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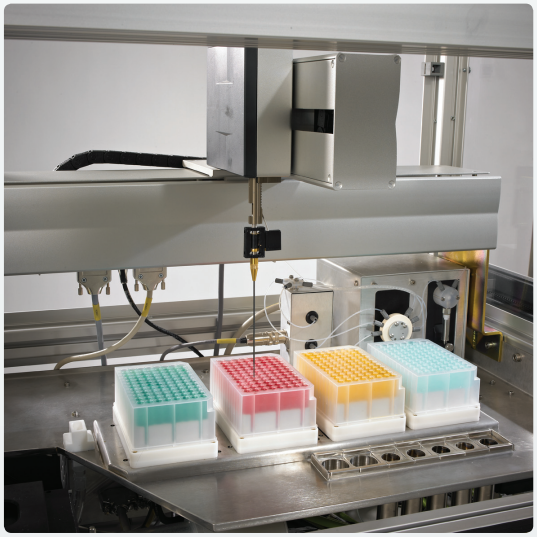

CHIRASCAN SERIES APPLICATION NOTE

Monitoring the Bilayer Reconstitution of a Membrane Protein by ACD

Abstract: This application note demonstrates the use of Automated Circular Dichroism (ACD) in monitoring the bilayer reconstitution of a membrane protein.

The bacterial K^+ channel protein, KcsA, is solubilised in detergent and reconstituted into an *E. coli* lipid vesicles by a stepwise increase the vesicle lipid concentration. Automation of the process allows many samples to be measured without the need to manually load, wash, and dry the sample cell.

The technique allows measurement of the reconstitution process whilst giving structural data about the protein. The results are validated by dynamic light scattering experiments.

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INTRODUCTION

From a pharmacological point of view, membrane proteins represent the most relevant class of biomolecules as they constitute 30% of all open reading frames in the human genome and are targeted by 50% of all drugs [1]. In spite of their importance, little is known about their detailed structures and functions, chiefly because they are notoriously difficult to produce, crystallise, and handle in biochemical and biophysical experiments. Since the abundance of most membrane proteins in their native membranes is too low for many *in vitro* assays, they first have to be overexpressed, purified, and reconstituted into well-defined membrane-mimetic systems [2]. The latter include detergent micelles, mixed bicelles, protein- or peptide-based nanodiscs, lipid vesicles, and free-standing or supported planar lipid bilayers [3].

During a stepwise reconstitution experiment, a purified, detergent-solubilised membrane protein is titrated with lipid vesicles prepared from crude lipid extracts or synthetic lipids [2]. In the simplest case, the reconstitution process can be described as a sequence of phase transitions following the so-called three-stage model [4]: Initially, at low lipid-to-detergent ratios, the vesicles added to the micellar protein solution are completely solubilised, and mixed detergent–lipid micelles form. Above a certain lipid concentration, mixed micelles and mixed vesicles coexist. At an even higher lipid-to-detergent ratio, the micelles completely disappear, and only bilayer vesicles persist.

KEYWORDS

▶ Membrane Proteins	▶ Proteoliposomes
▶ Automated	▶ Bilayer Reconstitution
▶ Circular Dichroism	▶ KcsA
▶ Vesicles	▶ Absorbtion Flattening

One of the major challenges in applying the above approach to membrane proteins is monitoring the reconstitution process in sufficient detail to accurately determine the lipid-to-detergent ratios at which the phase boundaries are located. This is, for example, important if a certain protein-to-lipid ratio in the resulting proteoliposomes is required for the actual experiment to be performed after reconstitution. The methods so far available for monitoring membrane-protein reconstitution include light scattering and turbidimetry [5], fluorescence spectroscopy [6], ^{31}P nuclear magnetic resonance spectroscopy [7], electron microscopy [8], and isothermal titration calorimetry [9,10]. However, none of these methods provides structural information on the membrane protein itself.

This gap is filled by automated circular dichroism (ACD) spectroscopy, which is excellently suited for monitoring the reconstitution of integral membrane proteins. Here, we demonstrate this approach using, as an example, the reconstitution of the bacterial K^+ channel KcsA [11] into *Escherichia coli* (*E. coli*) lipid vesicles by stepwise addition of lipid to KcsA solubilised in the detergent *n*-octyl-β-D-glucopyranoside (OG). Applying ACD spectroscopy, we followed the reconstitution process step by step and, at the same time, obtained structural data on the membrane protein. The results were validated by dynamic light scattering (DLS) experiments.

