



Circular Dichroism Spectroscopy of Membrane Proteins: A tutorial review

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Circular Dichroism Spectroscopy of Membrane Proteins

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Circular dichroism (CD) spectroscopy is a well-established technique for studying the secondary structures, dynamics, folding pathways, and interactions of soluble proteins, and is complementary to the high resolution but generally static structures produced by X-ray crystallography, NMR spectroscopy, and cryo electron microscopy. CD spectroscopy has special relevance for the study of membrane proteins, which are difficult to crystallise and largely ignored in structural genomics projects. However, the requirement for membrane proteins to be embedded in amphipathic environments such as membranes, lipid vesicles, detergent micelles, bicelles, oriented bilayers, or nanodiscs, in order for them to be soluble or dispersed in solution whilst maintaining their structure and function, necessitates the use of different experimental and analytical approaches than those employed for soluble proteins. This review discusses specialised methods for collecting and analysing membrane protein CD data, highlighting where protocols for soluble and membrane proteins diverge.

1.0 Introduction

1.1 Membrane proteins

Membrane proteins play essential roles in a wide variety of physiological functions, such as ionic regulation, molecular recognition, energy transduction and cell adhesion. It has been estimated that approximately 30% of all open reading frames in the human genome are membrane proteins and that they represent more than 60% of current drug targets¹.

Structural data are important for understanding how proteins achieve their functions, and therefore can provide insight into the nature of related diseased states, potentially leading to rational drug design; however the challenge of obtaining such data for membrane proteins is revealed by a quick review of the literature: At the time of writing this review, over 100,000 protein structures have been deposited in the Protein Data Bank (PDB)² of which less than 2000 are membrane proteins³. These figures are reflective of the many obstacles to studies of membrane proteins, including the difficulties in purifying membrane protein/detergent complexes in high yields and the challenges of maintaining functional viability and of coaxing amphipathic proteins into crystalline organisations. Consequently most structural genomics programmes have automatically excluded membrane proteins and consequently the proportion of known membrane protein structures relative to those of soluble proteins is decreasing. Hence the availability of other structural methods for characterising membrane protein structures and their interactions with ligands, lipids and other proteins is paramount. Circular dichroism (CD) spectroscopy is such a method, and has, to date, been utilised in more than 10,000 publications on

membrane proteins and peptides. This review discusses specific considerations that are essential for undertaking CD studies of membrane proteins, including the underlying issues that distinguish CD spectroscopic characterisations of soluble and membrane proteins, as well as special protocols for data collection and analyses when using CD spectroscopy to characterise membrane proteins.

1.2 Circular Dichroism Spectroscopy

CD spectroscopy is a well-established biophysical tool for the structural characterisation of biopolymers such as proteins and nucleic acids. The physical basis for the technique is the differential absorption of left- and right- handed circularly polarised light by molecules which either contain a chiral centre, or have a three dimensional structure that provides a chiral environment. Although the structural information obtained from CD is limited compared to that obtained by X-ray crystallography, NMR spectroscopy, or cryo electron microscopy, it is a valuable adjunct to these techniques and offers a number of advantages. These advantages include the ability to explore a wide range of solution conditions and temperatures, rapid data collection, and the consumption of relatively small amounts of sample material.

Protein secondary structural information can be derived from CD signals in the far ultraviolet (UV) wavelength region between ~240 and 190 nm due to the amide chromophores of the peptide bonds. There are two types of electron transitions responsible for the CD signals in this wavelength region, an $n \rightarrow \pi^*$ transition at around 222 nm, and $\pi \rightarrow \pi^*$ transitions (both parallel and perpendicular orientations) at ~208 and 190 nm. Far UV CD spectroscopy can not only be used to determine the secondary structure content of proteins but also to provide information on ligand-protein interactions and to monitor protein folding and unfolding. In addition, qualitative tertiary structural information can be obtained

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from the environmental-dependent CD spectra of protein aromatic residues in the near UV (250 nm to 300 nm) wavelength range.

Both the techniques and applications of CD and its enhanced version, synchrotron radiation circular dichroism (SRCD) spectroscopy, [see section 4.4] for the study of soluble proteins have been reviewed in recent years⁴⁻⁹ so this review concentrates on specific uses of the technique for studies of membrane proteins, and the unique experimental and analytical considerations (especially potential artifacts) that arise due to the particulate and hydrophobic nature of membrane protein samples, and how to minimise or mitigate these either experimentally or during analyses.

2.0 Differences Between CD Spectroscopy of Soluble and Membrane Proteins

2.1 Effects of Fold Characteristics of Membrane Proteins on Spectral Properties

Integral membrane proteins tend to fall into two major classes, alpha helical up-down bundles or beta barrels (Figure 1). The individual helices or beta strands tend to be longer than those found in typical soluble proteins, since they span the membrane, which is generally around 30-50 nm thick. This requires around 7.5 turns or more of an alpha helix (~25-30 residues), whereas the helices in soluble proteins tend to be on the order of ~10-12 residues or less. Likewise the beta sheets in membrane-spanning beta-barrel structures tend to be significantly longer than the average beta sheet lengths in soluble proteins. It was predicted early on that longer helices would produce hyperchromic effects in protein spectra¹⁰; however, later studies indicated that this effect is not very significant in peptides with secondary structural elements longer than 10 residues¹¹, so on the whole, it is unlikely to be a significant issue when analysing secondary structures from CD spectra of membrane proteins. The influence of this effect on the empirically-determined secondary structures of membrane proteins based on CD data can be further minimised if the reference dataset used includes a large variety of proteins with wide ranging examples of different secondary structural elements, as is commonly the case for modern bioinformatics-defined reference datasets^{12,13}.

2.2 Effects of Environmental Physical Characteristics of Membrane Proteins on Spectral Properties

CD spectroscopy has been widely used for the study of soluble proteins; however CD studies of membrane proteins have been more limited in their utility. A main reason has been that whereas soluble proteins form uniform isotropic solutions in aqueous environments, membrane proteins are sequestered into hydrophobic anisotropic and large particles such as liposomes or membrane fragments¹⁴, detergent micelles, small unilamellar vesicles¹⁵, and, more recently, nanodiscs¹⁶ and oriented lipid bilayers¹⁷. These types of objects can give rise to spectral artifacts which present problems during data collection, analysis, and interpretation. Three major types of effects need to be considered: solvent-induced wavelength

shifts, spectral effects of fold characteristics, differential light scattering, and absorption flattening⁶. The physical phenomena producing these effects, the effects they have on CD spectra and calculated secondary structures derived from CD data, and the means of mitigating these effects in membrane protein CD spectra are discussed in this section.

2.2.1 Solvent Shift Effect

2.2.1.1 The Phenomenon

Membrane and detergent environments tend to be hydrophobic and/or amphipathic, so they have different physical properties than aqueous solutions, producing different spectral characteristics for proteins embedded in them. Because secondary structure analyses usually entail empirical methods based on reference datasets of spectra derived from soluble proteins of known structures, they often do not take into account the different characteristics present in membrane proteins spectra. Most of the publically-available datasets only contain spectra of soluble proteins¹⁸ and any analysis of membrane protein spectra using them can be subject to large errors, due to the different "solvent" properties of the low dielectric medium of the bilayer.

The dielectric constant (~1-2) of the hydrophobic core of a detergent micelle or phospholipid bilayer in which a membrane protein is embedded is considerably lower than that of water (~80). This can cause both bathochromic and hypsochromic shifts in the CD spectrum of proteins measured in these environments compared to the spectra of proteins comprised of similar secondary structures but present in aqueous solution^{19,20}. The extent and nature of the shift depends on both which electronic transition in the peptide is examined, and on the relative location of the peptide bond with respect to the membrane environment. The direction and magnitudes of the shifts are ultimately related to the changes in the energy gap between the ground and excited states of the transitions, and the peak positions can vary substantially between the same type of secondary structure in aqueous solution and in membranes²¹. As the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions are differentially affected (Figure 2), the wavelength dependence on solvent dielectric is not a linear and hence cannot be corrected simply by shifting the entire spectrum. Such shifts in peak positions can have significant effects on secondary structure analyses, and tend to produce inaccurate results when using standard deconvolution methods with the commonly-used reference datasets derived from soluble proteins²¹.

