

Circular dichroism of oriented α helices. I. Proof of the exciton theory

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Moffitt's exciton theory for α helices, an important cornerstone of the circular dichroism (CD) theory for biopolymers, was recently cast in doubt by a linear dichroism measurement of electric field oriented polypeptides [Yamaoka *et al.*, J. Am Chem. Soc. **108**, 4619 (1986)]. In particular the prediction that the polarization of an exciton split component at 208 nm should be parallel to the α -helical axis was not borne out. This revealed the inadequacy of previous experiments which used long polypeptides to prove the theory. We performed two experiments to measure the effect of orientation of α helices on CD, one with a short membrane peptide, alamethicin, oriented in defect-free multibilayers and another with long polypeptides oriented with electric field. We found the result of the experiment with alamethicin to be consistent with the exciton theory. An elaborate procedure was established to measure the CD of protein molecules embedded in lipid multibilayers with light incident on bilayers at various angles (the helical sections of alamethicin are perpendicularly embedded in bilayers). Thus we are able to measure the polarization of the 208 nm band and prove that it is indeed parallel to the α -helical axis. The problem of electric field oriented polypeptides is discussed in paper II.

I. INTRODUCTION

Since the early 1960's when circular dichroism (CD) studies in the far UV of polypeptides and proteins first began, the negative CD band at ~ 208 nm and the positive CD band at ~ 192 nm of α helices had been assumed to be the result of an exciton splitting of the peptide $\pi\pi^*$ transition (~ 190 nm) where the 192 nm has been predicted to be polarized perpendicular and 208 nm polarized parallel to the α helix axis.^{1,2} This important feature of the exciton theory is very useful for the conformational analysis of membrane proteins whose transmembrane sections are often α helical.³⁻⁵ However the assumption that the CD bands at 192 and 208 nm are Moffitt's exciton components was recently cast in doubt by Yamaoka *et al.*⁶ who measured the electric dichroism of partially oriented α -helical polypeptides. This revealed the inadequacy of the previous experimental proof for the Moffitt exciton theory.⁷⁻⁹ The weaknesses of all the previous tests were that either the bulk orientation of the α helices could not be independently determined or the degree of orientation was insufficient. In the course of studying the molecular mechanism of the voltage-gated alamethicin ion channel, we found that alamethicin has a high degree of α -helix formation ($\sim 50\%$) when embedded in a phospholipid bilayer environment. Therefore, we embedded the alamethicin molecules in *defect-free* phospholipid multibilayer liquid crystals¹⁰ which allowed us to measure the far UV CD of uniformly aligned α helices at various angles between the direction of light and the helical axis. The result was consistent with the predictions of the exciton theory. In particular, the CD band at 208 nm was shown to be polarized parallel to the α -helical axis. In order to understand the disparity between the result of Yamaoka *et al.*⁶ and the others, we also performed an experiment measuring the CD of electric field

oriented polypeptides. Like Yamaoka *et al.*,⁶ we found an apparent disagreement between our results and Moffitt's prediction. We concluded that it is impossible to determine the polarization of an α helix with long polypeptides because of their bending flexibilities.

In the following, we will first review the background of the problem under consideration, and then present and discuss our experiment with alamethicin. It is well known that light scattering, linear dichroism and linear birefringence produce distortions in the CD spectra.¹¹⁻¹³ The procedure for avoiding these artifacts in our CD measurements will be elaborated on. The problem of electric field oriented polypeptides will be discussed in another paper (paper II).¹⁴

II. BACKGROUND

Ultraviolet circular dichroism spectroscopy has been used extensively in the analysis of the secondary structures of proteins (see review by Woody²). The principle of this structure probing technique is based on the experimental fact that the CD spectra (below ~ 250 nm) of polypeptides and proteins are dominated by the electronic transitions in the peptide units and are relatively independent of the side chains. The asymmetric and periodic arrangement of a polypeptide chain gives rise to its characteristic far UV CD spectrum. In particular, the α -helix conformation has a highly distinctive spectrum. Consequently the α -helix content of a protein can usually be determined from CD to within 10%.² Because of its inherent symmetry, the theory for the α helix is also simpler than those of other conformations. As early as 1956, Moffitt predicted that for every transition in its individual residues, a long helix has two transitions whose moments are, respectively, parallel and perpendicular to the helical axis. The theory has since been revised and improved.¹⁵⁻²¹ But the main feature of Moffitt's exciton splitting, i.e., a pair of parallel-polarized band and perpendicularly polarized bands with the former on the long

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wavelength side, is correct. Since 1964²² these exciton levels have been, respectively, assigned to the observed negative CD band at ~ 208 nm and the positive band at ~ 192 nm (this actually followed the assignments of the absorption bands by Gratzer *et al.*⁷). Because the precise theoretical calculations of the eigenenergies, widths, and amplitudes of the exciton bands are difficult, these assignments are by no means certain. It was clear from the beginning that the most definitive test of the exciton theory would come from the polarization measurement of partially or wholly oriented α helices.

