Published in final edited form as:

Biochemistry. 2012 February 7; 51(5): 1005–1008. doi:10.1021/bi300025c.

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

Hirak Chakraborty and **Barry R. Lentz***

Department of Biochemistry and Biophysics & Program in Molecular and Cellular Biophysics, University of North Carolina, Chapel Hill; Chapel Hill, North Carolina 27599-7260.

Abstract

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 non-homogeneous medium) is limited by i) high light scattering and ii) differential scattering of incident left and right circularly polarized light, especially at lower wavelengths (<200 nm). We report a novel methodology to estimate the distortion of circular dichroism (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]: std. 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 of β-turn content.

Keywords

light scattering; membrane-bound protein; correction of CD-spectra

Introduction

CD spectroscopy is the most widely used method in structural biology for examining the secondary structure of peptides and proteins and to assess folding and conformational changes in a homogeneous medium. It has proven invaluable for protein conformational characterization when other high-resolution techniques such as crystallography, NMR etc. are impractical or impossible. These methods are especially challenging for membrane associated proteins and peptides, but the applicability of CD spectroscopy to this class of macromolecules is limited due to distortion of CD spectra associated with differential scattering of right- and left-circular polarized light by scattering particles (1, 2). Differential scattering is especially important for particles whose dimensions are great than $1/20^{th}$ 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 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

*Corresponding Author Barry R Lentz, uncbrl@med.unc.edu, Department of Biochemistry and Biophysics & Program in Molecular and Cellular Biophysics, University of North Carolina, Chapel Hill; Chapel Hill, North Carolina 27599-7260. Phone: 919-966-5384, Fax: 919-966-2852.

different structural states of these proteins are stabilized in different detergent micelles or membranes of different composition, or perhaps because even detergent micelles scatter significant light at the low wavelengths required for accurate estimations of secondary structure.

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 at the lower wavelength (<200 nm) region. We use an aqueous soluble peptide [Ac]ANLKALEAQKQKEQRQAAEELANAK[OH] (std. peptide) as a reference peptide and measure individually the loss of right- and left-circularly polarized signal due to light scattering from different concentration 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/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 phosphate assay (5). Myoglobin from equine heart was purchased from Sigma-Aldrich, USA. KEAP1 and TrpRS have been expressed and purified by the Prof. Brian Kuhlman Lab and Prof. Charles W. Carter Lab respectively, at University of North Carolina at Chapel Hill, USA, and kindly gifted us for our work. All other reagents were of the highest purity grade available.

Methods

Vesicle Preparation

POPC in cyclohexane/methanol mixed solvent were freeze-dried under high vacuum overnight. Small, unilamellar vesicles (SUVs) on diameter 25 nm (from dynamic light scattering) were prepared as documented previously (6). The dried lipid powders were suspended in buffer for 1 hour above the phase transition temperature. All experiments were carried out in 10 mM Phosphate buffer at pH 7.4.

CD measurement

Aqueous solution of peptides or proteins of appropriate concentration were added to the SUV solution and CD spectra were measured in the Chirascan Plus® (Applied Photophysics) spectrophotometer. All the measurements were carried out at 23°C.

CD analysis

The CD machine outputs data as specific rotation ($< \theta >_{deg\ ree}$). These values were converted to molar ellipticity per residue ([Θ]) using the equation:

$$[\Theta]=\frac{<\theta>_{\deg ree}}{l \times n \times c}=3298.2 \times \Delta\varepsilon, \Delta\varepsilon=\varepsilon_L - \varepsilon_R \quad \text{Eqn. 1}$$

Where l = path length of the cell, n = number of residues and c = concentration of the peptide in mg/ml unit and Δε is the difference between the extinction coefficients for left (ε_L) and right (ε_R) circularly polarized light. Since scattering artifact means that scattering effects ε_L and ε_R differently (1), individual corrections to these quantities ($\delta\varepsilon_L$ and $\delta\varepsilon_R$) are necessary to correct Δε and <θ>. The correction factors we need are obtained as:

