Synchrotron radiation linear dichroism spectroscopy of the antibiotic peptide gramicidin in lipid membranes

Matthew R. Hicks,[a] Timothy R. Dafforn,[b] Angeliki Damianoglou,[a] Paul Wormell,[c] Alison Rodger*[a] and Søren V. Hoffmann[d]

We have developed synchrotron radiation linear dichroism (SRLD) to measure the insertion of peptides into lipid bilayers, significantly improving both signal-to-noise and wavelength range over existing methods. Our wavelength cut-off is currently determined by the quality of quartz in the cell, rather than the light source, with signal quality still high at the cut-off. We demonstrate the use of a lipid probe to measure the orientation of the lipid bilayers under flow and describe the way in which this can be used to further interpret SRLD data. The antibiotic peptide gramicidin is shown to exhibit drastically different kinetic and equilibrium behaviour when interacting with lipid membranes with different properties. The charge on the membrane is of interest because of differences in charge between human and bacterial membranes. For this reason we increased the negative charge on the membrane by changing the lipid composition. Increasing negative charge in the gel phase stabilises the liposomes but changes the kinetics of peptide folding. In a gel phase with no negatively charged lipids, gramicidin does not fold well and gives a small signal that indicates a change in orientation of the tryptophan side chains over time. In the fluid phase with no negatively charged lipids, there is initially >10-fold greater peptide signal relative to the gel phase indicating a highly folded and ordered gramicidin backbone. This is followed by liposome disruption. In the gel phase with negatively charged lipids the liposomes are resistant to disruption by gramicidin and exhibit different folding kinetics depending on membrane composition. In the fluid phase with negatively charged lipids there is little signal from either the peptide or the lipid probe indicating that the liposomes have been disrupted by the gramicidin in the time it takes to make the first measurement.

Introduction

Membrane proteins comprise ~30% of the proteins expressed in a cell, yet characterizing them structurally is a challenge because they generally require lipid environments for their structure and function. We have shown that we can use linear dichroism (LD) to determine the insertion, orientation and kinetic changes of membrane peptides and membrane proteins when they are mixed in aqueous solution with liposomes, a model membrane bilayer system. To date our data have been limited by the wavelength range we could access on benchtop spectropolarimeters. Intrinsic reductions in light intensity from a xenon arc lamp below 200 nm and the challenges of the light scattering by the liposomes combine to result in our data often only being reliable down to ~210 nm.[1]

Here we report LD measurements performed by aligning lipid membranes made from saturated phospholipids in solution and adding the antibiotic peptide gramicidin D. The light source is a high energy synchrotron radiation source rather than a xenon arc lamp. This gives an advantage of much higher light flux, particularly at wavelengths <200 nm, resulting in data even for light scattering samples (e.g. from lipids) being able to be measured because sufficient light still reaches the detector at all wavelengths. An additional advantage is that one can measure small signals that result from lipids that exhibit low efficiency of insertion of the peptide because of the excellent signal-to-noise compared with conventional instruments. The dynamic range of SRLD is at least an order of magnitude and often two higher than with benchtop instruments.

LD spectroscopy[2–8] is the difference in absorbance, by an aligned sample, of light polarized parallel and perpendicular to the alignment axis. The theory of this technique has been discussed in detail elsewhere[9] and is beyond the scope of this paper. The alignment required by LD spectroscopy can be carried out in various ways, e.g. stretching a film containing the chromophore, using magnetic fields or compressing a gel. However, we are generally interested in what happens in solution, so we have developed alignment systems that use shear flow following the early work of Wada and Kozawa[10] and later work of Nordén et al.[11] By placing the sample in the annular gap between a cylinder and a rod (orientated coaxially) and rotating either the cylinder

[a] Department of Chemistry, University of Warwick, Coventry, UK CV4 7AL. E-mail: a.rodger@warwick.ac.uk; matthew.hicks@warwick.ac.uk; Fax: +44 (0)2476 524112; Tel: +44 (0)2476 523234

[b] Department of Biosciences, University of Birmingham, Birmingham, UK B15 2TT. E-mail: t.r.dafforn@bham.ac.uk; Tel: +44 (0)121 4145881