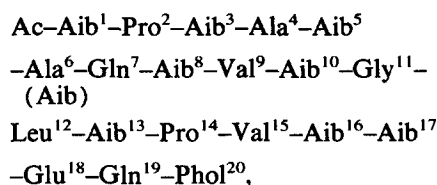
However, it is difficult to align polypeptides and even more so to determine the degree of alignment. The absorption bands assignment mentioned above⁷ was based on a polarization measurement of poly- γ -methyl-*L*-glutamate (PMLG) films. The films were made by evaporating polymer solution on quartz plates and orientation was achieved by unidirectionally stroking the solution with a needle while drying. From our related experience of preparing liquid crystals, we know that such a procedure would not provide a sufficient control on polymer orientation. We repeated this experiment. We found that it was easy to obtain birefringence and linear dichroism in such films, but we could not obtain films which gave a consistent and reproducible result. Furthermore, such polymer films, including the Langmuir monolayers of polymers used by Cornell,²² are liquid crystals. It is well known that in general the optical properties, including CD, of a liquid crystal are different from that of individual polymers (see, e.g., Patel and Dupre, 1980²⁴).

Another idea was to use an electric field to align polypeptides. A polypeptide in the straight α -helical form has a net dipole moment along its helical axis. Its magnitude depends on the side chain composition as well as on the solvent; it varies from about $0.7 e \text{ \AA}$ per residue (where e is the electron charge) to about 50% of that value.²⁵ In order to achieve a substantial degree of alignment, the electric energy (dipole moment times electric field) must be greater than the thermal energy $k_B T$ (the Boltzmann constant times temperature). However, in practice, it is difficult to maintain a dc field of more than, say, 50 kV/cm across a sample. Therefore, it is necessary to use long polypeptides. The polypeptides, PMLG and poly- γ -benzyl-*L*-glutamate (PBLG), used by Hoffman and Ullman,⁸ Mandel and Holzwarth,⁹ and Yamaoka *et al.*⁶ were about 1000 \AA or longer. We have systematically studied the CD of PBLG (260 000 Daltons) in ethylene dichloride and dioxane, with light in the direction of an applied dc electric field. Like all previous electric dichroism measurements, qualitatively the data seem to support the exciton theory. For example, the CD amplitudes at 208 and 224 nm decrease with increasing electric field. However, our quantitative analyses clearly show that at 208 nm there is nonvanishing transition moment perpendicular to the molecular dipole. This is the same conclusion reached by Yamaoka *et al.* by their electric linear dichroism measurement. If the long α -helical polypeptides are rod-like and rigid, the molecular dipole must be parallel to the straight helical axis by the symmetry argument; then the abovementioned results clearly invalidate the assignment of the 208 nm band as the parallel-polarized exciton compo-

nent. But there is evidence that long α -helical polypeptides are not rigid. Light scattering and hydrodynamic studies showed that the pitch per residue (the length of the projection of a monomer unit on the helical axis) of PBLG and other polypeptides is a decreasing function of the molecular weight.²⁶ The dipole moment per residue also decreases with increasing polymer length.²⁷⁻²⁹ We have calculated the effect of bending flexibility on the polarization of α -helical polypeptides and shown that our results do not contradict but are in fact consistent with the exciton theory (paper II). We also believe that if the early experiments on polypeptides could have been done more precisely, it would have been concluded to have disproved the exciton theory just like Yamaoka and his colleagues did. In other words, we believe that there should be no contradiction between the early and the recent experiments.

III. ALAMETHICIN

Alamethicin is a 20-amino-acid peptide naturally produced by the fungus *Trichoderma viridae*.²⁹ Natural alamethicin has been shown to be a mixture of various components; however, there are two major homologs which make up approximately 94% of the natural peptide mixture. The primary sequences of the two major homologs are



where Aib is α -aminoisobutyric acid and Phol is phenylalaninol. The two homologs differ only in position 6 where Ala is replaced by Aib. The residues are predominantly hydrophobic and the peptide has an acylated NH_2 terminus and a COOH terminal alcohol.