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

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

$$\delta\Delta\varepsilon=\Delta\varepsilon^{stdP,SUV}-\Delta\varepsilon^{stdP,buffer}=\varepsilon_L^{stdP,SUV}-\varepsilon_R^{stdP,SUV}-\varepsilon_L^{stdP,buffer}-\varepsilon_R^{stdP,buffer}=\delta\varepsilon_L-\delta\varepsilon_R \quad \text{Eqn. 3}$$

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 $[\Theta]^{corr}$ *versus* λ) as:

$$\Delta\varepsilon^{P,corr}(\lambda)=\Delta\varepsilon^{P,SUV}(\lambda)+\delta\Delta\varepsilon(\lambda). \quad \text{Eqn 4}$$

Results & Discussion

The CD spectra of the water soluble standard peptide [Ac]ANLKALEAQKQKEQRQAAEELANAK[OH] (std. peptide) in the absence and in presence of SUVs of different lipid concentration has been shown in Fig.-1. As the peptide does not interact with membranes, the CD spectrum is not expected to change due to the presence of membranes. However, we observed significant changes in peptide CD spectra (Fig. 1A) with increasing lipid concentration, especially for the strong electric-dipole allowed π→π* transition at 190nm. The effect of SUVs on the weak, magnetic-dipole-allowed n→π* transition was much less, indeed essentially non-existent (Fig. 1A). 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. 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 the programs CDSSTR, CONTIN and SELCON3 from Dichroweb (7, 8), using both the basis set-3 and 6. The best fit was obtained with the CDSSTR program and the average of the secondary structural elements obtained from the basis set-3 and 6 have been shown in Fig. 1b. Not surprisingly, the resulting estimates of secondary structural elements also varied with lipid concentration (Fig. 1b). In the absence of SUVs, the analysis shows comparable amounts of beta & turn, alpha, 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 theory. Their advantage for optical measurements is 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 small amount of proteins or other agents) scattering. In order to confirm structural stability, we checked the size of the SUVs, using dynamic light scattering, before and after treating with peptide or proteins and there is no change (data not shown) in size. In order to confirm reasonable constancy of scattering profile, we measured sample OD at 190nm for SUVs alone, SUVs in the presence of proteins, and proteins alone. For all concentration of lipids the optical density (O.D) of the protein-SUV samples was below 1.0 at 190 nm and was 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%:

$$\delta OD\% = \frac{OD_{SUV,protein} - (OD_{SUV} + OD_{protein})}{(OD_{SUV} + OD_{protein})} \times 100\%, \quad \text{Eqn. 5}$$

was on the order of + 4%, likely due to secondary absorption by proteins of light scattered by SUVs. Even for peptides that do interact with SUVs, this quantity was only about 6% larger than that seen with non-interacting proteins (unpublished observations of H. Chakraborty and B. Lentz with Influenza hemagglutinin fusion peptide and trans-membrane domain at lipid/peptide ratio of 300/1), indicating that the SUV scattering profile was not significantly perturbed by binding of a small amount of protein.

The peptide spectra in Fig. 1A were used to obtain scattering correction factors as described in the method section. The correction factor obtained using equation 3 is plotted in Figure 2 for various lipid concentrations. Comparing the right ordinate in Fig. 1A with the δΔε 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 δΔε at 190 nm with concentration of lipid. Though the correction is applicable to the entire wavelength region, it is clearly largest at 190nm, so we plotted δΔε for single wavelength (190 nm) to show the dependency of δΔε with lipid concentration. As expected, δΔε extrapolated to zero at limiting low SUV concentration, but increased dramatically up to 0.5mM lipid. For studies of membrane proteins, this range of lipid concentration is critical.