[c] School of Natural Sciences, University of Western Sydney, Richmond, NSW, 2753, Australia. E-mail: p.wormell@uws.edu.au; Tel: +61245701445

[d] Institute for Storage Ring Facilities, University of Aarhus, Ny Munkegade, Building 1520, DK 8000 Aarhus C, Denmark. E-mail: vronning@phys.au.dk; Fax: +45 8612 0740; Tel: +45 8942 3781

or the rod, molecules that have a sufficiently large axial ratio and stiffness then orient. In this alignment regimen, molecules are induced to align without any appreciable damage to their structures. Recent technical advances have enabled the use of small sample volumes (<50 μL)[6,12] thus facilitating the use of LD for biological samples where it was not previously practicable.

Ardhammar *et al.* showed that LD can be measured for small molecules bound to membranes.[13,14] We have subsequently shown that this can be extended to membrane peptides and proteins[1,8] and to peptides and proteins with small molecules.[7] The physical basis for the alignment that underlies these measurements is centred on the observation that spherical liposomes become ellipsoidal in shear flow thus orienting with their long axes arranged circumferentially around the cell. The degree of alignment of the membranes as well as the LD signal from the peptide increases the information that can be gleaned from LD measurements. For this reason we have, in addition to the peptide, used a probe molecule to determine the degree of alignment of the liposomes themselves. This helps us probe the effect of the peptide on the lipid environment as well as enabling calibration of the LD magnitude. The use of a probe molecule in the lipid bilayer gives information on the degree of alignment of the bilayer and greatly assists in the interpretation of LD data as explained in more detail below. Ideally, a probe molecule for this application should have a high extinction coefficient at high wavelengths away from the signal from peptides and aromatic side chains, but have a low extinction coefficient in the peptide/side chain region. After testing several candidates[7] such a molecule was found from a commercial source (Invitrogen, Paisley, UK): β-DPH-HPC (2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine).

Gramicidin

The choice of gramicidin as a model compound is motivated by the problem of increasing antibiotic resistance in hospitals and in the general population which has renewed interest in the discovery of new antibiotic compounds. One class of antibiotics are peptides, such as gramicidin, that insert into bacterial membranes and kill the bacteria by destroying the ion gradient across the membrane. Gramicidin consists of 15 amino acid residues of alternating D- and L-isomers capped by a formyl group at the N-terminus and an ethanolamine group at the C-terminus. Gramicidin D, used in this study, is a naturally-occurring mixture of gramicidins A (~85%), B (~10%) and C (~5%) with position 11 (denoted X) being tryptophan, tyrosine or phenylalanine: HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-X-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH.

Obtaining information on peptide–membrane interactions is important as it underlies the key aspects of antibiotic peptides whose mode of action involves insertion into membranes. In particular we need to determine into which membranes a putative antibacterial peptide does insert since an effective antibiotic peptide for therapeutic use must not only be efficient in killing bacterial cells, but must not kill the host (human) cells. LD is uniquely able to probe membrane insertion in a wide range of different lipids, as opposed to less direct methods such as fluorescence where one has to assume (not always correctly) that, for

example, the tryptophan environment change equals membrane insertion. However, to date, LD results of sufficient quality to make such assessments have not been easily obtained.

Experimental

Beamline and apparatus

The beamline used was the UV1 beamline at the synchrotron radiation facility ASTRID at the Institute for Storage Ring Facilities (ISA) at Aarhus University in Denmark. The experimental set-up of the cell on the beamline was as described previously.[15] Briefly, the beam from the UV1 beamline was polarized by an MgF$_2$ Rochon polarizer. A photo-elastic modulator (PEM, Hinds Instruments, USA) produced alternating horizontal and vertical polarized light at a frequency of about 100 kHz, and the light was refocussed onto the capillary of the micro-volume cell by a 100 mm focal length Suprasil quartz lens. The micro-volume temperature-controlled LD cell was supplied by Kromatek (great Dunmow, UK) and mounted in a purpose-built chamber fitted to the beamline. The transmitted light from the cell was recorded with a photo-multiplier tube (model 9406B, Electron Tubes Limited, UK). The LD signal was recovered from the detector output using a lock-in amplifier.