2.2.1.2 Solutions/Corrections for the Phenomenon

Although an early study attempted to deal with this issue by deconvoluting and separately shifting the individual transitions²², this procedure was both computationally costly and difficult to enact due to the presence of both soluble and transmembrane domains in most membrane proteins. A more effective and simple solution was the creation of a reference dataset containing the spectra of membrane proteins. The SMP180 reference dataset [standing for soluble and

membrane proteins including data down to 180 nm – and hence usable also for SRCD data]²³ is comprised of 98 soluble protein spectra and 30 membrane protein spectra. It is available on the DichroWeb server²⁴ (<http://dichroweb.cryst.bbk.ac.uk>) for use with a wide range of deconvolution algorithms. Because it contains both soluble and membrane proteins, this reference dataset is also suitable for polytopic proteins that contain both membrane-embedded and water-soluble (extramembranous) subunits or domains.

2.2.2 Differential Light Scattering Effect

2.2.2.1 The Phenomenon

Suspended particles with dimensions that are large relative to the wavelength of the incident light will scatter a proportion of the incident light away from the forward direction thereby reducing the transmitted light reaching the detector in a spectrophotometer, thus increasing the apparent absorbance due to the sample¹⁴ (Figure 3). The extent of scattering is dependent upon both the size and the shape of the particle, as well as the refractive indices of the scattering object and the solvent. Light scattering is also wavelength-dependent and affects the low wavelength (high energy) data more drastically than the higher wavelength data. This manifests itself in a UV (unpolarised) spectrum as apparent absorbance in the wavelength regions where there is no chromophore absorption, and tends to produce a continuous spectrum with a sixth order wavelength dependence. This is commonly seen in UV spectra of membrane proteins which are measured in order to quantitate the amount of protein present from the A_{280} peak [see section 3.2.1]. It also has the effect of decreasing the signal-to-noise ratio of the data in the UV region and raising the low wavelength cutoff of the data [lowest wavelength at which the data can be measured with statistically-significant accuracy]. The effects of light scattering are more serious in CD spectra than unpolarised UV spectra, as the refractive indices of an optically active sample are different for left- and right- circularly polarised light, leading to differential light scattering, which can change both the magnitudes and ratios of the different peaks, thereby distorting the shape of the spectrum¹⁵ (Figure 4).

2.2.2.1 Solutions/Corrections for the Phenomenon

For (unpolarised) absorbance, the scattering component can be dealt with as follows: In the region above 310 nm (where there is no peptide chromophore absorption) the apparent absorption due to scattering $A_s(\lambda)$ at wavelength λ , can be expressed as a single power dependence: $A_s(\lambda) = k\lambda^{-n}$. A plot of $\log A_s(\lambda)$ versus $\log(\lambda)$ extrapolated into the far UV will give values for the constants n and k to provide a correction factor¹⁴. However, this is not useful for CD measurements, because of the different extinction coefficients for the left- and right- circularly polarised absorbances. So direct physical changes to the sample or experimental setup are required to minimise or eliminate the problem.

The simplest methods involve reducing the size of the particles so they are much smaller ($\sim 1/10$) than the wavelengths of the

UV light used in the investigation. Most detergent micelles are too small to exhibit substantial scattering in the far UV region of the spectrum. However, there may be some concern about the conformation of a protein in a micellar environment being different from that in a membrane due to the packing constraints imposed by the relative size, shape and charge of the detergent head groups and the length and composition of the hydrophobic tail groups²⁵. Hence whilst the dimensions of the micelle may be ideal for eliminating scattering, their physical characteristics such as their geometry may have deleterious effect on the protein structure. Furthermore, the functions of many types of membrane proteins (eg. ion channels) cannot be assessed in micelles so there is no way to ensure their structure is not perturbed.

Small unilamellar vesicles (SUVs)^{14,15} (~ 25 nm diameter) also tend to produce little scattering in the far UV region, and provide another solution. Such samples can be produced by mechanical means such as sonication or extrusion, but in very small vesicles the protein structure may also be distorted due by the process of producing the vesicles or by the curvature of the membranes. Alternatively the protein can be examined in bicelles, or nanodiscs, which can also have small dimensions relative to the wavelength of light used for CD studies. Again, however, there may be issues associated with protein integrity in such environments, and because the intra- and extra-cellular surfaces are not in separate compartments, some proteins such as channels also cannot be functionally assayed in these types of samples.

Alternatively it has been suggested that scattering effects may not preclude measurements in large lipid unilamellar vesicles (LUVs), a conclusion largely based on comparisons of a soluble protein in the presence and absence of lipid vesicles²⁷. However, in the test samples, the scattering particles (LUVs) were not themselves chiral, nor were the chiral objects (proteins) scatterers, meaning that the scattering would be non-chiral, and would naturally not be seen in the CD spectrum. Hence this may not have been an entirely suitable test for a chiral membrane protein in a scattering lipid vesicle. Nevertheless, their conclusions²⁷ that protein CD spectra obtained in the presence of LUVs could be used if the data were limited to wavelengths above 200 (or 215 nm, depending on the size of the LUVs) provides another option for avoiding the effects of light scattering. However, it is noteworthy that virtually all secondary structure analysis algorithms require the availability of data down to at least 190 nm (due to the number of eigenvectors of information present)^{28,29} so quantitative analyses in LUVs would not be accurate, but may be useful for the qualitative examination of large differences in more native-like environments.

A simple physical solution to the scattering problem is to locate the sample as close to the instrument detector as possible so that the light scattered at relatively large angles can be captured, eliminating the apparent effect on the spectrum. The detector acceptance angle (θ) geometry is defined in Figure 3. Different commercial CD instruments have different default θ angles, but most can be modified to enable large values of θ by moving the sample (or detector) so that

the sample cell is adjacent to the detector face. Most SRCD beamlines have this type of geometry as their default. The effectiveness of this procedure depends on the geometry of the scattering object, with empty and filled spheres, filaments, and discs producing very different 3-dimensional scattering profiles. However for “empty spheres” such as lipid vesicles, the scattering is generally within 90 degrees of the forward direction¹⁴, an angle that can usually be achieved by the appropriate positioning of the cell and detector.

A practical consideration issue when moving the detector to obviate the scattering measured from the sample is the sample cell geometry. All of the scattered light from a particular angle θ must reach the detector and not be subtended by the side edges of the sample cell. This thus requires that the use of circular rather than rectangular cells, as the sides of the latter would intersect some of the scattered light in the forward directions.

2.2.3 Differential Absorption Flattening Effect

2.2.3.1 The Phenomenon

Spectroscopic characterisations

of solutions assume a uniform distribution of chromophores within the sample (Figure 5, top). Hence for every slice (thickness element, or t) of the sample in the direction perpendicular to the light beam, the same number, on average, of proteins will be encountered. However, in membrane samples, proteins are usually not uniformly dispersed in the sample; rather they are localised to discrete regions of membrane, where the chromophores are concentrated, while the rest of the solution is depleted of proteins (Figure 5, bottom). This means that even though the proteins may be uniformly distributed in the membrane particles, and the membrane particles uniformly distributed throughout the solution, the local protein concentrations within the sample are not uniformly distributed, leading to a breakdown of the Beer-Lambert law. As a consequence, the absorbance (or in the case of CD, the CD signal) will be diminished or “flattened” relative to the signal for a corresponding amount of protein in a dispersed solution^{14,30} (Figure 6).