As a test of the method for soluble proteins not known to interact with membranes, we recorded in phosphate buffer (10mM, pH 7.4) the CD spectra of myoglobin from equine heart, Kelch-like ECH-associated protein 1 (KEAP1), and tryptophanyl-tRNA synthetase (TrpRS) in the presence and absence of 2.0 mM SUVs of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Figure 3). As for std. 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% quantity was small. These spectra were then corrected using Eqn. 4. The secondary structural elements 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 (pdb 1MBN), while KEAP1 is a β-barrel (pdb 1ZGK), and TrpRS contains a mix of α and β structural elements (pdb 1D2RT). Clearly, our method quite successfully restores the 190 nm positive CD of the myoglobin spectrum and thus returns a good estimate of helical content. The same is true for TrpRS. It is also clear from the KEAP1 spectra that it under-corrects 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 versus 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 estimation of β-sheet and turn contributions unless these structural elements are present in very small amount, in which case CD is not use for their estimation even in the absence of membranes. However, it must be confirmed sing the quantity 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-elastics scattering, but need not be, since 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.

Acknowledgments

This research was supported by NIGMS grant 32707 to BRL.

References

1. Dorman BP, Maestre MF. Experimental differential light-scattering correction to the circular dichroism of bacteriophage T2. Proc Natl Acad Sci U S A. 1973; 70:255–259. [PubMed: 4509658]
2. Gregory RP, Raps S. The differential scattering of circularly polarized light by chloroplasts and evaluation of their true circular dichroism. Biochem J. 1974; 142:193–201. [PubMed: 4441376]
3. Bustamante C, Tinoco I Jr, Maestre MF. Circular differential scattering can be an important part of the circular dichroism of macromolecules. Proc Natl Acad Sci U S A. 1983; 80:3568–3572. [PubMed: 6574499]
4. Wallace BA, Lees JG, Orry AJ, Lobley A, Janes RW. Analyses of circular dichroism spectra of membrane proteins. Protein Sci. 2003; 12:875–884. [PubMed: 12649445]
5. Chen PS, Toribara TY, Warner H. Microdetermination of Phosphorus. Anal Chem. 1956; 28:1756–1758.
6. Lentz BR, Carpenter TJ, Alford DR. Spontaneous fusion of phosphatidylcholine small unilamellar vesicles in the fluid phase. Biochemistry. 1987; 26:5389–5397. [PubMed: 3676258]
7. Whitmore L, Wallace BA. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Res. 2004; 32:W668–W673. [PubMed: 15215473]
8. Whitmore L, Wallace BA. Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. Biopolymers. 2008; 89:392–400. [PubMed: 17896349]