Spectra were measured between 170 and 450 nm at 1 nm steps, using the Low Energy Grating of the UV1 beamline at a spectral resolution of about 1 nm.

Spectra were measured at a cell rotation speed of 3000 rpm and a non-rotating baseline spectrum was subtracted from this to account for the inherent LD signal of the system originating from the optics and the detector. SRLD spectra are reported in the output signal from the lock-in amplifier (in mV). In terms of Delta Absorbance units, 1 mV is approximately 6×10^{-4} ΔA.

Liposomes and gramicidin

In order to make liposomes, lipids (Avanti Polar Lipids, Alabaster, Alabama, US) were first dissolved in chloroform (99.8% spectrophotometric grade) (Sigma-Aldrich) at 10 mg/mL. The lipids used were 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DPPG). The lipid probe was β-DPH-HPC (2-(3-(diphenylhexatrienyl)propanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine) (Invitrogen, Paisley, UK) and was dissolved in ethanol at 1 mg/mL.

The lipid chloroform stocks were mixed to the required proportions and the lipid probe was added to a final concentration of 1% (w/w). The lipid/probe chloroform/ethanol mixtures were evaporated under a stream of nitrogen to give a thin film in a glass vial. The film was placed under vacuum overnight to remove residual organic solvent. Water (MilliQ 18.2 MΩ.cm) was then added to give a total lipid concentration of 5 mg/mL. The aqueous lipid suspensions were subjected to 5 freeze–thaw cycles between a dry-ice/ethanol bath and a 50 °C water bath with manual agitation during thawing. The lipid samples were then extruded 13 times at 50 °C with a Lipofast Extruder (Avestin, Ottawa, Ontario, Canada) fitted with 100 nm polycarbonate membranes.

Gramicidin D (Sigma-Aldrich) was used as supplied (assay 960 μg gramicidin/mg) and dissolved at 1 mg/mL in

trifluoroethanol (TFE; 99+%) (Sigma-Aldrich). Kinetic experiments were performed by mixing 2 volumes lipid to 7 volumes water to which was added and rapidly mixed 1 volume of gramicidin. This gave final concentrations of 1 mg/mL lipid, 0.01 mg/mL probe and 0.1 mg/mL gramicidin in 10% (v/v) TFE.

Stretched film experiments

Several 100 μL aliquots of chloroform were dropped onto a film of polyethylene, from a Gilson pipette tip bag (Anachem Ltd, Luton, UK) that had been stretched to an axial ratio of 3 using a custom-built film stretcher and it was then placed in a Jasco J-815 spectropolarimeter at room temperature with the long axis horizontal (defined as the parallel direction). The LD spectrum was measured using a scanning speed of 100 nm/min with a response time of 1 second. The band width was 2 nm and the data pitch was 0.2 nm. This spectrum was used as the baseline measurement. The probe molecule (β-DPH-HPC) was dissolved in chloroform at a final concentration of 0.1 mg/mL. Several 100 μL aliquots of probe dissolved in chloroform were placed on the same film and allowed to dry in air and the LD spectrum was measured in the same way as the baseline. The reported LD spectrum is the sample's spectrum minus the baseline spectrum.

Results and discussion

Probe molecule transitions

In order to measure the direction of the transition moments of the probe molecule we performed stretched film experiments on a conventional LD instrument as described in the Experimental section. The molecule is oriented with its long axis in the parallel polarization direction on the stretched film. The stretched film LD signal is positive for all of the transitions in the region from 300 to 400 nm (Fig. 1A). LD is defined as

$$LD = A_{\text{parallel}} - A_{\text{perpendicular}} = A_{\text{isotropic}} \frac{3S}{4}\left(1 - 3\cos^2\beta\right) \quad (1)$$

where β is the angle the transition moment of interest makes with the normal to the cylinder surface (*i.e.* to the lipid long axis) and S is the orientation factor that denotes the fraction of the liposome that is oriented as a cylinder perfectly parallel to the flow direction.

This means that the transition moments in this region are polarized along the long axis of the molecule (*i.e.* a large absorbance in the parallel polarization direction). Thus, if the lipid probe is inserted into the lipid bilayer with its long axis parallel to the membrane normal (*i.e.* in the perpendicular polarization direction) the probe should then give a negative signal in aligned membranes (Fig. 1B). Conversely, if it binds on the membrane surface then the signal will be positive.

