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A Simple Method for Correction of Circular Dichroism Spectra Obtained from Membrane-Containing Samples

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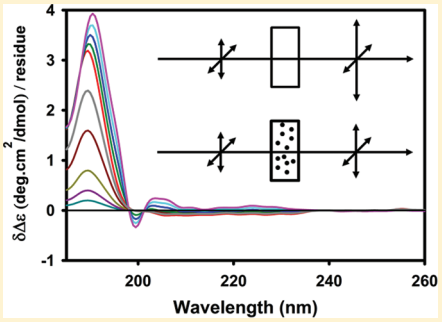
ABSTRACT: Circular dichroism (CD) spectroscopy is an important technique in structural biology for examining folding and conformational changes of proteins in solution. However, the use of CD spectroscopy in a membrane medium (and also in a nonhomogeneous medium) is limited by (i) high light scattering and (ii) differential scattering of incident left and right circularly polarized light, especially at shorter wavelengths (<200 nm). We report a novel methodology for estimating the distortion of CD spectra caused by light scattering for membrane-bound peptides and proteins. The method is applied to three proteins with very different secondary structures to illustrate the limits of its capabilities when calibrated with a simple soluble peptide ([Ac]ANLKALEAQKQKEQRQAAEELANAK[OH], standard peptide) with a balanced secondary structure. The method with this calibration standard was quite successful in estimating α-helix but more limited when it comes to proteins with very high β-sheet or β-turn content.



Circular dichroism (CD) spectroscopy is the most widely used method in structural biology for examining the secondary structure of peptides and proteins and for assessing folding and conformational changes in a homogeneous medium. It has proven to be invaluable for protein conformational characterization when other high-resolution techniques such as crystallography, nuclear magnetic resonance (NMR), etc., are impractical or not possible. These methods are especially challenging for membrane-associated proteins and peptides, but the applicability of CD spectroscopy to this class of macromolecules is limited because of the distortion of CD spectra associated with differential scattering of right and left circularly polarized light by scattering particles.[1,2] Differential scattering is especially important for particles whose dimensions are greater than 1/20th of the wavelength of light.[3] The effect of light scattering on CD measurements has not been well-studied, but the signal loss due to scattering and depolarization of light is unambiguous. Different approaches have been taken to overcome spectral distortions associated with scattering (e.g., reduction of sample−PMT distance[1] or optical methods for collecting scattered light[2]), but they have been found to be inadequate for obtaining a good spectrum below ∼200−210 nm. Membrane protein secondary structure has been difficult to predict, perhaps because basis sets specific for membrane proteins are needed,[4] perhaps because different structural states of these proteins are stabilized in different detergent micelles or membranes with different compositions, or perhaps because even detergent micelles scatter significant light at the low wavelengths required for accurate estimations of secondary structure content.

Here we report a remarkably simple and effective method for correcting CD spectra for the distortions resulting from scattering from small unilamellar vesicles (SUVs) of an average size of 25 nm. Because of their small size and spherical shape, these vesicles are most often used for CD studies of membrane-bound proteins, though they scatter significantly in the shorter wavelength (<200 nm) region. We use an aqueous soluble peptide [Ac]ANLKALEAQKQKEQRQAAEELANAK[OH] (standard peptide) as a reference peptide and measure individually the loss of right and left circularly polarized signal due to light scattering from different concentrations of SUVs as a function of wavelength. As the peptide does not interact with the membrane, the change in these quantities with addition of SUVs is attributed to differential scattering, the possible reasons for which have been discussed but are still ambiguous.[1,2] Separate correction factors for the right and left circularly polarized signals for a given spectrometer are then easily constructed using the peptide in the presence and absence of membranes. These are easily applied to the right and left circularly polarized signals from an unknown protein or peptide in the presence of SUVs. The method yields excellent correction for α-helical content, which is the structural component severely underestimated in scattering samples, but is predictably less reliable for β-structure.

MATERIALS

Chloroform stock solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. The concentration of the stock lipids was determined by a phosphate assay.[5] Myoglobin from equine heart was purchased

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from Sigma-Aldrich. KEAP1 and TrpRS have been expressed and purified by the laboratories of B. Kuhlman and C. W. Carter, respectively, at the University of North Carolina and kindly given to us as gifts for our work. All other reagents were of the highest purity grade available.