Alamethicin when added to phospholipid bilayers forms voltage-dependent ion translocating pores.³⁰⁻³² The conducting properties of alamethicin resemble the voltage-gated channels in actual biological membranes. Therefore, much effort by researchers has been devoted to understanding the structure and mechanism of alamethicin. The conformation of alamethicin in crystalline form has been determined by x-ray analysis.³³ The solution conformation in methanol was studied by measuring the NMR coupling constants between amide protons and α -carbon protons of alamethicin.³⁴ The crystal and methanol solution conformations differ; however, both conformations show the first seven to ten residues to form a stable α -helical section. Therefore, approximately 40%–50% of the peptide is in the α -helical form in these environments. The CD of alamethicin in dilauroylphosphatidylcholine (DLPC) vesicles (Fig. 1) is that of a typical α helix.² The amplitude at 224 nm indicates that $\sim 50\%$ of the residues are in the α -helical form in this environment as similarly found for the crystal and methanol conformations; it seems that the CD contributions of the nonhelical residues are negligible due to mutually de-

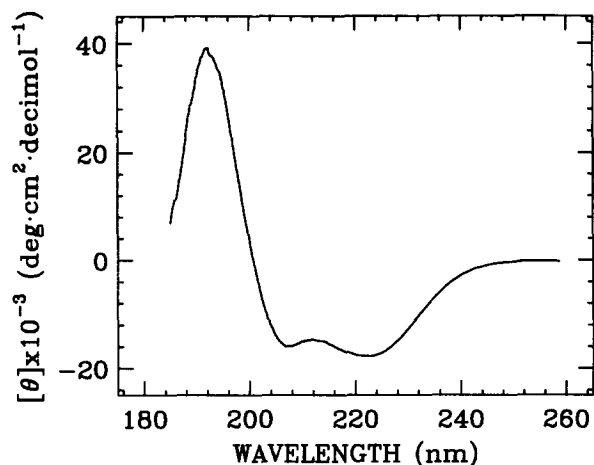


FIG. 1. Circular dichroism of alamethicin in DLPC vesicles, with the background signal of pure DLPC removed. The protein/lipid molar ratio is 1/80. The lipid concentration is 4 mg/ml. The suspension was prepared as described in Ref. 10. No effect of light scattering was detected.

structive superpositions. Although there are in general no selection rules for the exciton theory applied to α helices of finite length, as few as five to ten residues should still exhibit exciton splitting characteristics.^{17,20,21} Therefore, alamethicin embedded in DLPC multibilayers is suitable for testing the exciton theory.

IV. DEFECT-FREE MULTIBILAYERS

In the past year we have developed a technique of preparing thick defect-free phospholipid multibilayers with membrane peptides embedded in them.¹⁰ The multibilayers are smectic lyotropic liquid crystals of lipid bilayers intercalated with H₂O or D₂O. The alignment technique has since been improved so that samples with dimensions up to 80 μ thick \times 130 mm² can be routinely made. The improvements made in the alignment procedure will be elaborated on in the following experimental section. Since our samples are free of smectic defects, the alamethicin molecules embedded in the multibilayers provide a bulk phase of uniformly aligned α helices; the helical sections are presumably embedded in the bilayers with the helical axis perpendicular to the bilayer plane.³³ Therefore, it is possible to perform a well defined CD measurement of the orientational effect.

V. EXPERIMENTAL

A. Materials

DLPC and alamethicin were purchased from Sigma Chemical Co., St. Louis, MO. Only DLPC indicating a purity better than 99.5% by thin-layer chromatography (TLC) was used. Alamethicin was used without further purification. A stock solution of alamethicin was prepared in CHCl₃:CH₃OH (1:1) at a concentration of 1 mg/ml and stored at 4 °C. A stock solution of DLPC was also prepared in CHCl₃:CH₃OH (4:1) at a concentration of 10 mg/ml and stored at 4 °C. All solvents used in the sample preparation procedure were of spectroscopic or HPLC grade and always filtered before use.

Fused silica plates (1 in. \times 1 in. \times 0.5 mm) and fused silica cylinders (one cylinder end cut at the desired tilt angle of either 15°, 30°, and 45°) were supplied by Optical Instruments Inc., Houston, TX. Also, fused silica plates (1 in. \times 1 in. \times 0.16 mm) were purchased from Esco Products, Inc., Oak Ridge, NJ. Either 0.5 or 0.16 mm thick plates were used successfully to sandwich the multibilayer samples. Mylar sheets, used to define the sample thickness and to seal the sample, were supplied by DuPont, Wilmington, DE.

The circular dichroism was measured on a JASCO J500-A spectropolarimeter. 0.05% (w/v) androsterone in dioxane was used to calibrate the CD amplitude assuming an ellipticity of $[\theta]_{304\text{ nm}} = +11\ 170^\circ$. The 586 nm peak of neodymium glass and the 287.7 nm peak of holmium glass were used to calibrate the wavelength. Calibrations were checked daily during the course of the experiment.