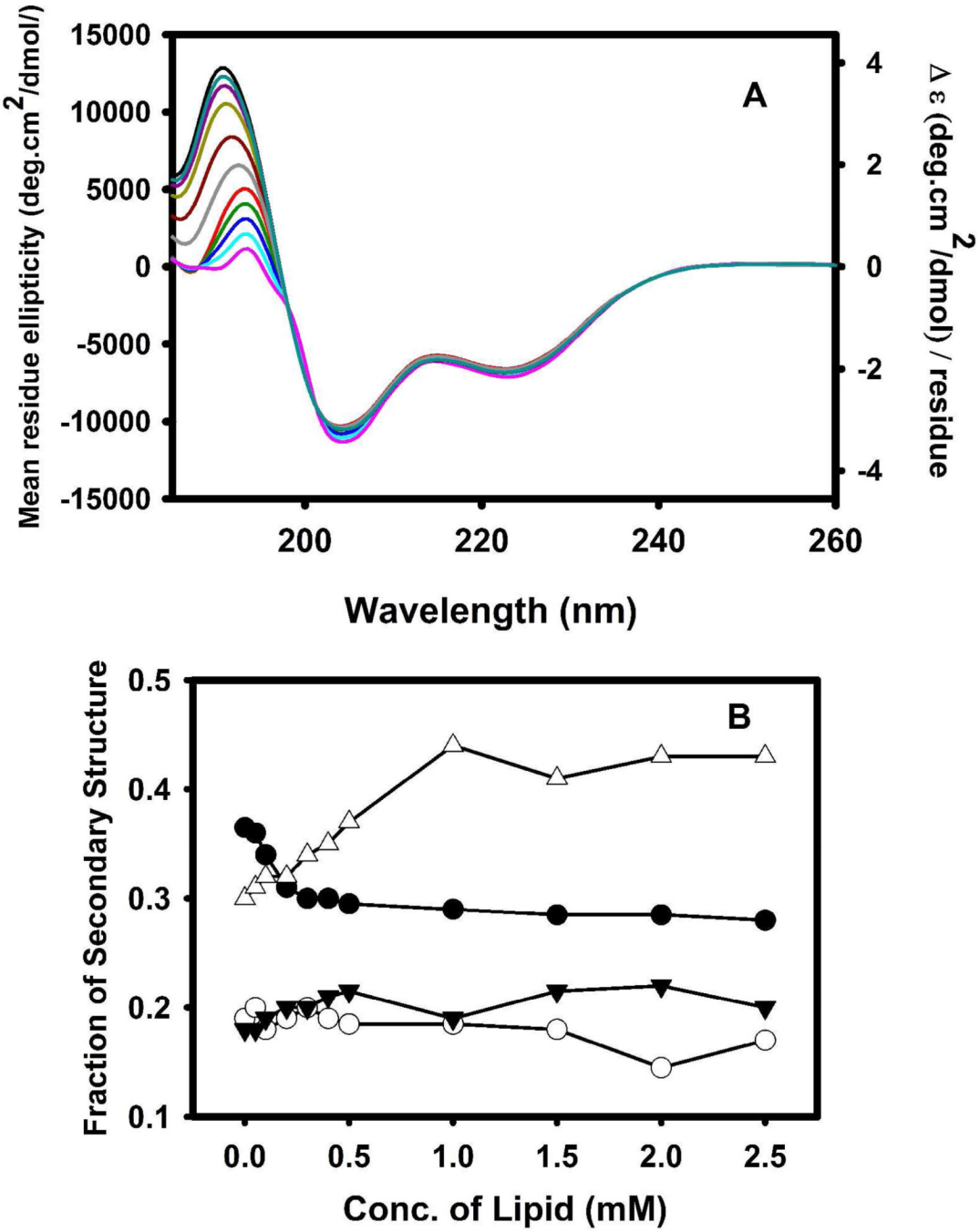

Figure 1.

(A) Plot of mean molar ellipticity of std. peptide in absence (black) and in presence of 0.05 mM (dark cyan), 0.1 mM (dark pink), 0.2 mM (dark yellow), 0.3 mM (dark red), 0.4 (dark grey), 0.5 mM (red), 1.0 mM (green), 1.5 mM (blue), 2.0 mM (cyan) and 2.5 mM (pink) of POPC SUVs. Measurements have been carried out at 10 mM phosphate buffer 23°C temperature. The average diameter of the SUVs used in our all experiment was 25 nm. **(B)** Plot secondary structural elements, i.e., helix (solid circle), beta-sheet (solid down triangle), turn (open circle) and unordered (open up triangle) of std. peptide at various concentration of POPC.