The amount of flattening is proportional to the extent of the sample non-uniformity. This would be less problematic if the effect were uniform across the wavelength range of the spectrum, in which it could simply be compensated for by a scaling factor. However, this is not the case because absorption (and thus flattening) is a function of the extinction coefficient of the sample at a given wavelength. Hence, the spectrum will not be uniformly flattened at all wavelengths. In an optically active sample the extinction coefficients at a given wavelength are different for left circular polarised light (CPL) and right CPL. As a consequence, the CD peaks are not only depressed with respect to samples at lower concentrations, but further distorted by this differential effect. Differential flattening will be more apparent for CD peaks with higher extinction coefficients (in most cases, this is the electron transition at 190 nm). Put simply, the higher the absorbance,

the more the flattening, thus not only making the overall spectral magnitude smaller, but also the magnitude of different peaks smaller by different ratios, thereby distorting the shape of the spectrum. The extent of the flattening will also depend on the relative concentration of the proteins in the particles, with higher concentrations producing more flattening, and on the geometry of the particles³¹.

An extreme example is the purple membrane containing the membrane protein bacteriorhodopsin^{10,30,31}, in which the proteins are close-packed into two-dimensional crystals. Relative a solution of isolated bacteriorhodopsin molecules, the spectrum of bacteriorhodopsin in purple membranes is not only much smaller, but the more intense peaks at ~190 and 208 nm are significantly depressed relative to the less intensely absorbing peak at ~222 nm. When compared with a dispersed sample of the protein in SUVs, or the back-calculated spectrum from the crystal structure, the spectrum of the highly concentrated protein patches are very different¹⁴ (Figure 6).

The extent of the flattening (q) at any wavelength can be expressed as the ratio of the absorbance (or ellipticity in the case of CD) of the spectrum of the protein in the membrane particle (A_M) divided by absorbance (or ellipticity) of the spectrum of the same protein in a completely dispersed form (A_I).

2.2.3.2 Solutions/Corrections for the Phenomenon

Obviously the simplest means of correcting for this phenomenon would be to completely disperse the protein in a form where there was one protein per particle. If the protein can be incorporated particles (detergent micelles, lipid vesicles, bicelles, nanodiscs, or membrane fragments) containing a single protein then the dispersed condition can be met^{30,31}. However, that condition is challenging to meet, even for SUVs. It cannot be achieved simply by sonication of larger particles, as decreasing the particle size whilst maintaining the lipid-to-protein ratio will not significantly affect the distribution of absorbing proteins. The complete elimination of flattening in SUVs requires lipid-to-protein molar ratios of around 2000. [see section 2.3]. However the CD signal from a sample with such a high lipid-to-proteins ratio will generally be compromised by the absorption of the phospholipid carboxyl groups which absorb strongly in the wavelengths of interest. So for membrane proteins there is often a trade-off of concerns for detergent effects versus spectral distortions produced by the particulate nature of lipid membranes.

2.3 Effects of Lipid-to-Protein Molar Ratios

There will inevitably be a compromise between the requirement to reduce solvent flattening by using high lipid-to-protein ratios, and the need to generate a measurable CD signal without overly raising the sample absorption due to high concentrations of lipid. Although lipids generally do not produce significant CD signals in the UV wavelength range used to study proteins, they do contribute to the overall (non-chiral) absorbance, and hence lead to less light reaching the detector after passing through the sample, and thus noisier

spectra. Using conventional CD instruments, lipid/peptide molar ratios are often used at 50:1 (or even lower) depending on the molecular weight of the protein or peptide under study, so that data may be collected down to 190 nm. However, such high protein (low lipid) concentrations may not be appropriate representations of what are found in a biological membranes, and may also encourage the aggregation of proteins within the bilayers, or may (especially in the case of surfactant peptides and proteins) result in membrane lysis³². [see section 4.4].

However, the high light flux of SRCD beamlines (which enables light penetration through relatively opaque samples) can mitigate against these problems by enabling the measurement of SUVs which have lipid-to-protein ratios of 250:1 or even as high as 2000:1⁶.

3.0 Preparations and Protocols for Measuring CD Spectra of Membrane Proteins

3.1 Sample Preparations

The ability to accurately measure and quantify membrane proteins samples clearly depends on removing or diminishing the optical artifacts noted in section 2.0. However, there are other important considerations when producing samples suitable for CD measurements. These include the spectral contributions of the non-protein components in the solution, and the protocols by which spectra are collected.

3.1.1 Detergents

Membrane particle size can be reduced by the solubilisation of membrane proteins in detergents to form protein-detergent micelles or protein-lipid-detergent mixed micelles, both of which tend to have considerably smaller dimensions than even SUVs. Some proteins remain structurally and functionally intact in detergent micelles. Although transporters and channels cannot usually be assayed functionally in these environments, if they or other membrane proteins are capable of binding ligands, this can be used to form the basis for a “functionality” assay. Many membrane-bound enzymes retain their catalytic functions in the presence of micelles.

The use of detergents during isolation and purification has been comprehensively reviewed²⁵ and will not be repeated here, except to note that detergents which are good for isolating and maintaining protein integrity are not always suitable for spectroscopic studies. Often mild (non-ionic) detergents are used for solubilising membrane proteins since they tend not to be denaturing. These include sugar based molecules such as *n*-dodecyl- α -D-maltopyranoside (DDM) and *n*-octyl glucoside (OG). Alternatively, zwitterionic detergents such as lauryl dimethylamine oxide (LDAO) or anionic detergents such as sodium cholate are suitable spectroscopically. However, these detergents are often not efficient for solubilising membranes, and hence for CD studies the protein may need to be exchanged into other more suitable detergents during the purification procedures. A number of popular detergents used for characterisation or crystallisation of membrane proteins, such as HEGA10, and the Triton series, need to be avoided however, as their structures contain chromophores that absorb in the far UV region.

Sodium dodecyl sulphate (SDS), whilst a harsh detergent that usually denatures proteins [see section 4.1.1], is suitable spectroscopically; however, it is unlikely to produce results that are biologically-relevant. Ultimately, the suitability of using any detergent should be assessed in advance of solubilising the protein, by measurements of protein-free detergent micelles on their own, and it is often useful to keep a list of suitable detergents that have been tested.

3.1.2 Buffers and Salts

For CD measurements in general, the components of a buffer system that absorb strongly in the wavelength region of interest should be minimised or substituted with a non-absorbing equivalent. This is more crucial when high concentrations of phospholipid also contribute to the sample absorption. Buffers for maintaining low pH such as citrate, acetate and glycine have carbonyl groups that absorb strongly in the far UV and should be used at the lowest possible concentration. Neutral or slightly acidic buffers such as tricine, MES, MOPS, and HEPES, which are often popular in protein preparations, also absorb strongly. On the other hand, phosphate buffers, which can be used at virtually any useful concentration, and bicarbonate or borate buffers can be suitable for many samples. Tris acidified with HCl can be used at relatively low concentrations (10-20 mM).

Chlorine anions are also problematic as they absorb strongly below 200 nm; even 100 mM sodium chloride in a 0.01 cm optical pathlength cell may increase the low wavelength cutoff to 190 nm or above, especially when combined with other absorbing compounds. NaF can be a useful substitute for NaCl (unless this interferes with the protein structure or function), as it exhibits very low absorbance in the far UV range. However, even higher salt concentrations are often needed in order to stabilise membrane proteins in solution. In all cases the buffer absorbance problem increases with optical pathlength, so carefully choosing a combination of concentration, pathlength and buffer/salt/detergent environment is essential for achieving low wavelength data with membrane proteins.

3.2 Spectral Conditions: Concentration and Pathlength

Protein concentration and optical cell pathlength are two (interdependent) parameters that need careful consideration whenever a CD experiment is performed. With shorter optical pathlengths there is less sample (and hence less buffer) in the light path, with a consequent reduction in buffer absorbance that can allow the collection of data to lower wavelengths. This is one of the simplest means of enabling data collection in the presence of high(er) salt and buffers. Commercially available standard Suprasil quartz cuvettes usually range from 1 cm pathlengths down to pathlengths of 0.1 cm. Demountable cells rather than sealed cuvettes of pathlengths down to 0.001 cm are available, and fortunately for the scattering issues described above, circular rather than rectangular geometries are available. This means the amount of buffer/detergent/salt in the light pathway will be 10³ times lower than in the sealed rectangular cuvettes, so the

absorbance of the baseline buffer will drop accordingly. However there will also be less protein present, and hence a smaller CD signal unless there is a compensatory increase in the required protein concentration.