A computer-controlled 360° rotator with a stepping motor from Oriol Corp., Stratford, CT was mounted in the CD sample compartment. The rotator allowed 360° rotation of the sample about the light direction \hat{k} . Special sample mounts (one for each tilt angle; namely, 0°, 15°, 30°, and 45°) were made which fit into the 360° rotator and allowed the optic axis of the sample to be tilted at the desired angle with respect to \hat{k} . Since the multibilayer samples are uniaxial crystals with their optic axis directed perpendicular to the plane of the bilayers, the tilted samples will possess linear birefringence and linear dichroism which will distort the CD signal. It is therefore necessary to be able to rotate the tilted samples about the \hat{k} direction and average the CD signals for equally distributed rotations around a circle.^{11–13,23,25}

B. Sample alignment

The sample preparation as described in Huang and Olah, 1987¹⁰ was used with some slight modifications. An amount of alamethicin stock solution was added to an appropriate amount of DLPC (~200 mg) stock solution so as to give a molar ratio of 1/80. (Alamethicin conformation in a phospholipid environment depends slightly on peptide/lipid molar ratio as observed by CD of both vesicle and multibilayer samples with different molar ratios. Both sample types have a systematic decrease in the CD of the 208 nm dip relative to the 224 nm dip for molar ratios greater than ~1/50. Our interpretation of this effect will be presented in a future paper. Molar ratios less than ~1/50 show no difference in CD signal with respect to each other and appear to have slightly higher α -helical content; therefore, a molar ratio of 1/80 is optimal.) The mixture was placed in a 100 ml round-bottom flask, rotary evaporated so as to remove the solvent, and dried under vacuum (< 10 μ m) for 2 h. Approximately ~1 ml/gm of benzene was then added and the mixture was lyophilized and left under vacuum for 48 h.

The sample batch was removed from vacuum and placed in a container which in turn was placed in a small flask slightly filled with H₂O or D₂O. The small flask was purged with nitrogen, sealed and kept at 15 °C. The top of the container containing the sample batch was left open so that the sample would be in contact with the high humidity (~85%–90% relative humidity at 15 °C which can be con-

trolled by using salt solutions instead of pure water). When the sample appears to be a clear gel instead of a fluffy powder the container is removed from the flask, purged quickly with nitrogen, capped and stored at 15 °C. Sample batches with this clear gel texture are easiest to align and are thought to be equilibrated and fully hydrated ($\sim 20\%$ – 26% water by total sample weight).

For each sample batch with alamethicin prepared, an identical sample batch with only DLPC was also prepared by the same procedure. A circular hole of 13 mm diameter was made in 1.5 μm thick Mylar spacers to provide a cavity for the samples. A small amount of each sample batch was then sandwiched between either 0.5 or 0.16 mm thick fused silica plates (cleaned in hot sulfuric/chromic acid, rinsed with distilled water and dried under nitrogen) with the spacer. Samples were made less than 2 μm thick in order to minimize linear birefringent and linear dichroic effects on the CD signal for samples tilted with respect to \hat{k} . When the samples are this thin, accuracy in knowing the sample thickness is forfeited. Therefore, to accurately determine the sample thicknesses, we made duplicate samples with 40 μm thick spacers. Assuming the 40 μm sample thicknesses to be accurate to within 1%, we determined the thickness of the thinner samples by normalizing the 0° tilt spectra to that of the 40 μm thick samples. True sample thicknesses were found to vary between 1.6–1.8 μm .

The same compression/dilation and low temperature annealing (< 60 °C) procedure was used for aligning the samples as discussed previously.¹⁰ However, for samples less than ~ 50 μm thick, we found that slightly shearing the sample along with compression/dilation and low temperature annealing improved the speed as well as the quality of alignment. Areas as large as 130 mm^2 could be achieved in a few hours. The ~ 2 μm thick samples took about 15–30 min to perfectly align. The alignment procedure was monitored via a polarized microscope.¹⁰

Four different samples with alamethicin and four different samples without alamethicin were experimented with. The orientational effects of all samples were the same showing this measurement to be reproducible. The results of the four samples with alamethicin were not averaged since the exact molar ratio varied slightly between samples. Therefore, the data presented in the figures are for the relative changes due to tilting in only one of the four samples.

C. CD measurement

As already briefly alluded to, CD measurements of anisotropic materials can be overshadowed by linear birefringent (Δn) and linear dichroic (LD) effects. Corrections can be made for these effects in the tilted samples by rotating the sample about \hat{k} and averaging the CD spectra for an equal distribution of rotation angles around a circle. The rotator and tilted sample mounts mentioned in the materials section were used for this purpose. Typically, a single CD scan of a sample was the average of 16 rotations where rotation angles were 0°, 22.5°, 45°, 67.5°, ..., 337.5°. ^{12,23,35} The rotation angles were measured with respect to the initial linear polarization direction of the light before it is modulated into left- and

right-circular polarized light, so that at 0° rotational angle, the bilayer planes of the sample are tilted (15°, 30°, or 45°) with respect to the initial linear polarization direction. It should be noted, however, that if Δn and LD are too large, the rotational averaging would not sufficiently eliminate the distortion effects. Hence very thin samples need to be used.

A simple test for minimizing and determining the extent of distortion of the CD signal due to Δn and LD effects was as follows:

(a) A standard was prepared consisting of PMLG (150 000 Daltons) in hexafluoroisopropanol (HFIP). PMLG is a typical α -helical former in HFIP. The concentration of PMLG in HFIP was adjusted so that its CD spectra (185–300 nm) was about the same order of magnitude as that of the multilayer samples. Call the CD spectra obtained for the standard alone spectra A.