Gramicidin in zwitterionic lipids

The effect of the phase transition temperature on gramicidin insertion into lipid bilayers was investigated using liposomes made from the zwitterionic, saturated lipid DPPC. The gel-to-liquid phase transition temperature for this lipid is 41 °C.[16] When gramicidin was added to the DPPC liposomes at 30 °C (below the phase transition temperature) a negative LD signal around 234 nm is observed at time $t = 0$ (Fig. 2A). This shows that the
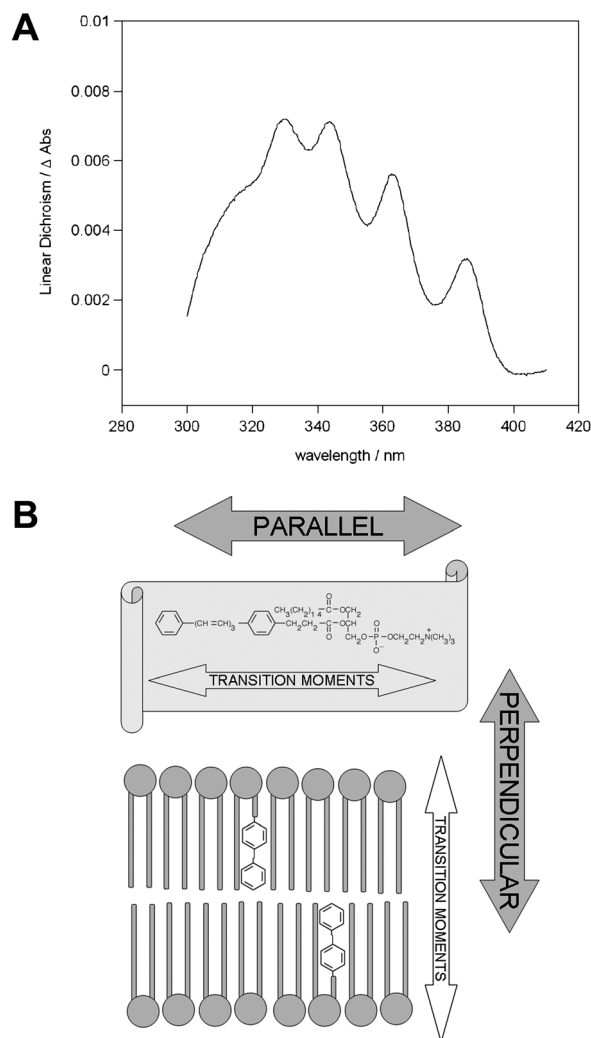

Fig. 1 Probe molecule. (A) LD spectrum of β-DPH-HPC on a stretched polyethylene film. The long axis of the probe molecule is aligned with the parallel polarization direction. The positive LD signals mean that the transition moments are along the long axis of the probe molecule. (B) Schematic of the probe orientation in stretched film (positive LD) and in lipid membranes (negative LD signal).