METHODS

Vesicle Preparation. POPC in a cyclohexane/methanol mixed solvent was freeze-dried under high vacuum overnight. Small, unilamellar vesicles (SUVs) 25 nm in diameter (from dynamic light scattering) were prepared as documented previously.[6] The dried lipid powders were suspended in buffer for 1 h above the phase transition temperature. All experiments were conducted in 10 mM phosphate buffer (pH 7.4).

CD Measurement. An aqueous solution of peptides or proteins of appropriate concentration was added to the SUV solution, and CD spectra were recorded in the Chirascan Plus (Applied Photophysics) spectrophotometer. All the measurements were taken at 23 °C.

CD Analysis. The CD machine outputs data as specific rotation ($\langle\theta\rangle_{degree}$). These values were converted to molar ellipticity per residue ($[\Theta]$) using the equation

$$[\Theta] = \frac{\langle\theta\rangle_{degree}}{lnc} = 3298.2 \times \Delta\varepsilon,\ \Delta\varepsilon = \varepsilon_L - \varepsilon_R \quad (1)$$

where l is the path length of the cell, n is the number of residues, c is the concentration of the peptide in milligrams per milliliter, and $\Delta\varepsilon$ is the difference between the extinction coefficients for left (ε_L) and right (ε_R) circularly polarized light. Because the scattering artifact means that scattering affects ε_L and ε_R differently,[1] individual corrections to these quantities ($\delta\varepsilon_L$ and $\delta\varepsilon_R$, respectively) are necessary to correct $\Delta\varepsilon$ and $[\Theta]$. The correction factors we need were obtained as

$$\delta\varepsilon_L(\lambda) = \varepsilon_L^{stdP,SUVs}(\lambda) - \varepsilon_L^{stdP,buffer}(\lambda);$$
$$\delta\varepsilon_R(\lambda) = \varepsilon_R^{stdP,SUVs}(\lambda) - \varepsilon_R^{stdP,buffer}(\lambda) \quad (2)$$

where $\varepsilon_L^{stdP,buffer}$ and $\varepsilon_L^{stdP,SUVs}$ are the extinction coefficients of left circularly polarized light for the standard peptide in the buffer and SUVs, respectively. Because only SUVs contribute significantly to scattering (i.e., the standard peptide does not), we can define a peptide-independent correction factor for each wavelength (λ) of incident light:

$$\begin{aligned}\delta\Delta\varepsilon &= \Delta\varepsilon^{stdP,SUVs} - \Delta\varepsilon^{stdP,buffer}\\ &= \varepsilon_L^{stdP,SUVs} - \varepsilon_R^{stdP,SUVs} - \varepsilon_L^{stdP,buffer}\\ &\quad - \varepsilon_R^{stdP,buffer}\\ &= \delta\varepsilon_L - \delta\varepsilon_R \end{aligned} \quad (3)$$

It is now easy to show that, for any protein, P, that also does not itself contribute significantly to scattering or significantly alter the scattering profiles of SUVs, we can obtain a corrected CD spectrum ($\Delta\varepsilon^{P,corr}$ or $[\Theta]^{corr}$ vs λ) as

$$\Delta\varepsilon^{P,corr}(\lambda) = \Delta\varepsilon^{P,SUVs}(\lambda) + \delta\Delta\varepsilon(\lambda) \quad (4)$$

RESULTS AND DISCUSSION

The CD spectra of the water-soluble standard peptide [Ac]ANLKALEAQKQKEQRQAAEELANAK[OH] (standard peptide) in the absence and presence of SUVs with different lipid concentrations are shown in Figure 1. As the peptide does not interact with membranes, the CD spectrum is not expected