(b) The CD of the multilayer sample was run and averaged over the 16 rotational angles. In a wavelength range (~ 300 nm for instance), if the base line was not flat after rotational averaging, then the sample was thinned until a flat base line was obtained. Call this averaged CD spectra due to the multilayer sample alone spectra B.

(c) Run a rotationally averaged CD spectra of the multilayer sample and the standard together. The standard was placed closest to the photodetector. Call this spectra C.

(d) If the Δn and LD distortion effects were not present then we would expect,

$$\text{spectra C} - \text{spectra B} = \text{spectra A.}$$

Indeed, only if the error in the above relation was less than 1% did we trust that the CD of the multilayer samples spectra B, was devoid of gross distortions. If noticeable error was present, then the sample was further thinned and the above procedure [(a)–(d)] was repeated until the spectral relation held. (We could not thin the pure DLPC multilayers sufficiently to obtain a spectrum at 45° tilt which met this criterion.)

The Δn and LD distortions due to the multilayer samples can be handled by the above procedures. However, another problem still remains. Since the samples are sandwiched between fused silica plates and the normal to the plates is tilted with respect to \hat{k} , the incident light will be refracted in the silica plates according to Snell's law. The light emerging from the plates will therefore be shifted slightly and impinge on the photodetector at a different place than if the silica plates were not tilted. This is a problem since the photodetector is areal sensitive. Also, due to the tilt of the fused silica plates the reflectance of the light will increase relative to no tilt and the transmitted light will be partially polarized due to this reflectance. About 8% of the incident light would be reflected in the worst case of 45° tilt with a degree of polarization of the transmitted light of $\sim 4\%$. Out of these three sources of error; namely, refraction, reflection, and polarization, the refraction is the most significant. All three problems were eliminated by using fused silica cylinders which have one face cut at the desired tilt angle as shown in Fig. 2. The index of refraction of the fused silica is 1.456 and the multilayer sample has an average index of ~ 1.45 .¹⁰ Water was added to glycerol to match its index to

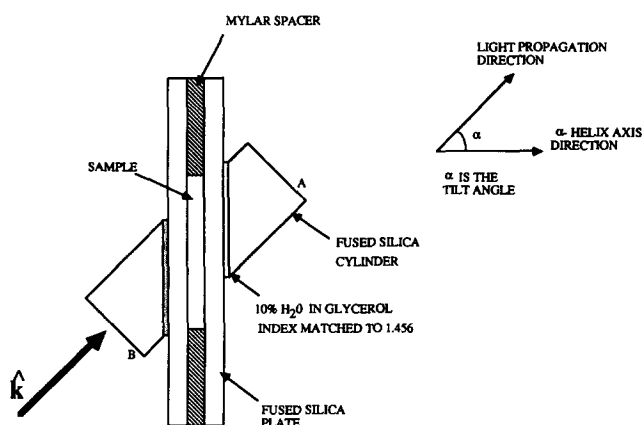


FIG. 2. Schematic diagram of a tilted multibilayer sample. It is crucial to make surfaces A and B perpendicular to \hat{k} .

fused silica and then applied between the fused silica pieces. It is crucial that the two flat surfaces of the cylinders are parallel. A He–neon laser was used to make this critical alignment. The cylinder/sample assembly was mounted on a rotator which allowed the assembly to be rotated about the assembly cylinder axis. A well collimated laser light (6 mm diameter beam) was directed down the axis of the assembly and projected onto a screen. If the two flat cylinder faces are not perfectly aligned then the laser light would be refracted and the projection on the screen would move when the assembly is rotated.

D. Neutron Bragg reflection

The H_2O or D_2O content of a multibilayer sample can be determined from the bilayer repeating distance and the latter can be measured by neutron Bragg reflection. The experiment was performed in Brookhaven National Laboratory (Upton, Long Island, NY) using the H3 beam line at HFBR. Two samples, one with and one without alamethicin, have practically the same bilayer repeating distance if they are kept under the same environmental conditions. When they are fully hydrated (after storage in high humidity environment), the repeating distance is 51.5 Å. If the samples are exposed to air for two days (storage in low humidity environment), the repeating distance is 44.5 Å. The DLPC bilayer thickness was previously determined to be 39.5 Å.³⁶ The mosaic spread of the multibilayers is smaller than the instrumental resolution.

VI. RESULTS AND DISCUSSION

Tinoco and Hammerle³⁷ showed that in an anisotropic medium, if the direction of light propagation \hat{k} is along an optic axis of the medium, the CD can be expressed as $\langle \hat{k} \cdot \mathbf{G} \cdot \hat{k} \rangle$, where \mathbf{G} is a second-rank tensor depending only on the properties of the molecules and $\langle \rangle$ denotes an average over the distribution of molecular orientations with respect to \hat{k} . Our measurements are always averaged over the complete azimuthal rotation around \hat{k} , so that the Tinoco–Hammerle formula is applicable. This rotational average also serves the purpose of deleting the effects of linear dichroism and linear birefringence, as is described in Sec. V.