polarization of the transition(s) at this energy is pointing more parallel than perpendicular to the membrane normal. Intensity in this region is due to a combination of the n–π* transition of the peptide bond and transitions of the many tryptophan residues[17] which are probably coupling to shift them to longer wavelength. For a discussion of this phenomenon see Woody.[18] The 234 nm negative signal is almost certainly dominated by the tryptophan B_b band, indicating that the tryptophan average long axis is initially parallel to the membrane normal. After two hours the tryptophans have reoriented so that their long axis is now perpendicular to the membrane normal. Note that the absence of aromatic signals around 270–290 nm (expected to be about 10% of the intensity of the B_b band) would be consistent with the tryptophans being tilted so their short axis is perpendicular to the membrane surface (and the transition moments lying close to the magic angle of 54.7°, where $3\cos^2\beta = 1$ (eqn (1))). The small signals at low wavelength are consistent with a lack of structure
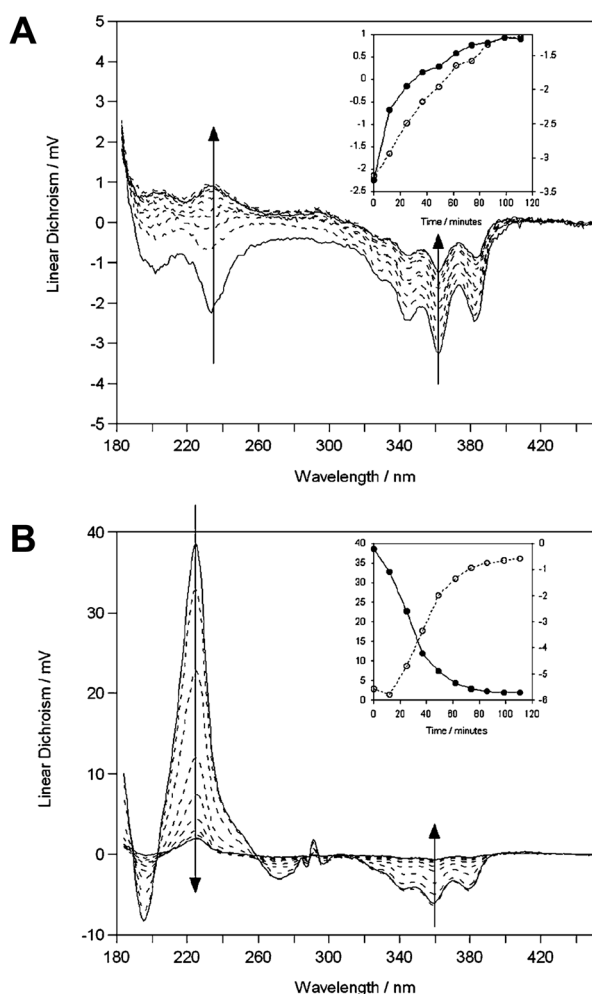
Fig. 2 (A) Kinetics of insertion of gramicidin D into DPPC/β-DPH-HPC (100 : 1 w/w) liposomes at 30 °C. The inset shows the signal at 234 nm (—●—, left hand scale) and 362 nm (---○---, right hand scale) as a function of time. The signal from 300 to 400 nm is from the β-DPH-HPC alignment probe. (B) Kinetics of insertion of gramicidin D into DPPC liposomes at 50 °C. Inset shows the signal at 225 nm (—●—, left hand scale) and 362 nm (---○---, right hand scale) as a function of time.

in the peptide backbone and/or a lack of orientation of the peptide relative to the lipid bilayer. This may be a result of the closely packed palmitoyl chains in the lipid (below the phase transition temperature for DPPC) excluding the peptide from the bilayer.

When the experiment with DPPC was repeated at 50 °C (*i.e.* above the phase transition temperature), the gramicidin LD signal is much bigger than that observed at 30 °C (Fig. 2B). The order of magnitude of the probe signal, however, is similar at 30 and 50 °C, showing that the change is due to the peptide–lipid interaction not the liposomes themselves. The signals from both the peptide and the lipid probe reduce in magnitude with a sigmoidal profile and a $t_{1/2}$ of around 30 minutes (Fig. 2B, inset). In this case there is no evidence of change in peptide orientation within the membrane. Also clearly visible in the SRLD is the aromatic signal (L_b band) with vibronic contributions around 280–290 nm[17] with several peaks indicating that there is significant orientation of the tryptophans in contrast to

the 30 °C experiment. The 272 nm negative LD of the L_a band indicates that the line through the indole nitrogen in the plane of the tryptophan is more parallel than perpendicular to the membrane normal; the bond from the tryptophan to the helix axis (which is approximately the 290 nm L_b polarization) is more perpendicular to the membrane normal. The 225 nm band is positive which is consistent with the n–π* backbone transition being perpendicular to the membrane normal as required for insertion of a helix. The contribution from the tryptophan B_b transition is likely to be small because for the L_a and L_b transitions to be strongly negative and positive respectively, the B_b transition will be polarized around the magic angle.

The large signal obtained for the 50 °C experiment means that, coupled with the good signal-to-noise that we obtain with the SRLD optical set-up, we can investigate a significantly wider peptide concentration range than when using conventional LD instrumentation. The concentration dependence of the rate of binding/insertion into the membrane is itself important as it enables one to deduce whether steps within the process are association- or dissociation-driven.[1] Given the limited pathlengths available for Couette flow LD (typically 0.1–1.0 mm), the dynamic range of SRLD is important in order to build up a profile of how the peptide kinetics change with concentration.