EXPERIMENTAL SECTION

Protein Production and Purification

The DNA coding for KcsA was cloned into a pET-30 Ek/LIC expression vector from Merck-Novagen (Darmstadt, Germany). Overexpression was done in *E. coli* BL21 (DE3) pLysS cells using auto-induction medium from Merck-Novagen. Cells were grown for 6h at 37°C. For protein production, the temperature was lowered to 30°C for another 18h. Harvested cell pellets were resuspended in buffer (150 mM KF, 10 mM KH_2PO_4/K_2HPO_4, 10 mM imidazole, pH 8.0), disrupted using a high-pressure homogeniser from Avestin (Mannheim, Germany), and incubated with 2% decyl-β-D-maltopyranoside for 2h on a shaker at 4°C to solubilise KcsA. The cell suspension was centrifuged at 50'000 *g* for 30 min at 4°C, and the supernatant containing KcsA was loaded onto an immobilised-metal affinity chromatography system from Bio-Rad (Munich, Germany) for purification. Detergent exchange into 80 mM OG (150 mM KF, 10 mM KH_2PO_4/K_2HPO_4, pH 7.1) was done using a P-6 desalting column from Bio-Rad. The final OG concentration in KcsA samples was determined by thin-layer chromatography according to the procedure of Eriks *et al*. [12].

Vesicle Preparation and DLS Measurements

E. coli polar lipid extract from Avanti Polar Lipids (Alabaster, USA) was dissolved to a final concentration of 50 mM in buffer (150 mM KF, 10 mM KH_2PO_4/K_2HPO_4, pH 7.1) by vortexing for 15 min. The lipid suspension was sonicated using a Sonopuls HD 3200 from Bandelin (Berlin, Germany) equipped with a water/ice-cooled BR 30 beaker resonator. Sonification was performed for 15 min at 100% amplitude (10 s on, 1 s off). The vesicle size distribution was determined by DLS using a Zetasizer Nano S90 from Malvern (Herrenberg, Germany) at 2 mM *E. coli* lipid and 20°C. Samples were allowed to equilibrate for 1 min in the sample holder. For DLS measurements on KcsA–detergent–lipid mixtures, the temperature was lowered to 8°C to enhance protein stability, and the equilibration time was increased to 6 min.

Sample Preparation for Protein Reconstitution

KcsA solubilised in 80 mM OG was mixed with buffer (150 mM KF, 10 mM KH_2PO_4/K_2HPO_4, pH 7.1) and *E. coli* lipid vesicles to yield a final OG concentration of 30 mM and lipid concentrations of 0, 0.2, 0.4, 0.8, 1.0, 1.25, 1.5, 2.0, 3.0, 4.0, and 5.22 mM. Samples with a final KcsA concentration of 0.5 µM were allowed to equilibrate for 1h at 8°C and were first measured by DLS and subsequently by ACD.

ACD Reconstitution Experiments

Protein–detergent–lipid mixtures and buffer containing 30 mM OG were manually dispensed into a 96-well plate in an alternating pattern. The plate was positioned on a plate holder on the ACD system deck and thermostatted at 8°C. After automated, user-unattended sample transfer from one of the wells in the 96-well plate into the flow-through cell, each sample was allowed to equilibrate for 6 min at 8°C. Spectra were scanned from 260 nm to 195 nm using a spectral bandwidth of 1 nm, a wavelength increment of 1 nm (190–240 nm) or 2 nm (240–260 nm), and a digital integration time of 6 s. Each sample was scanned three times, and the averaged protein spectrum was corrected by subtracting the spectrum of the preceding buffer blank and zeroing the resulting difference spectrum by subtracting the CD intensity averaged over the range 250–260 nm, which was treated as offset.

RESULTS AND DISCUSSION

Figure 1 depicts CD spectra of 0.5 µM KcsA solubilised in 30 mM OG at concentrations of *E. coli* lipid in the range 0–5.22 mM. The CD spectrum of detergent-solubilised KcsA in the absence of lipid exhibits a shape typical of an α-helical protein with two lipid characteristic minima at 208 and 222 nm. Titration with lipid leaves the CD spectrum virtually unchanged up to a lipid concentration of 1.0 mM. However, increasing the lipid concentration beyond this threshold leads to a gradual loss in signal intensity, which comes to a halt at a lipid concentration of 2.0 mM, as revealed in **Figure 2A**. Importantly, this reduction in CD signal intensity is not due to a loss of KcsA, as confirmed by independent protein quantification methods (not shown), but rather to increased differential light scattering and differential absorption flattening as vesicles form at concentrations >1.0 mM [13]. DLS experiments summarised in **Figure 2B** indicate that this is indeed the lipid concentration range in which vesicles begin to form, as manifested in a ~10-fold increase in both the light scattering intensity and the average particle diameter.