In each sample, the phospholipid bilayers are parallel to the fused silica surfaces as is proven by conoscopy and polarized microscopy.¹⁰ We shall assume that the α -helical sections of alamethicin are embedded in the bilayers and are normal to the bilayer plane; and examine the consequences of this assumption. Let α be the angle between \hat{k} and the normal to the fused silica plates or the bilayer plane. In the following, the components parallel to the normal will be denoted by a subscript \parallel , while the perpendicular components by \perp . (Note that the parallel CD component is perpendicularly polarized and vice versa.) For an aligned sample, the CD signal $\theta(\alpha)$ at angle α , for either α helices or DLPC molecules, obeys the following angular dependence:

$$\theta(\alpha) = G_{\parallel} \cos^2 \alpha + G_{\perp} \sin^2 \alpha, \quad (1)$$

whereas CD of the corresponding vesicular sample obeys

$$\theta_v = G_{\parallel} \langle \cos^2 \alpha \rangle + G_{\perp} \langle \sin^2 \alpha \rangle = \frac{1}{3}G_{\parallel} + \frac{2}{3}G_{\perp}. \quad (2)$$

G_{\parallel} and G_{\perp} in Eq. (1) and those in Eq. (2) are the same, if they are normalized to the same unit.

Figure 3 shows the CD of one and the same aligned sample of alamethicin/DLPC at $\alpha = 0^\circ$ (A), 15° (B), 30° (C), and 45° (D); the spectra were normalized by a factor $\cos \alpha$ to the 0° thickness. Figure 4 shows the spectra of the corresponding aligned sample of pure DLPC at $\alpha = 0^\circ$ (A), 15° (B), and 30° (C). These two samples have the same water concentration as proven by having the same bilayer repeating distance measured by neutron diffraction. Since the exact density of either sample is unknown, initially the scales are not normalized to the molar unit; we simply adjusted the scale of DLPC to that of alamethicin/DLPC by thickness. (Later we will find a calibration between the aligned samples and the vesicle samples of known densities; and we will be

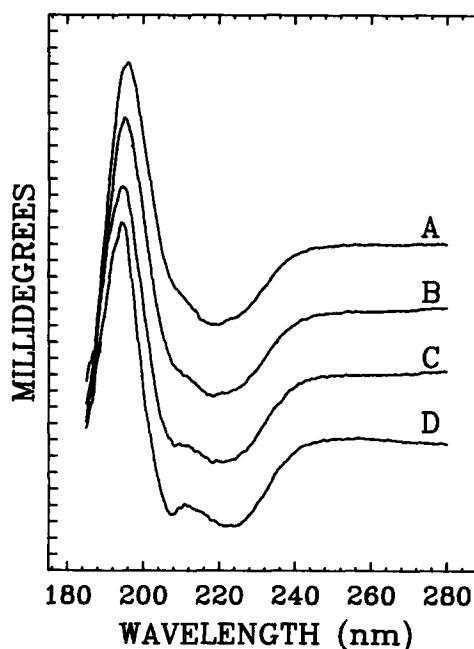


FIG. 3. Circular dichroism of one and the same alamethicin/DLPC multibilayer sample at the tilt angle $\alpha = 0^\circ$ (A), 15° (B), 30° (C), and 45° (D). The sample thickness is $1.6 \mu\text{m}$ and the alamethicin/DLPC molar ratio is $1/80$. The spectra are normalized by the factor $\cos \alpha$ to the 0° thickness and are displaced for clarity.

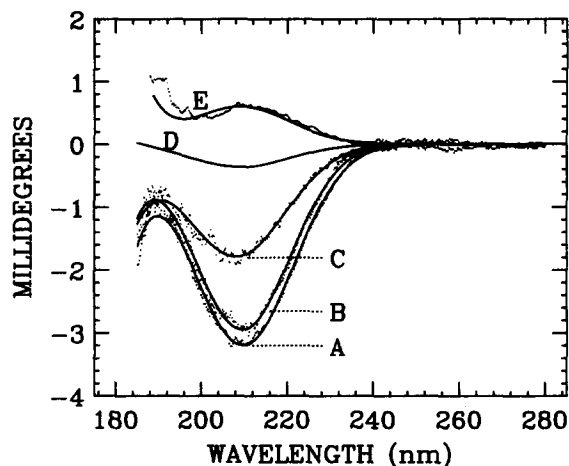


FIG. 4. Circular dichroism of one and the same pure DLPC multilayer sample at the tilt angle $\alpha = 0^\circ$ (A), 15° (B), and 30° (C). The solid lines A, B, and C are the least-squares fits with two Gaussians. The solid lines D and E are the extrapolations by Eqs. (1) and (2). Dotted line E is the spectrum of DLPC vesicles calibrated to the multilayer sample as described in the text.