Gramicidin in negatively charged lipids

To investigate the changes in peptide–lipid interaction that may be affected by changing the charge on the surface of the membrane,[19] we prepared liposomes with DPPC (zwitterionic), DPPG (negatively charged) and β-DPH-HPC probe in the ratio 90 : 10 : 1, respectively. The spectral shape and kinetic profile obtained when gramicidin was added to these negatively-charged liposomes were different from those obtained in the absence of a net charge on the membrane. The peptide backbone transitions were at 189, 203 and 225 nm indicating that there is a different structural form of gramicidin in the mixed membrane compared with the DPPC/probe membrane (Fig. 2 *vs.* Fig. 3A). The kinetic profile of the 30 °C insertion of gramicidin into the DPPC/DPPG/probe membrane is not a simple process (Fig. 3A, inset). The peptide backbone LD first increases and then decreases. The probe molecule decreases then levels off. Note that the peptide signal continues to change after the probe signal has stopped changing. Since the probe molecule gives a measure of liposome alignment that can be affected by changes in the size of liposomes, this shows clearly that the changes in the backbone LD signal are not just due to changes in liposome alignment. Liposomes containing negatively charged lipids are not stable in a 50 °C experiment since we observe no signal from the probe or from the peptide (Fig. 3B). Note that without the use of the lipid probe one might conclude that there was simply no insertion of the peptide.

Upon increasing the negative charge on the lipid membranes the kinetics in the gel phase change again, with stable liposomes (measured by the probe signal around 362 nm) but a small and decreasing signal from the gramicidin (Fig. 4A). At 50 °C, again we see that in the presence of negative charge the liposomes are not stable (Fig. 4B) with no appreciable signal from either the peptide or the probe.
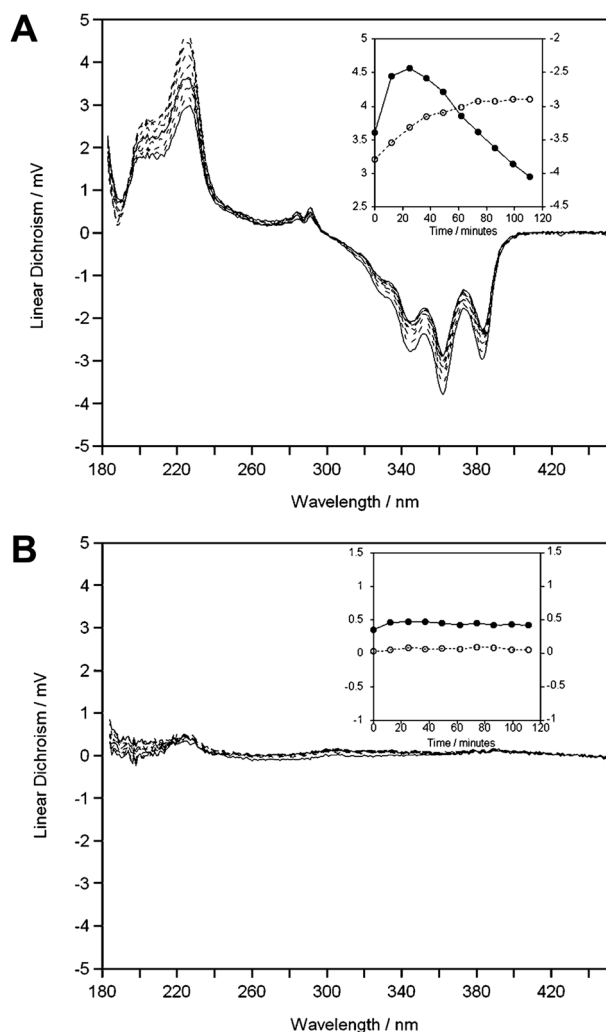
Fig. 3 Kinetics of insertion of gramicidin D into DPPC/DPPG/β-DPH-HPC (90 : 10 : 1 w/w) liposomes. Inset shows the signal at 225 nm (—●—, left hand scale) and 362 nm (---○---, right hand scale) as a function of time. The measurements were performed at (A) 30 °C and (B) 50 °C.