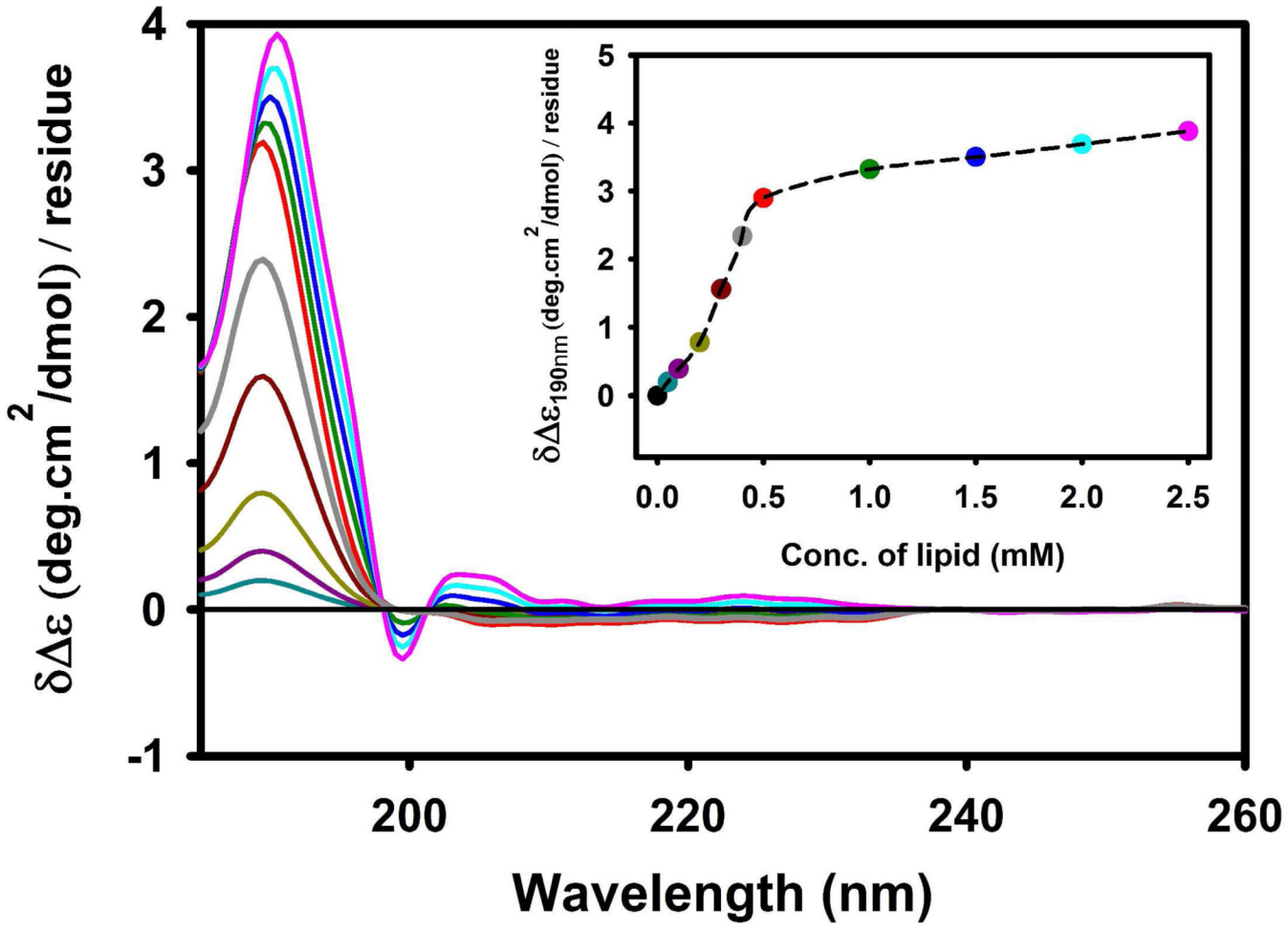

Figure 2.

Plot of δΔε for std. peptide in the absence (black) and presence of 0.05 mM (dark cyan), 0.1 mM (dark pink), 0.2 mM (dark yellow), 0.3 mM (dark red), 0.4 (dark grey), 0.5 mM (red), 1.0 mM (green), 1.5 mM (blue), 2.0 mM (cyan) and 2.5 mM (pink) POPC SUVs. **Inset:** Plot of $\Delta\Delta\varepsilon^{P.corr}$ at 190 nm vs. concentration of lipid. Smooth line drawn through the points to guide the eye. All measurements have been carried out at 10 mM phosphate buffer 23°C temperature. The average diameter of the SUVs used in our all experiment was 25 nm.