The optimum concentration for soluble proteins in a transparent buffer such as sodium phosphate is in the range of ~1-2 mg/ml in a 0.005 cm cell.⁹ Since integral membrane proteins usually give rise to relatively large CD signals compared to the average soluble protein, a helical transmembrane protein at a concentration as low as 0.3 mg/ml may generate an acceptable signal in the same 0.005 cm cell, and will, under most circumstances, allow the resolution of the 190 nm peak. The spectral magnitudes of transmembrane beta sheet proteins are generally two to three times smaller and given a similar concentration useful data would only be obtained 0.01 cm cell.

The volumes required for 0.1 cm cuvettes can be as high as 200 μ l, whereas the volumes for the shortest demountable cells may be on the order of 15-20 μ l. These can still require prohibitive amounts of protein; however, specialty calcium fluoride cells with very short pathlength designed originally for use in SRCD spectroscopy, have the advantage of requiring only 3-5 μ l of solution for pathlengths of 0.002 or less³². With concentrations as low as 2 mg/ml needed, these are amounts of membrane proteins that are often achievable.

If the only option is to use the shorter pathlength cells but a high protein concentration cannot be produced without causing precipitation or aggregation, the small noisy CD signal can be enhanced by lengthening the averaging time or increasing the number of repeat scans, bearing in mind that the signal-to-noise ratio is proportional to the square root of the data collection time. However a spectrum with an overall magnitude of a few millidegrees cannot be entirely rescued.

3.2.1 Concentration Determination

The most common source of magnitude error during data analysis is due to inaccurate concentration measurements. Common methods such as Lowry, BCA and Coomassie blue binding can be precise but usually require some kind of calibration against other methods to render them accurate. Whilst quantitative amino acid analysis (QAA) provide a gold-standard method for quantitation, usually this is not possible for membrane proteins as the lipids and detergents present in the samples tend to clog up the analytical columns used, and hence commercial services are reluctant to analyze them.

However, the absorbance of aromatic amino acids at 280 nm (A_{280}) is a useful method that can be done on aliquots of the same sample that is used for the CD measurements, when one of the currently available devices using very small volumes such as the Nanodrop are used. The calculated extinction coefficient can be obtained from the protein amino acid composition using the ExPASy website (<http://web.expasy.org/protparam/>) and used to determine the protein concentration. However caution must be taken in the measurements of lipid-containing samples: rather than simply measuring simple the A_{280} , a far-UV spectrum (310-270 nm) must be obtained, as frequency-dependent light scattering from the lipids will increase the A_{280} , and hence the apparent absorption of

the protein. This can be remedied by the addition of 10% sodium dodecyl sulphate (SDS) to the liposomes, which will enable the measurement of A_{280} signal, but will also render the treated sample unusable for parallel CD measurements, as this detergent will denature the protein.

Before measuring the protein concentration (or indeed, the CD spectrum), samples should be micro-centrifuged to separate undissolved particles and precipitated protein, but not at such high g values as to cause a concentration gradient in the sample tube. In addition, if sample solutions are sent by airfreight, it is also advisable to degas the samples prior to measurements, as changes in air pressure can enable the dissolution of gases from the atmosphere, which later can slowly escape during data collection.

3.2.2 Optical Cell Pathlength Determination

Since deviations from optical cell pathlengths stated by manufacturers can represent another major source of error in calculated magnitudes of CD spectra during analyses of CD data, it is advisable to check the pathlength using either the dilution method (for long pathlengths) or the interference method³⁴ for pathlengths shorter than 0.01 cm. Often deviations of as much as 50% from the expected (provided) values can occur for short pathlength cells, although such deviations are rare for longer pathlength cells. The obvious extension of this is that the calculated ellipticity of the spectrum will error by a concomitant amount.

3.2.3 Monitoring the HT (or Dynode Voltage, or Absorbance) Signal

CD and SRCD instruments measure and provide graphical displays of the high tension (HT) or dynode voltage signals in real time, along with the CD spectrum. The HT maintains a constant signal from the detector as the photon flux varies with wavelength. It is therefore proportional to absorbance of the sample. When the measured absorbance increases above ~1.5 units, an insufficient number of photons reach the detector for accurate measurements to be made. Each CD instrument will have a characteristic HT cutoff point that corresponds to this level of absorbance. Usually this is around 500-600 mV, well below the HT saturation point where no detectable photons are reaching the detector.

When measuring soluble proteins under optimal conditions, the peptide absorbance at around 190 nm produces a wide peak in the HT signal and if this exceeds the cutoff point the CD peaks in this region will be depressed; this can be rectified by lowering the sample concentration or optical cell pathlength³ (see above). If those changes are not possible, then the CD spectrum reported should be truncated at the HT cutoff point determined for the instrument. This point can be ascertained experimentally by placing the same sample in two (or more) different pathlength cells, and after scaling to each other based on the pathlength, if the low wavelength peak(s) are diminished relative to the higher wavelength peaks, then it can be ascertained where the cutoff value lies (Figure 7).

For membrane protein samples, the absorbance of the lipids or detergents may mask the peptide absorbance at 190 nm and

the HT signal will have a smoothly rising profile showing a rapid increase to the saturation value at low wavelengths. A useful rule of thumb is any CD peaks situated at wavelengths less than 5 nm below the HT cutoff point should be suspect.

In addition, because membrane protein samples can tend to aggregate, precipitate or settle with time, or even be light-sensitive, it is important to monitor the CD and HT values at the lowest wavelength obtained during data collection. If either of these values varies during repeat scans of the same sample, then they may reflect changes to the nature of the sample, and should be a cause for concern with respect to integrity of the protein/lipid/detergent complexes, and a reason to identify new conditions for data collection. Indeed, good practice suggests that when repeat scans are made with the intention of averaging multiple spectra to improve the signal-to-noise levels, saving individual spectra during data collection rather than just the average spectrum, will allow monitoring of any changes that occur during data collection.

3.3 Data Collection and Processing/Analysis of Membrane Protein Spectra

When collecting CD data it is important to measure (and separately save) at least three replicate scans of both the sample and baseline. As well as enabling detection of sample changes as described above, the separate files can be used to monitor the presence of instrumental artifacts which may arise in a single scan and to calculate error bars associated with the instrumental measurements. The latter will be especially important in studies comparing two samples/conditions to determine if any differences seen are statistically significant. It is also important to ensure the sample and baseline are obtained relatively closely temporally, so if there is any instrumentation variation over time, it does not affect the net spectrum.

The next consideration is that the baseline should overlay the sample spectrum in the wavelength region between ~250 and 273 nm, where there is normally no detectable CD signal due to protein. If the spectra in this region are not co-incident, this indicates the presence of a difference where none should be present. Such differences can arise if the orientation of the sample cell is not the same for both the sample and baseline. Obviously using the same cell for both the sample cell for both sample and baseline is important because there is always some inherent dichroism signal due to the cell windows, however small; so it must be present to the same extent in both sample and baseline spectra, in order for it to be removed from the net spectrum by the processing step of subtraction. Other experimental features that can lead to non-matching samples and baselines are when the two plates of demountable cells are not assembled in the same relative orientations or aligned in the same way with respect to the light beam. A useful suggestion can be to put small fiducial marks on both plates of a demountable cell (outside the light pathway) and ascertain they are lined up for each usage. For demountable cells, which are particularly useful for the small samples usually available for membrane proteins, it is recommended that a list of the fill volumes (and identity) of each cell be kept so that these can be

accurately reproduced each time (since the fill volume can sometime influence the effective pathlength).

Measurement parameters, which depend on the type of instrument and the nature of the experiment, and good practice considerations have been the subject of a number of reviews^{4,7,9}. The wavelength range will optimally include a high wavelength of around 280 nm (to include the baseline matching region), to just below the lowest wavelength where HT saturation occurs.

Spectra can be processed using the software provided with the CD instrument, using commercial spreadsheets, or with specialised CD processing and analysis software such as CDTool³⁵. Guidelines for processing described in the CDtools manual available online (<http://cdtools.cryst.bbk.ac.uk/manual.pdf>) can be useful whatever software is used.