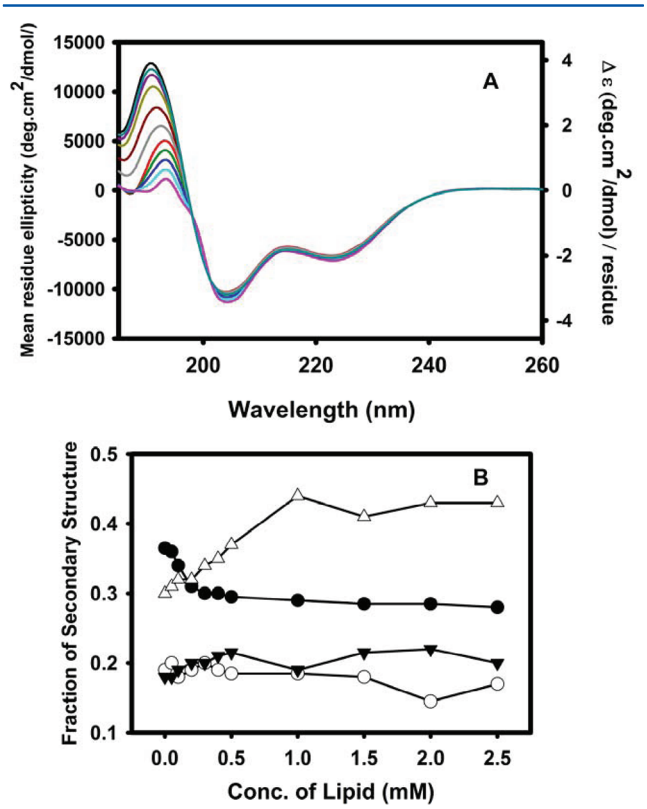

Figure 1. (A) Plot of mean molar ellipticity per residue of the standard peptide in the absence (black) and presence of 0.05 (dark cyan), 0.1 (dark pink), 0.2 (dark yellow), 0.3 (dark red), 0.4 (dark gray), 0.5 (red), 1.0 (green), 1.5 (blue), 2.0 (cyan), and 2.5 mM (pink) POPC SUVs. Measurements were taken in 10 mM phosphate buffer at 23 °C. The average diameter of the SUVs used in our all experiment was 25 nm. (B) Plot of secondary structure content, i.e., helix (●), β-sheet (▼), turn (○), and unordered (△), of the standard peptide at various POPC concentrations.

to change because of the presence of membranes. However, we observed significant changes in peptide CD spectra (Figure 1A) with increasing lipid concentrations, especially for the strong electric dipole-allowed $\pi \rightarrow \pi^*$ transition at 190 nm. This is not surprising as we think of visible light scattering as reflecting perturbations of electronic distributions (i.e., charge distribution) of the scattering materials. The effect of SUVs on the weak, magnetic dipole-allowed n $\rightarrow \pi^*$ transition was much weaker, indeed essentially nonexistent (Figure 1A). Because these changes in CD spectra near 190 nm increased with SUV concentration, they logically are attributed to scattering from the membranes. We analyzed the CD spectrum using CDSSTR, CONTIN, and SELCON3 from Dichroweb,[7,8] using basis set 3 and 6. The best fit was obtained with CDSSTR, and the averages of the secondary structural contents obtained from basis set 3 and 6 are shown in Figure 1B. Not surprisingly, the resulting estimates of secondary structural content also varied with lipid concentration (Figure 1B). In the absence of SUVs, the analysis shows comparable amounts of β and turn, α, and unordered secondary structure, making it a reasonable standard for correction.

SUVs are Tyndall scatterers in the visible range but in the 190 nm range take on properties of spherical shells in Mie

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theory. Their advantage for optical measurements is the fact their fairly rigid, limiting spherical shape results in low, fairly simple (Tyndall or spherical shell Mie), and stable (structure not significantly perturbed by interactions with a small amount of proteins or other agents) scattering. To confirm structural stability, we checked the size of the SUVs, using dynamic light scattering, before and after treating the samples with peptide or proteins, and there was no change (data not shown) in size. To confirm the reasonable constancy of the scattering profile, we measured the sample OD at 190 nm for SUVs alone, SUVs in the presence of proteins, and proteins alone. For all concentrations of lipids, the optical density (OD) of the protein−SUV samples was below 1.0 at 190 nm and even lower at 210 nm, where the peptide absorption was reduced by 30%. For the proteins considered here that do not interact with membranes, the quantity δOD%

$$\delta\mathrm{OD}\% = \frac{\mathrm{OD}_{\mathrm{SUV,protein}} - (\mathrm{OD}_{\mathrm{SUV}} + \mathrm{OD}_{\mathrm{protein}})}{\mathrm{OD}_{\mathrm{SUV}} + \mathrm{OD}_{\mathrm{protein}})} \times 100\% \quad (5)$$

was on the order of 4%, likely because of secondary absorption by proteins of light scattered by SUVs. Even for peptides that do interact with SUVs, this quantity was only ~6% larger than that seen with noninteracting proteins (unpublished observations of H. Chakraborty and B. R. Lentz with influenza hemagglutinin fusion peptide and transmembrane domain at a lipid:peptide ratio of 300:1), indicating that the SUV scattering profile was not significantly perturbed by the binding of a small amount of protein.