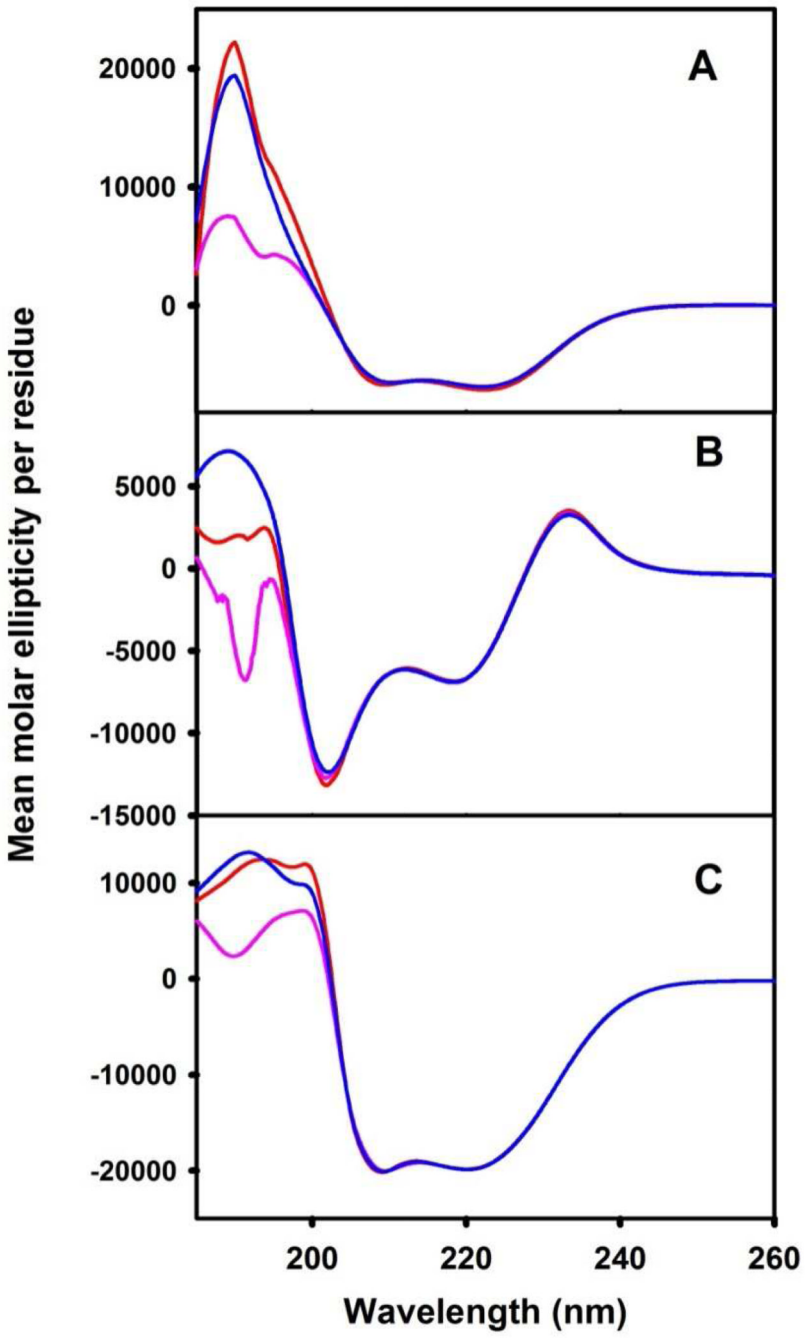

Figure 3.
Plot of mean molar ellipticity of (A) myoglobin, (B) KEAP1 and (C) TrpRS in phosphate buffer (red), in presence of 2.0 mM SUVs (pink) and after correction in presence of 2.0 mM SUVs (blue). All the measurements have been carried out in 10 mM Phosphate buffer at pH 7.4 at 23°C.

Table 1

Calculated secondary structure (Dichroweb (7, 8)) of different proteins in buffer, SUVs and after correction of the CD spectrum in SUVs using our correction factor. All experiments have been carried out in 10 mM Phosphate buffer at pH 7.4.

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

Biochemistry. Author manuscript; available in PMC 2013 February 7.