Empirical secondary structural analyses are reviewed in detail in reference 17. Least squares methods, principal component analysis or machine learning techniques are used in combination with a reference dataset of protein spectra to calculate a spectrum that best matches the experimental spectrum. The secondary structures of the reference proteins, derived from their crystal structures, are then used to produce the secondary structure content of the query protein. A goodness-of-fit parameter, such as the normalized root mean squared deviation (NRMSD), is calculated as a measure of the correspondence between the experimental spectrum and the calculated best fit spectrum. A low NRMSD value (<0.1) is a necessary but not sufficient condition for identifying an "accurate" answer. In general, the most accurate secondary structure determinations are obtained when the reference dataset used contains protein spectra similar to the spectrum under scrutiny; hence reference datasets containing membrane proteins tend to be most appropriate for analyzing membrane protein spectra for the reasons described in section 2.2.1. There are a number of online servers or downloadable programs that enable secondary structure analyses, but at present only DichroWeb²⁴ and CDPro³⁶ have reference datasets that include membrane proteins (30 out of 128 proteins in the case of the SMP180 reference dataset in DichroWeb, and 13 out of 56 proteins in the SMP56 dataset in CDPro).

4.0 Specialist Methods for Studying Membrane Proteins using CD Spectroscopy

4.1 Drug/Ligand Binding, Protein Folding, and Mutational Studies

CD spectroscopy can be used to monitor ligand binding if the binding results in a change in the secondary structure of the protein. Frequently, however, ligand binding does not have a significant effect on the secondary structure of the protein. However, ligand binding does often stabilise the structure of a protein, and this can be monitored using either chemical or physical denaturation methods.

Likewise CD can be used to make comparisons of wild-type and designed mutant membrane proteins (often created to test theories of ligand binding sites or functional roles of certain residues). Mutants often exhibit either no significant differences in their CD spectra from the wildtype protein (if the mutation affects a very local environment around the side chains without any major effects to the polypeptide backbone

conformation) or else they result in significant unfolding of the native conformation. In the former case, the CD spectrum alone will not provide useful information on the nature of the interactions, but as in ligand binding, mutations can often shift the equilibrium between conformations and stabilise or destabilise the protein fold. Several types of folding/unfolding studies (see below) can be used to detect such interactions. In the latter case where mutations shift the CD spectrum to that of a partially or fully unfolded protein⁸, CD can be a useful diagnostic for determining the suitability of the mutant for structure/function studies.

Other methods for studying drug and ligand binding of membrane proteins by CD spectroscopy, include using the near UV region of the spectra where signals due to the aromatic residues can be monitored to identify either changes in tertiary structure, or local changes around specific residues. Such types of studies have recently been reviewed³⁸.

4.1.1 Chemical Unfolding (Stability) Studies

Chemical denaturants such as guanidine hydrochloride and urea are commonly used to assess the stability of either apo-versus ligand-bound soluble proteins and/or effects of mutations (both with and with ligands added). They may or may not be effective in unfolding membrane proteins, and must be tested on a case-by-case basis. Ionic detergents such as SDS may also be used for such purposes with membrane proteins, but may have limited utility as they tend to irreversibly unfold membrane proteins. Ironically, for some proteins, the addition of SDS tends to produce more helical structures, as identified by CD spectroscopy, so just an increase in helicity may not be indicative of a correctly folded protein. In general, beta-barrel proteins tend to be more refractory to unfolding/refolding than are transmembrane bundles.

If the unfolding transition is fully reversible, such studies have greater value as they can be used to determine thermodynamic parameters associated with the protein stability³⁷. However, denaturants are not universally effective for all integral membrane proteins, and thus conditions must be determined experimentally for each protein.

Another concern for such studies on membrane proteins is that in order to completely unfold a protein, denaturants such as urea (and especially guanidine) may need to be used at concentrations that produce too high an absorbance to enable measurements below 210 nm, making them unsuitable for detailed studies using CD spectroscopy. Nevertheless, they can often be effectively used for monitoring changes to the helical content using the peak at 224 nm. An alternative, described in detail below, is to monitor the thermal stability of the membrane protein to detect changes associated with ligand binding and/or mutations.

4.2 Thermal Unfolding (Stability) Studies

Often ligand binding produces little change in the peak magnitudes at room temperature, and thus cannot be used to assess whether or not the compound has bound. However, ligand binding often acts to stabilise the protein (and can

discriminate between mutations that do and do not affect the binding interactions). This stabilisation can be assessed by thermal denaturation studies. Although in many cases the end result of thermal denaturation is irreversible aggregation, the relative stabilities of proteins in the system under scrutiny can still be determined.

Thermal denaturation studies have the advantage over chemical denaturation studies of leaving other environmental conditions such as ionic strength and pH intact (assuming the pH of the buffer itself is not temperature dependent!).

There are two general methods of conducting and analysing thermal assays: In the first a single spectral peak is chosen and its CD value monitored with increasing temperature. In this type of study it is important to ascertain in advance that the peak in question does not shift during the course of heating/unfolding the protein. The instrument software may even provide an option for monitoring a number of peaks simultaneously. An advantage of this method is that it is relatively rapid, and thus quite small temperature increments can be monitored. The second method is to measure the entire spectrum at each temperature (Figure 8). Time constraints usually impose a minimum limit on the size of the temperature steps; however a set of analysable CD spectra are obtained complete with associated HT signals providing much more information including differences in secondary structure content at each temperature. In addition, the presence of problems arising due to bubble formation during heating or precipitation that would not be obvious from a single wavelength scan can be revealed in the HT trace as described below. Obtaining three repeat scans at each temperature also provides evidence that the sample has equilibrated at each temperature since the replicate spectra should overlay. Another major advantage of measuring entire spectra is revealed by Figure 8. Here the protein becomes increasingly disordered with temperature and monitoring the peak near 224 nm would not be ideal because the peak wavelength is temperature dependent. Neither of the other peaks is a good candidate: the one at ~208 nm is both shifted and not clearly defined at higher temperatures and the 190 nm peak may be compromised by high absorption. However the entire set of spectra can be analysed using singular value decomposition⁴⁵, a mathematical method which reduces the data to a small number of orthogonal basis curves which, in various proportions, make up each spectrum³⁵. The advantage of this is that it can be determined if the unfolding of the different types of secondary structures are concerted, or sequential in nature.

4.2.1 Protocols for Thermal Stability Studies

It may be preferable to perform the experiment in a sealed circular cell because upon heating the changes in the contacts between plates in demountable cells may vary as the sample is heated, potentially leading to leakage [this can be monitored in the HT spectrum]. Alternatively, if the amount of sample volume available is limited or a shorter pathlength is required, it is possible to use demountable cells by including 5% glycerol in the buffer to increase the sample viscosity, providing a

remedy to sample egress. The glycerol will increase the sample absorption and may increase the T_m [the thermal denaturation midpoint] slightly (usually only by a fraction of a degree), but as most thermal assays are comparison studies of similar proteins or the same protein under different conditions, the addition of glycerol to all samples and baselines should mean that the samples can be effectively compared.

When using the method of monitoring single wavelengths, it is helpful to first measure the complete spectra at the lowest and highest temperatures in order to determine the trajectory of the various peaks, if there is enough sample. The concentration and pathlength can then be optimised to maximise the magnitude of the wavelengths to be monitored during the temperature ramp, and to determine if the peak positions shift as a function of temperature/unfolding. In the latter situation, single wavelength monitoring will not be an effective technique for data collection. As the peaks at 208 and 222 nm tend to decrease as the protein denatures with increasing temperature, a starting spectral magnitude of >10 mdeg at the peaks is advisable. It is often not possible to monitor the 190 nm peak throughout a melt experiment since the HT cutoff tends to increase by around 6 nm when the temperature increases from 20°C to 85°C, and, even if the 190 nm peak is resolvable in the first spectrum, it may become compromised by absorption flattening at higher temperatures. The lowest temperature is normally the ambient temperature, but can be lower, and the maximum temperature can be as high as $\sim 90^\circ\text{C}$, depending on the instrument. Data can best be fitted to a sigmoidal function if there are plateaux at both the beginning and end of the melt, bearing in mind that data is normally far from this ideal. Single wavelength experiments are relatively rapid, and hence, fairly narrow temperature steps can be used to accurately define where the transition is. After the highest temperature has been measured, and before cooling the sample, a full wavelength should be made so that the high and low temperature spectra can be compared, secondary structural analysis performed, and any anomalies in the HT signal of the hot spectrum taken into consideration. For thermodynamic analyses, the process should then be repeated in reverse, or at least the spectrum of the protein collected after the sample has been cooled to the initial temperature, to determine the extent of reversibility in the unfolding process. Comparisons of the HT spectra at the beginning and end of the experiment are useful indicators of whether the sample has aggregated or precipitated.