The peptide spectra in Figure 1A were used to obtain scattering correction factors as described in Methods. The correction factor obtained using eq 3 is plotted in Figure 2 for

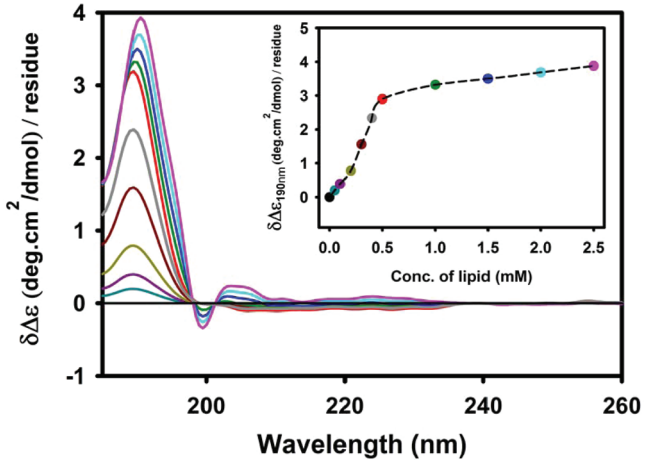

Figure 2. Plot of $\delta\Delta\varepsilon$ for the standard peptide in the absence (black) and presence of 0.05 (dark cyan), 0.1 (dark pink), 0.2 (dark yellow), 0.3 (dark red), 0.4 (dark gray), 0.5 (red), 1.0 (green), 1.5 (blue), 2.0 (cyan), and 2.5 mM (pink) POPC SUVs. The inset shows a plot of $\delta\Delta\varepsilon^{\mathrm{P,corr}}$ at 190 nm vs lipid concentration. A smooth line is drawn through the points to guide the eye. All measurements were taken at 10 mM phosphate buffer at 23 °C. The average diameter of the SUVs used in our all experiment was 25 nm.

various lipid concentrations. Comparing the right ordinate in Figure 1A with the $\delta\Delta\varepsilon$ values in Figure 2 reveals that the correction at 2 mM lipid constitutes roughly 10% of the uncorrected observations. The inset of Figure 2 shows the plot of $\delta\Delta\varepsilon$ at 190 nm versus lipid concentration. Though the correction is applicable to the entire wavelength region, it is clearly largest at 190 nm, so we plotted $\delta\Delta\varepsilon$ for a single wavelength (190 nm) to show the dependence of $\delta\Delta\varepsilon$ on lipid concentration. As expected, $\delta\Delta\varepsilon$ extrapolated to zero at limiting low SUV concentrations but increased dramatically up to 0.5 mM lipid. For studies of membrane proteins, this range of lipid concentrations is critical.

As a test of the method for soluble proteins not known to interact with membranes, we recorded the CD spectra of myoglobin from equine heart, Kelch-like ECH-associated protein 1 (KEAP1), and tryptophanyl-tRNA synthetase (TrpRS) in phosphate buffer (10 mM, pH 7.4) in the presence and absence of 2.0 mM SUVs of POPC (Figure 3). As for the

