L/P ratio of 50 in both samples. 20 mol % DMPC was exchanged for DMPC-d_{54}. For samples aligned perpendicular to B_0 no lanthanide was added, and to align bicelles parallel ytterbium was added at approximately 1.5:1 with DMPE-DTPA. To confirm ^{31}P NMR studies of bicelle alignment, 2H NMR spectra were recorded with a quadrupole echo sequence, with 4 μs π/2 pulses and a 40 μs inter-pulse delay. The spectra confirm the alignment observed in ^{31}P NMR studies and reveal comparable degrees of alignment with a S_{bic} of 0.61 and 0.83 for the samples aligned perpendicular and parallel to the magnetic field.

Supplementary Figure 7



Supplmentary Figure 7. SRCD spectra of fukutin TMD in DMPC/DHPC/DMPE-DTPA (3.5/1/0.023) at 20 % w/v in the presence of a magnetic field at 40 °C. Spectra are overlaid from Figure 9

Supplementary Material 8

We can estimate the effect of this distribution in the CD spectra using the order parameter from EPR, which has a closer field to the MCD instrument than NMR. Using the splitting from the EPR spectra in Figure 6 the order parameter S_{mol} can be calculated according to[1]:

$$S_{mol} = S_{33} [(3 \cos^2 \theta - 1) / 2]^{-1}$$
$$S_{33} = ((A_{\parallel} - A_{\perp}) / (A_{zz} - A_{xx})) (a_N / a'_N)) \quad (1)$$

where S_{33} defines the motion of the CLS long axis with respect to bilayer normal, a_N is the isotropic hyperfine splitting (1/3 (A_{xx} + A_{yy} + A_{zz})) and a'_N is the solvent polarity correction factor (1/3 ($A_{\parallel}$ + 2 $A_{\perp}$)), with the principle hyperfine tensors of CSL taken as 6, 6 and 32 G for A_{xx}, A_{yy} and A_{zz} respectively[1]. Since the angle between the bilayer normal and the CSL long axis (θ) is ~0°, $S_{33} \approx S_{mol}$. For the samples here we found S_{mol} = 0.38, which indicates there is significant deviation of the bilayer from the magnetic field director, giving a maximum θ of approximately 40°. Coupled with the cone opening angle of fukutin TMD in the bicelles, which the NMR data indicates could be ~30° it is easy to see that a wide distribution of orientations is possible. Although the field of the MCD instrument is greater than the EPR, even in Q band EPR (~1.2 T) an order parameter for a similar lipid mixture has been calculated as 0.56[2].

References

(1) Dave, P. C.; Nusair, N. A.; Inbaraj, J. J.; Lorigan, G. A. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2005**, *1714*, 141.
(2) Ghimire, H.; Inbaraj, J. J.; Lorigan, G. A. *Chem Phys Lipids* **2009**, *160*, 98.