For experiments in which complete spectra are obtained at each temperature, measuring three replicate scans at each temperature will not only enhance the data but also provide evidence of whether equilibration has been reached at each temperature (ie. first and last spectrum at each temperature increment are identical). The disadvantage of this method is the time required for data collection, so larger step sizes (5°C or 10°C is standard) are typically used.

4.2.2 Processing/Analysing Thermal Stability Data

Single wavelength thermal melt data can be facily processed using spread sheets such as Excel or Origin to make plots using a sigmoidal fit (for a two-state process with no long-lived intermediates), or a three (or more) parameter fit when such intermediates exist to examine the nature of the unfolding using single wavelength data. Alternatively, the changes present in the entire spectrum can be assessed using singular value decomposition techniques such as that available with the CDTool³⁵ software. The latter is advantageous in that it can be used if a concerted (overall) change occurs or if different elements in the structure melt independently.

4.3 Oriented CD Spectroscopy (oCD)

In an alpha helix, exciton splitting of the $n\rightarrow\pi^*$ transition centered around 200 nm results in vector components parallel and perpendicular to the helix axis, giving rise to a negative 208 nm peak and the positive 190 nm peak. Isotropic solutions, where the protein can adopt all orientations with respect to the light beam, produce the typical alpha helical spectra with the 190 nm peak being roughly twice the magnitude of the 208 peak (if there are no significant amounts of other types of secondary structure present). In an anisotropic sample with helices oriented in specific directions with respect to the incoming light, the differently oriented transitions are either reinforced or diminished (Figure 9). As the relative magnitude of a transition vector depends upon its orientation with respect to the electric field of the incident beam, it is greatest when the transition is parallel to the electric field and zero when the vector is perpendicular to the electric field. The signal is therefore dependent on the relative orientation of the vector components and the direction of the electric field of the incident beam.

This is the underlying basis of the method of oriented CD spectroscopy of helical peptides in lipid bilayers⁴⁰, an increasingly popular method that can be used to define the orientation of a helical (or sheet) component in an oriented stack of bilayers containing membrane proteins or peptides. This method is the subject of an excellent recent review¹⁷ and so will not be discussed in detail here.

In general the sample preparation usually involves spreading a thin film of a concentrated sample on a quartz (or calcium fluoride plate, drying it and then rehydrating it by controlling the humidity of the environment. Generally, the phospholipids form stacks of bilayers parallel to the plate surface, with the peptides or proteins either aligned parallel or perpendicular to the membrane stacks⁴¹ (Figure 9), and the relative magnitudes of the peaks can be used to define the orientation and/or tilt with respect to the membrane. Transmembrane helices are oriented perpendicular to the electric field of the incident light and therefore the 208 transition, also perpendicular, has a low magnitude relative to the parallel transition. If the helices interact with the lipid bilayer such that they lie parallel to the magnetic field, then the opposite occurs. The average angle of the helix axis with respect to the membrane normal can be calculated from the ratios of the peaks^{40,42}. This has proven to be a very valuable method for examining antimicrobial and other surfactant peptides¹⁷.

4.3.1 Protocols for Collecting Oriented CD Data

Circular flat plates made of Suprasil quartz or calcium fluoride (the latter especially appropriate for SRCD measurements) are suitable substrates for creating oriented membrane samples. As these two types of materials have different wetting properties, use of one or the other may be more appropriate depending on the charged, uncharged or zwitterionic nature of the lipids in the sample, and whether the peptide/protein orients in a parallel or perpendicular manner. Films (especially for proteins) can be made by spreading aqueous suspensions of liposomes directly onto the plate and allowing them to dry or partially dry under ambient (or other) conditions. However, surface tension tends to draw the sample to the centre of the plate as the water evaporates, potentially resulting in films of uneven thickness and shape. More uniform films can be produced by drying peptide/lipid mixtures dissolved in organic solvents such as trifluoroethanol or ethanol/trichloromethane, but this of course, depends on the sensitivity of the peptides to the solvents. In this case, the film is dehydrated under vacuum for 2-3 hours before being placed in an environment with controlled humidity to produce specifically hydrated samples. This can be achieved by placing a small tray of a saturated salt solution [eg. potassium sulphate will produce a ~98% humidity environment, potassium chloride ~88%; sodium chloride ~75%] in a sealed container along with the film for 24 hours. The plate can then be placed in a specially constructed cell holder such as the one in ref³³, which has a central chamber for the saturated salt solutions, between two plates (one with sample and one without).

After the initial spectrum is measured, the cell holder is turned on its central axis by 45 degrees and another spectrum obtained. This is repeated turning the cell holder successively by 45° increments. This will both provide an indication of the film uniformity, and a comparison of the spectra at all angles can be used to determine if the sample exhibits any linear dichroism (which would contribute to and distort circular dichroism spectra). Many SRCD beamlines have automatically rotating cell holders, which can also be made as accessories for conventional CD instruments, although such rotations can be simply made manually, as long as the cell holder contains fiducial marks to monitor the rotation positions. Ideally the baseline should be a hydrated film of lipid without protein; however it is important to note that baseline samples consisting of the lipid alone can sometimes behave differently on the plate surface from the lipid/peptide complex; this case, a baseline of the plate alone (rotated as before) may be adequate as, in principle, the lipid should not contribute to the dichroism absorbance.

4.4 Synchrotron Radiation Circular Dichroism Spectroscopic Studies of Membrane Proteins

Over the past 15 years circular dichroism beamlines have been developed at storage ring facilities in the US, Europe and Asia, with a number of additional beamlines currently in the development/planning phase⁴³. Using synchrotron radiation as a light source greatly enhances the quality and quantity of data

that can be obtained from an experiment. Applications of SRCD spectroscopy for the study of soluble proteins have been the subject of a previous review⁵. However, CD spectroscopy using synchrotron radiation light sources has also been especially valuable for the study of membrane proteins, and its early advantages for membrane proteins were reviewed previously⁶.

In particular, the high light flux in the far and vacuum ultraviolet wavelength ranges enables measurements to lower wavelengths and drastically improves the signal-to-noise levels, which means that for membrane proteins which are often hard to purify in large quantities, small amounts of sample can be used, and the influence of other absorbing components (detergents, lipids) is less severe. The higher signal-to-noise levels that are attainable also mean that more subtle differences, for example in ligand binding studies, can be detected with confidence. As indicated in section 2.3, the high penetration of the intense light also means that higher lipid-to-protein ratios that can be examined, which has important consequences not only for diminishing absorption flattening effects, but also enabling proteins to be examined under conditions more comparable to those found in native membranes.

A number of novel types of membrane protein structural studies that would not have been possible with conventional CD spectroscopy, have been enabled by the use of SRCD spectroscopy: determination of the complete residue-by-residue structure of the CTD of a sodium channel⁴⁴ (Figure 10), dissection of the stabilities of transmembrane and extramembraneous domains for an integral membrane protein⁴⁵, and the novel use of SRCD as a molecular ruler for a membrane-protein substrate⁴⁶. In addition, SRCD studies of oriented samples (o-SRCD) provide much more accurate measurements of tilt angles of helices in membranes⁴². These suggest a strong potential for using SRCD to push the boundaries of the types of membrane samples that can be examined, and the types of questions that can be addressed with the technique.