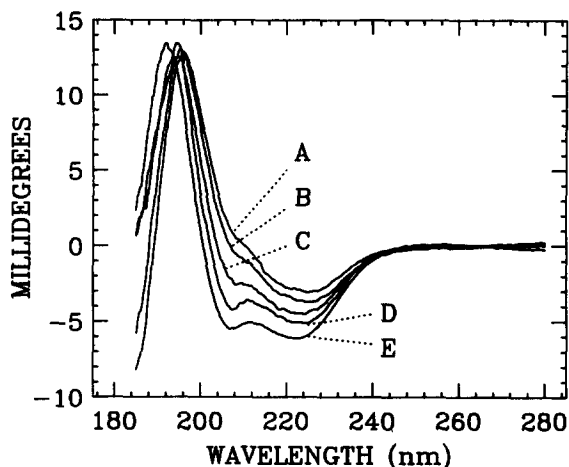


FIG. 5. Circular dichroism of oriented alamethicin at tilt angle $\alpha = 0^\circ$ (A), 15° (B), 30° (C), and 45° (D), obtained by subtracting the solid lines A–D in Fig. 4 from the spectra A–D in Fig. 3. E is the CD of randomly oriented alamethicin (Fig. 1) calibrated to this figure as described in the text.

able to convert the scales of Figs. 3 and 4 to the molar units.) One unexpected finding of this experiment is the CD of pure DLPC, which turns out to have a peak near 208 nm and contributes a substantial background to the spectra of alamethicin/DLPC sample. However, we should point out that the increase of the 208 nm dip with the tilt angle (Fig. 3) (the expected result of the exciton theory) cannot be the consequence of the DLPC background, because the latter decreases with increasing α (Fig. 4).

A. CD of DLPC

Spectra A, B, and C of Fig. 4 were decomposed by the least-squares fit into two Gaussians, one centered at ~ 209 nm and the other at ~ 162 nm (Table I). The fits are shown in solid curves (Fig. 4). The three amplitudes of each Gaussian (their widths are about the same) satisfy Eq. (1) (three equations for two unknowns) within 5%, with G_{\parallel} and G_{\perp} shown in the last row of Table I. Thus we are able to extrapolate the data with confidence to predict the spectrum for 45° and the spectrum for a corresponding DLPC vesicles (Fig. 4, solid lines D and E, respectively).

As we mentioned in Sec. V, we were not able to obtain a reliable spectrum at $\alpha = 45^\circ$ due to strong birefringence. Therefore the extrapolated spectrum D will be used as the

background for the 45° spectrum of alamethicin/DLPC. Through a calibration procedure to be explained later, we were able to normalize the experimental data of vesicles to Fig. 4 (dotted line E). The close agreement between the extrapolation and the data indicates that the calibration is reasonable; i.e., 1 millidegree in Fig. 4 corresponds to $36 \text{ deg cm}^2/\text{decimole}$. Thus the 209 nm band of DLPC is characterized by $G_{\parallel} = -1.2 \times 10^2$ and $G_{\perp} = 9.3 \times 10$ in $\text{deg cm}^2/\text{decimole}$.

B. CD of oriented alamethicin

The smooth spectra of pure DLPC (Fig. 4 solid lines A–D) are subtracted from the corresponding spectra of alamethicin/DLPC (Figs. 3A–3D) to obtain the spectra of oriented alamethicin (Figs. 5A–5D). We note the systematic changes in the amplitudes between 200 and 240 nm as a function of angle α . But the height of the peak at ~ 192 nm remains relatively unchanged for four different α 's. Therefore, we use this peak to calibrate the scale of the aligned sample. First, we plot Figs. 1 and 5 on the same scale (millidegrees), then multiply a constant (0.34×10^{-6}) to the spectrum of alamethicin in vesicles (Fig. 1), so that the amplitude of its 190 nm peak is the same as those of the aligned sample (Figs. 5A–5D); the result is the spectrum E. In this

TABLE I. Gaussian decompositions of DLPC spectra.

α	$\theta = a_1 \exp[-(\lambda - \lambda_1)^2/\sigma_1^2] + a_2 \exp[-(\lambda - \lambda_2)^2/\sigma_2^2]$					
	a_1 (millidegrees)	λ_1 (nm)	σ_1 (nm)	a_2 (millidegrees)	λ_2 (nm)	σ_2 (nm)
0°	-3.26	209.7	16.5	-16.4	162	14.4
15°	-2.95	209.5	15.8	-14.0	161	14.5
30°	-1.79	208.0	16.4	-7.8	163	15.4
$\theta = G_{\parallel} \cos^2 \alpha$ $+ G_{\perp} \sin^2 \alpha$	$G_{\parallel} = -3.26 \text{ millidegrees}$ $G_{\perp} = +2.53 \text{ millidegrees}$			$G_{\parallel} = -16.4 \text{ millidegrees}$ $G_{\perp} = +34.3 \text{ millidegrees}$		

way we find 1 millidegree in Fig. 5 to be equivalent to 2.9×10^3 deg cm²/decimole. The aligned lipid spectra are calibrated by the same multiplication factor. Taking the protein/lipid molar ratio (1/80) into account, we obtained the unit for Fig. 4 as given in the last section.