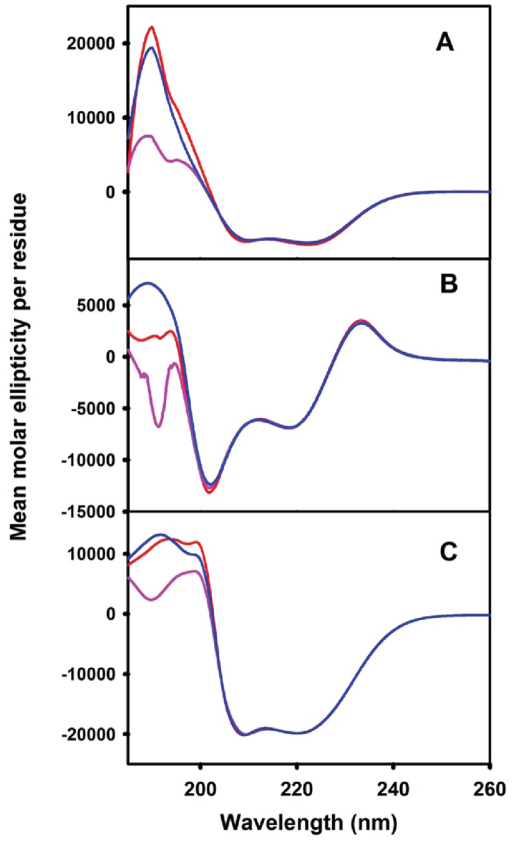

Figure 3. Plot of mean molar ellipticity per residue of (A) myoglobin, (B) KEAP1, and (C) TrpRS in phosphate buffer (red), in the presence of 2.0 mM SUVs (pink), and after correction in the presence of 2.0 mM SUVs (blue). All the measurements were taken in 10 mM phosphate buffer (pH 7.4) at 23 °C.

standard peptide, we also confirmed using dynamic light scattering that the presence of myoglobin, KEAP1, and TrpRS did not cause SUV aggregation and that the δOD% was small. These spectra were then corrected using eq 4. The secondary structural contents obtained by analysis of these corrected spectra are shown in Table 1 along with those derived from spectra obtained in the absence of membranes. Myoglobin is well-known to have mainly α-helical secondary structural elements [Protein Data Bank (PDB) entry 1MBN]; KEAP1 is a β-barrel (PDB entry 1ZGK), and TrpRS contains a mix of α and β structural elements (PDB entry 1DRT). Clearly, our method quite successfully restores the 190 nm positive CD of the myoglobin spectrum and thus returns a good estimate of

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Table 1. Calculated Secondary Structure (Dichroweb[7,8]) of Different Proteins in Buffer, in SUVs, and after Correction of the CD Spectrum in SUVs Using Our Correction Factor[a]

protein	medium	helix	β-sheet	turn	unordered
myoglobin	buffer	0.69	0.04	0.08	0.22
	SUVs	0.42	0.07	0.20	0.33
	SUVs after correction	0.68	0.10	0.10	0.12
KEAP1	buffer	0.0	0.34	0.23	0.42
	SUVs	0.0	0.37	0.23	0.40
	SUVs after correction	0.0	0.32	0.24	0.42
TrpRS	buffer	0.51	0.21	0.11	0.16
	SUVs	0.40	0.19	0.12	0.26
	SUVs after correction	0.52	0.19	0.12	0.17

[a]All experiments were conducted in 10 mM phosphate buffer (pH 7.4).

helical content. The same is true for TrpRS. It is also clear from the KEAP1 spectra that it undercorrects for β-sheet and turn contributions in the region of 185−200 nm. However, Table 1 reveals that the sums of β-sheet and turn contributions, while not restored to estimates in the absence of membranes, are in all cases corrected in the proper directions. Because estimates of β-sheet and turn contributions from CD must always be interpreted with some skepticism as compared to reasonable reliability for α-helical structure, this result is neither unanticipated nor a significant drawback for the method proposed.

In summary, the proposed method is very useful for obtaining correct helical content and does not interfere with the estimation of β-sheet and turn contributions unless these structural elements are present in very small amounts, in which case CD is not useful for their estimation even in the absence of membranes. However, it must be confirmed using δOD% that the presence of proteins or peptides does not significantly alter the scattering profile or structural integrity of the SUVs employed in a study. Structural integrity can also be confirmed by quasi-elastic scattering, but need not be, because a large ΔOD will reliably reveal SUV aggregation or fusion. Because this condition will not be met for large unilamellar vesicles or for biological membranes, the method cannot be properly applied in these instances. Nonetheless, if proteins can be associated with SUVs, the method outlined here represents a significant improvement in technology for examining membrane proteins.

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Notes

The authors declare no competing financial interest.

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