It is particularly noteworthy that a large number of the high quality spectra of membrane proteins deposited and publically available in the Protein Circular Dichroism Data Bank (PCDDDB)⁴⁷ have been obtained using SRCD spectroscopy, a further indication of the utility and potential of this enhanced CD instrumentation for the study of membrane proteins.

Conclusions

Circular dichroism and synchrotron radiation circular dichroism spectroscopies can provide valuable information on the structure, function, and dynamics of membrane proteins in micelles, liposomes, nanodiscs, bicelles and oriented bilayers. However, careful attention must be paid to the experimental conditions and consideration of potential artifacts (such as scattering, absorption flattening, and spectral shifts) that can arise due to the physical nature of the samples.

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Figure Legends

Figure 1. Circular dichroism spectra typical of membrane proteins composed of different secondary structural types¹²: Predominantly antiparallel alpha-helical bundle (red: a sodium channel pore⁴⁸); predominantly beta barrel (blue: BTUB outer membrane cobalamin transporter²³), Mixed helical, beta sheet and unordered structure (green: WZA translocon for capsular polysaccharides²³). The CD spectra correspond to PCDDb⁴⁷ IDs CD0004012000, CD0000102000, CD0000128000, respectively. Inset are the crystal structures (PDB IDs 4F4L, 1NQE, and 2J58, respectively) of these proteins depicted in the same colour scheme.

Figure 2. Demonstration of spectral shifts observed for each of the different electronic transitions in membrane protein spectra relative to those in soluble proteins spectra. Membrane proteins (black spectra) and soluble proteins (grey spectra) were selected to have matching secondary structures in each case²¹. Left: Predominantly helical proteins. Right: Predominantly beta sheet proteins. In both examples the arrows indicate the peak positions (in black and grey, respectively) for the membrane and soluble proteins. It is notable that not all of the peaks shift in the same direction, nor to the same extent.

Figure 3. Diagram indicating the nature of the phenomenon of light scattering. I_0 is the light incident onto the scattering sample (red circles represent membrane particles). I_t is the transmitted light that impinges on the detector and is used to measure the light absorbed by the sample. I_s is light scattered in a direction that does not intersect with the detector, and contributes to the additional "apparent" (but not actual) absorbed light. φ is the acceptance angle of the detector and describes the angle of the scattered light that impinges on the detector.

Figure 4. Light scattering effects in CD spectra¹⁵: Effects of changing the detector acceptance angle (θ). Left: Sample (bacteriorhodopsin in octyl glucoside micelles) that does not exhibit scattering, measured at two different values of θ (2 degrees: dashed line and 90 degrees: solid line). It can be seen that the spectra are essentially identical. Right: Sample (bacteriorhodopsin in purple membranes) that exhibits scattering (2 degrees: dashed line and 90 degrees: solid line). In this case the spectra are very different, both in magnitudes and peak positions of the wavelength maxima/minima.

Figure 5. Diagram indicating the nature of the phenomenon of absorption flattening. The top panel depicts an isotropic sample, whereas the bottom depicts a membrane sample. The small circles represent proteins, whilst the large circles represent membrane particles. t is the cell pathlength, I_0 is the incident light on each sample. I_t is the transmitted light by the isotropic sample, whereas I_M is the transmitted light for the

membrane sample. $I_M/I_t = q$ (the flattening coefficient). In the limit of one protein per membrane, $q=1$.

Figure 6. Spectra depicting the effects of absorption flattening on the spectra of a membrane protein, bacteriorhodopsin, which has a primarily helical secondary structure¹⁴. Purple membrane fragments (large particles with low lipid-to-protein ratios, dotted line) where flattening is large, and small unilamellar vesicles (SUVs) (with high lipid-to-protein ratios, solid line) where flattening is negligible. These are compared with the spectrum calculated from the protein crystal structure (dashed line), which would correspond to an "unflattened" spectrum. It clearly matches the SUVs spectrum, but not the spectrum of the membrane fragments.

Figure 7. Depiction of the spectral effects for a sample in which the HT values are too high⁹. The SRCD spectrum (solid line) for the sample in a long pathlength cell (0.0020 cm) at 15 mg/ml and its corresponding HT curve (dashed line) are plotted in grey. The SRCD and HT spectra for the same sample concentration but in a shorter pathlength cell (0.0006 cm) are in black. The short pathlength spectrum is well below the cutoff limit at all wavelengths, whereas for the long pathlength cell the HT limit has been exceeded at wavelengths below ~ 210 nm ($HT > 5$). The black CD spectrum has been scaled to the grey CD spectrum at 224 nm, so that the relative depression in the CD signal for the high HT (~ 190 nm) peak can be seen. The cutoff effect on the CD spectrum for the long pathlength sample produces a significant overall distortion, with both smaller and narrower low wavelength peaks (corresponding to the high HT values).

Figure 8. Thermal unfolding experiment⁴⁹ for a membrane protein (Na,K-ATPase). Spectra were measured from 20°C to 80°C in 5°C steps. The largest CD spectrum (black) corresponds to the lowest temperature, and the smallest CD spectrum (also black) to the highest temperature. Intermediate temperatures are in grey. The CD peaks shift position as the protein changes conformation with increasing temperature. The HT curves corresponding to the CD curves progress from left to right with increasing temperature (ie. the HT signals at the low wavelengths increase with temperature), thereby changing the cutoff values (in this case occurring at a value of ~ 5) to higher wavelengths (~ 185 nm). In order to maximise the wavelength range measurable, the data was collected by SRCD spectroscopy with the concentration of the protein 4.01 mg/ml, and using a 0.0015 cm pathlength cell.

Figure 9. Oriented SRCD spectra, showing the effect of different helix orientations. The spectra⁴¹ are the synthetic peptides KALP (transmembrane orientation, solid line) and LAH4 (orientation in the plane of the membrane, dotted line), and of an isotropic, unoriented solution. The orientations of the peptides are depicted in the diagram above the spectra. The relative magnitudes of the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ peaks are modulated due to the direction of the beam relative to the orientation of the electric field (top diagram, right).

Figure 10. Illustration of a novel application possible only using SRCD spectroscopy⁴⁴. Spectra of the wildtype NaChBac sodium channel (solid line) and a mutant that was truncated by 21

residues (out of 274 residues total) (dotted line), showing the sensitivity of the method to small differences in structure. The error bars (1 s.d.) from three repeats show the precision/reproducibility of the measurements. Although the differences in the spectra are small they are detectable, and interpretable on a molecular level. The right panel is a magnified view of the 193 nm peak, which clearly shows the differences. These can be translated into secondary structural types of the missing residues.

Tables

Table 1. Suggested cell pathlength and concentration combinations for membrane proteins.

| Pathlength (cm) | 0.001 | 0.002 | 0.005 | 0.01 |
|--|-------|-------|-------|-------|
| Concentration (mg/ml) for mainly helical proteins | 2-10 | 1-5 | 0.3-2 | 0.2-1 |
| Concentration (mg/ml) for β -barrel proteins | 4-10 | 2-5 | 0.6-2 | 0.4-1 |