Fig. 4 Kinetics of insertion of gramicidin D into DPPC/DPPG/β-DPH-HPC (50 : 50 : 1 w/w) liposomes. Inset shows the signal at 225 nm (—●—, left hand scale) and 362 nm (---○---, right hand scale) as a function of time. The measurements were performed at (A) 30 °C and (B) 50 °C.

Conclusions

We present data that demonstrate a significant improvement in spectral quality and range for peptide/liposome systems with data being measured down to the quartz cut-off at 183 nm. In addition, high signal-to-noise and time resolution was achieved over the whole wavelength range with no fall-off in data quality. This enables one to see low wavelength electronic transitions, detect small signals and to see how these change with time – all of which have the potential to revolutionise the way we analyse the mechanism of action of antibiotic peptides that act on lipid membranes. We have, for example, been able to see the different behaviour of gramicidin as a function of lipid and temperature. By using an independent spectroscopic lipid probe, the behaviour of the peptide can be uncoupled from the effects of temperature or peptide on the liposomes. The gramicidin insertion behaviour can be characterized over a wide concentration range facilitating the use of LD in analysis of binding and insertion mechanisms for such systems.

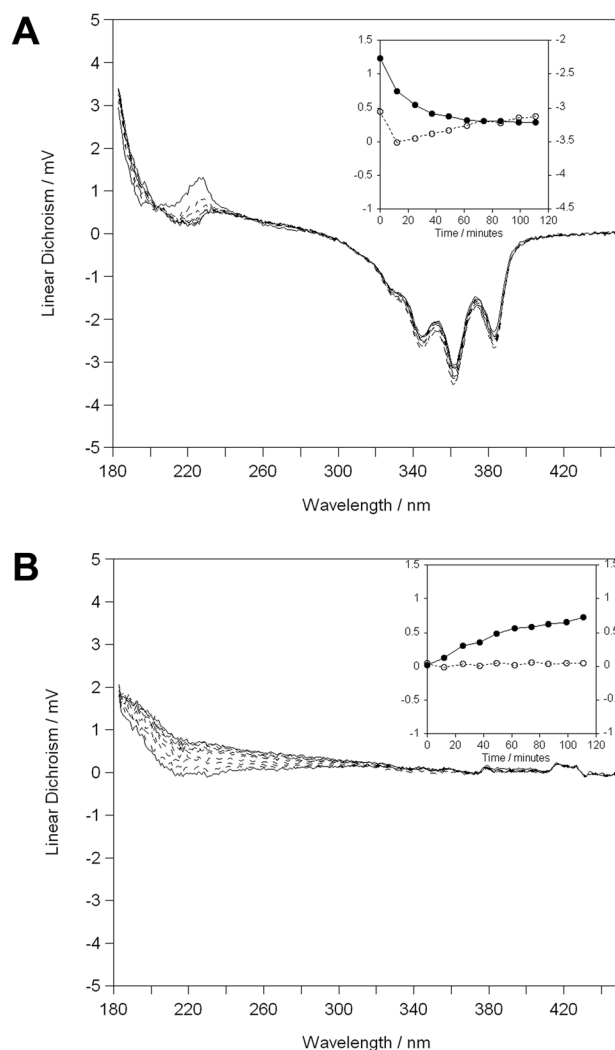Coupling the approaches outlined here with other biophysical techniques such as dynamic light scattering (DLS), circular dichroism (CD), fluorescence and Fourier transform infra-red (FT-IR) spectroscopy provides a way forward for determination of mechanism and kinetics of peptide–membrane interactions. Applications are not limited to antibacterial peptides, however, and we foresee that the speed and specificity of the LD experiment, together with access to the wider wavelength range and high quality data provided by the synchrotron source, will be particularly useful for screening potential antibiotics. The significantly improved LD data reported in this work with regard to both signal-to-noise and wavelength range open up the way for quantitative analysis of the secondary structure and orientation of membrane-inserting peptides using a methodology such as that developed for protein fibres in Dichrocalc by Bulheller et al.[2]

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