Thus we have in Fig. 5 the spectra of uniformly oriented and randomly oriented α helices to be compared with theory. Qualitatively the spectra exhibit the following systematic orientational dependence as the α -helix changes from 0° to 45° (A–D) in angle α and finally to a random distribution (E): (1) an amplitude increase and a ~ 4 nm blue shift from ~ 226 to ~ 222 nm in the ~ 224 nm band; (2) vanishingly small amplitude of the 208 nm band developing into a well defined negative band; (3) constant amplitude but a ~ 4 nm blue shift in the ~ 192 nm band from ~ 197 to ~ 192 nm. Conventionally the 224 nm band is identified with the amide $n\pi^*$ transition whereas the 208 and 192 nm bands are, respectively, the parallel- and perpendicular-polarized exciton components of the amide $\pi\pi^*$ transition. The observed orientational dependence is completely consistent with the theoretical descriptions of these three bands.^{9,17,21}

However much of the quantitative descriptions are inevitably model dependent. The most clearcut theoretical prediction is about the directions of polarization of the exciton split components. Particularly the 208 nm band is predicted to be a single band with the transition moment parallel to the α -helical axis. Although this feature was originally predicted for an infinitely long α helix, studies by Woody and Tinoco,¹⁷ and Madison and Schellman²⁰ showed that helices of ten or more residues have similar spectra with well separated parallel- and perpendicular-polarized bands. This prediction is indeed borne out by the spectrum of alamethicin.

According to the theory, the CD spectrum of an α helix consists of a positive Gaussian and a non-Gaussian band both centered around ~ 192 nm, a negative Gaussian near 208 nm and another negative Gaussian near 224 nm. Unfortunately, it is impossible to obtain a unique decomposition of our spectra into four bands. Depending on the restrictions used in the fitting routine, we obtained a multitude of different decompositions all of which fit the data with comparable reduced chi squares.

Supposing the exciton theory is correct, then the orientation dependence shown in the 208 nm region—i.e., the amplitude diminishes as the helical axis aligns with the probing light—clearly implies that the spectrum in this region is dominated by a parallel-polarized transition. Accordingly we assume that the spectral amplitude near 208 nm is proportional to the rotational strength of the parallel-polarized band; that means $G_{\parallel} = 0$ at 208 nm in Eqs. (1) and (2). Therefore, if both the theory and our assumptions are correct, the 208 nm amplitude should be proportional to $\sin^2 \alpha$. To minimize the fluctuations, we take the average amplitude between 207 and 208 nm (the peaks of spectra D and E fall in this region) for each spectrum in Fig. 5. The results are shown to be proportional to $\sin^2 \alpha$ [for spectrum E, $\sin^2 \alpha$ is replaced by 2/3 according to Eq. (2)] in Fig. 6. The positive value for the 0° amplitude is most likely due to the overlap of the 192 nm band. As the tilt angle α increases, the 192 nm

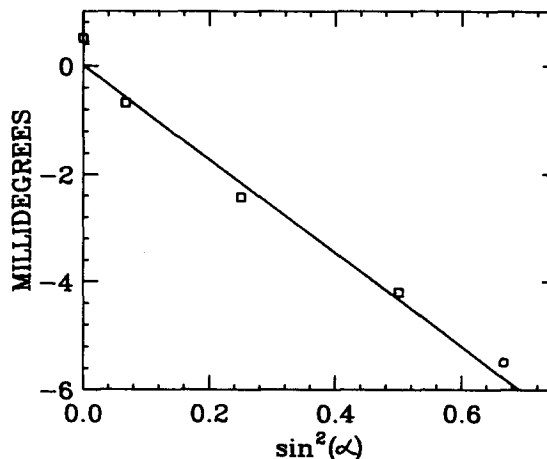


FIG. 6. The average amplitude between 207 and 208 nm of spectra A–E in Fig. 5 vs $\sin^2 \alpha$. For the spectrum E, $\sin^2 \alpha$ is replaced by 2/3 according to Eq. (2). The exciton theory predicts the amplitude to be linearly proportional to $\sin^2 \alpha$.

band shifts to the shorter wavelength and the overlap becomes less important.

In conclusion we have unequivocally confirmed the prediction of the exciton theory on the polarization of the 208 nm band. The result also shows that the α -helical section of alamethicin is at least approximately perpendicular to the bilayer membrane.

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