Figure 1

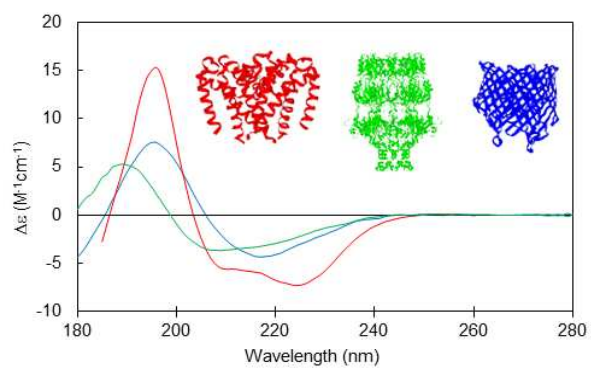


Figure 2

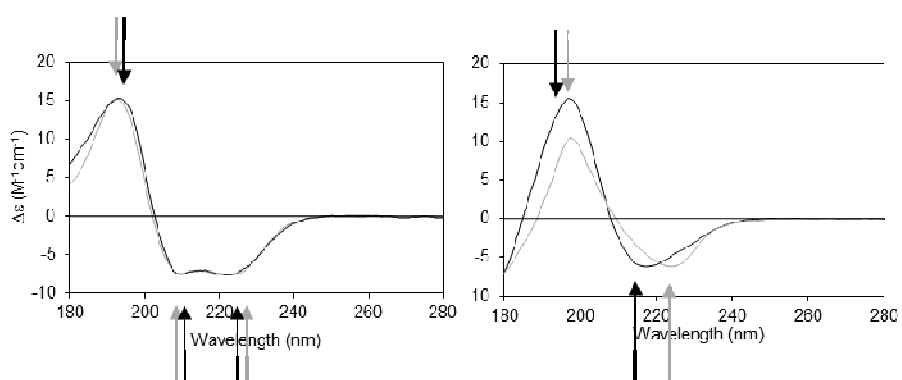


Figure 3

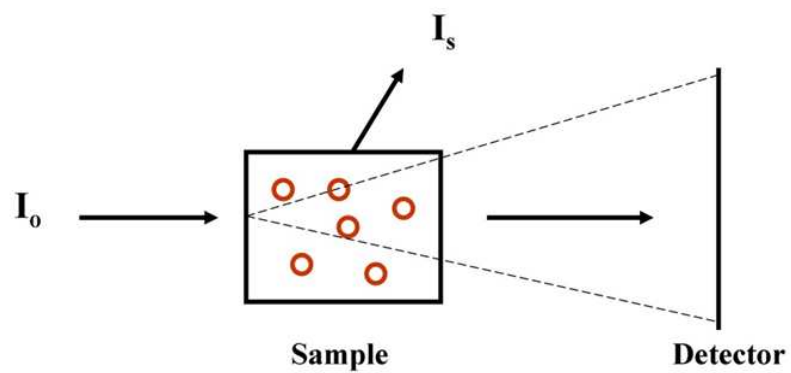


Figure 4

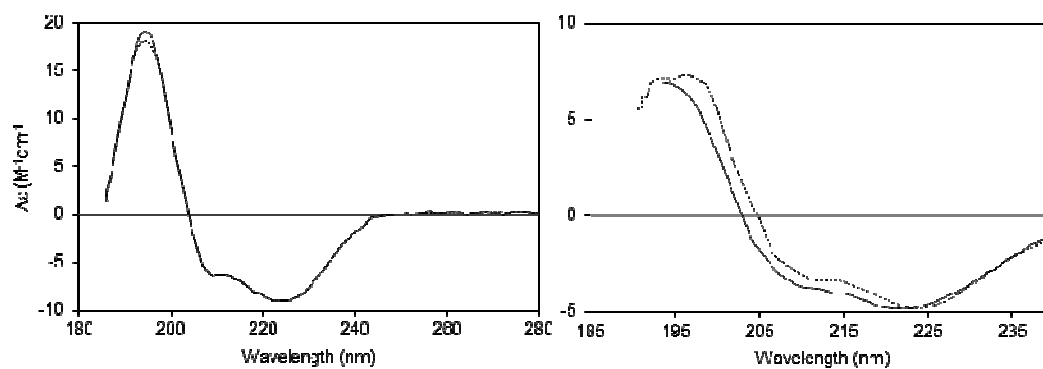


Figure 5

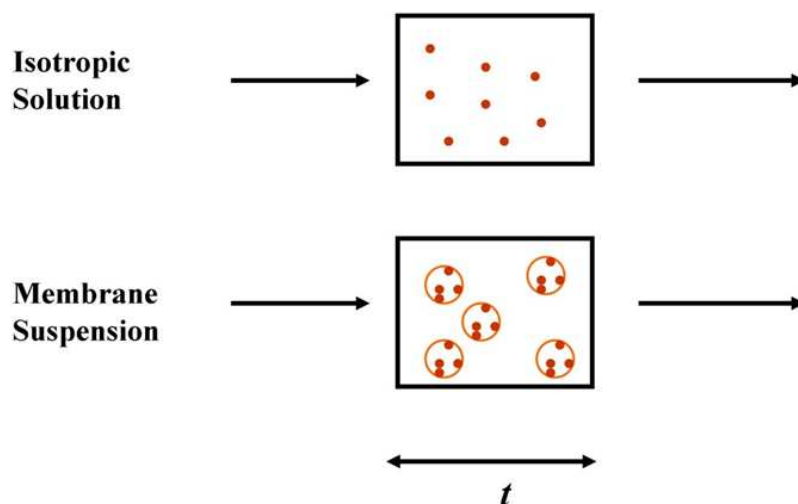


Figure 6

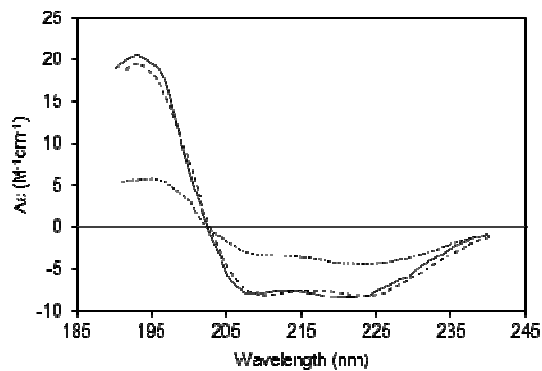


Figure 7

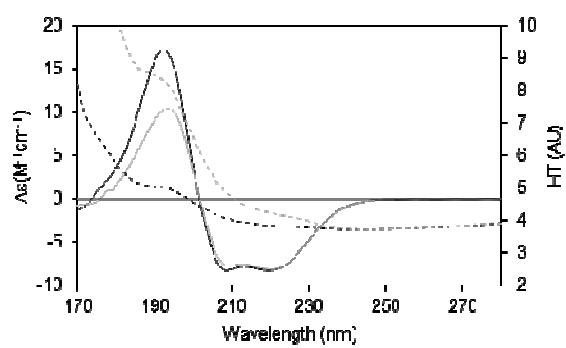


Figure 8

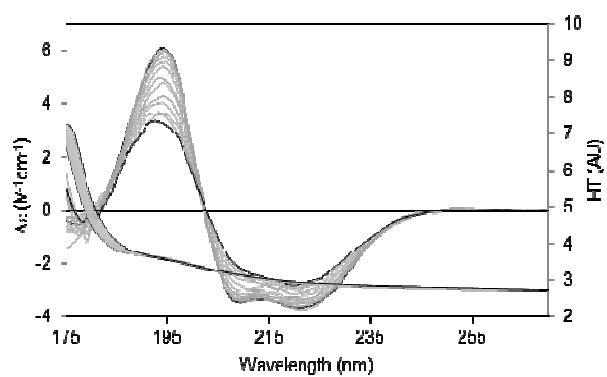


Figure 9

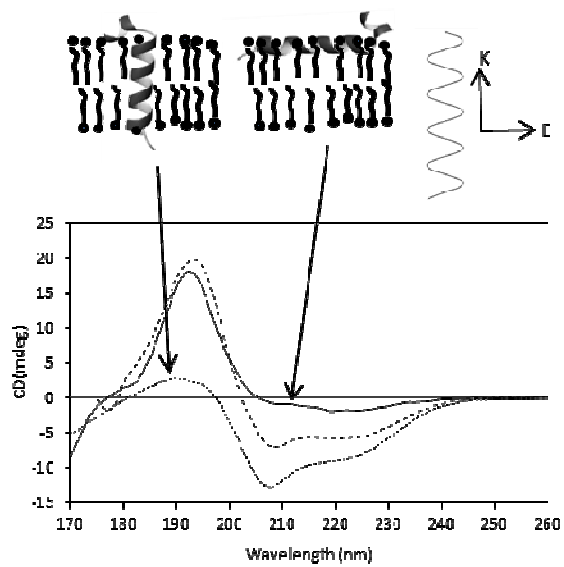


Figure 10