It should be noted that the light scattering intensity increases already at lower lipid concentrations, namely, in the range 0–0.5mM. This is most likely due to a transition from spherical micelles to cylindrical and tubular ones, a phenomenon previously described for micellar OG–lipid mixtures [14]. However, the corresponding CD spectra of KcsA were not affected by this transition (**Figure 2A**).

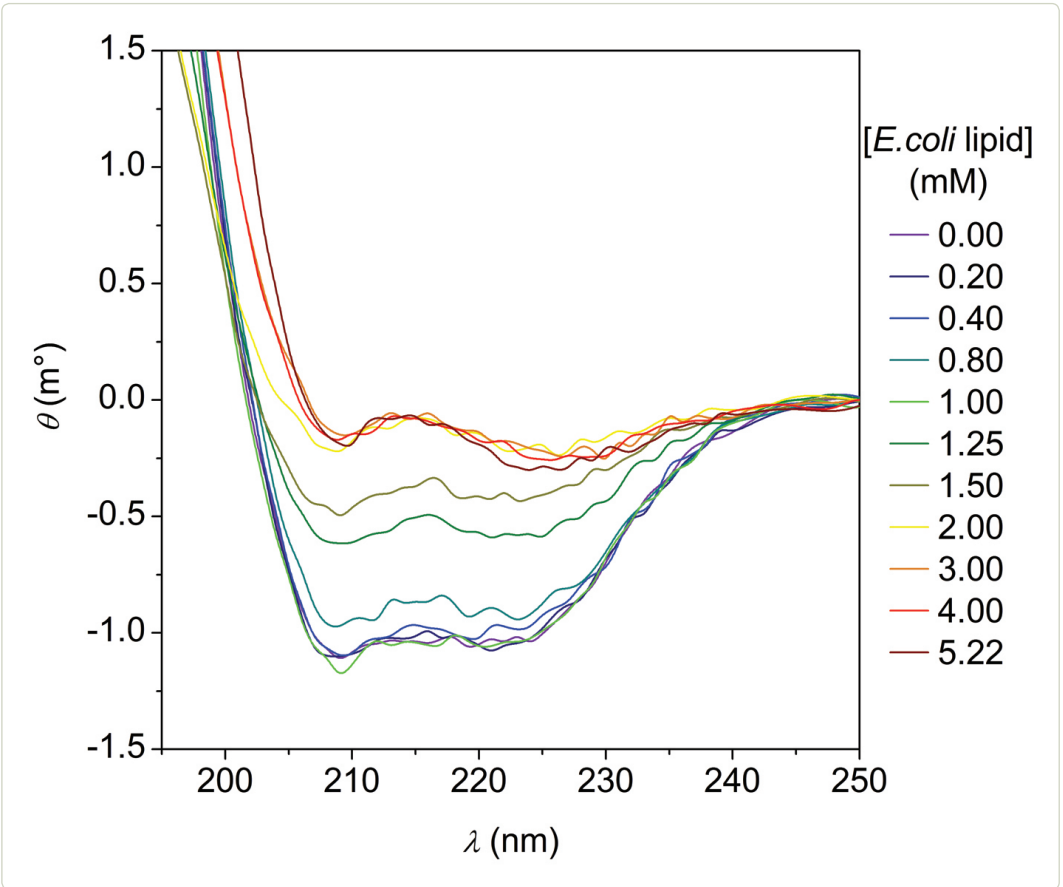

Figure 1. ACD spectra of 0.5 µM KcsA solubilised in 30 mM OG at increasing concentrations of *E. coli* lipid (see legend). The rather poor signal-to-noise ratio originates from the low protein concentration used and, at lipid concentrations >1.0 mM, from light scattering caused by vesicles and absorption flattening.

In addition to changes in CD signal intensity, close inspection of **Figure 1** reveals spectral distortions over the same lipid concentration range, with a pronounced flattening of the lower-wavelength edge of the far UV CD spectrum. Differential light scattering and differential absorption flattening lead to such effects because their effects increase dramatically with decreasing wavelength and increasing absorbance intensity, respectively [13,14]. This strong wavelength dependence is manifested in the ratio of the CD signal intensities at the two characteristic minima, $\theta^{208\ nm}/\theta$222nm, which changes from ~1.0 in the absence of lipid to ~0.5 in the presence of 5.22 mM lipid.

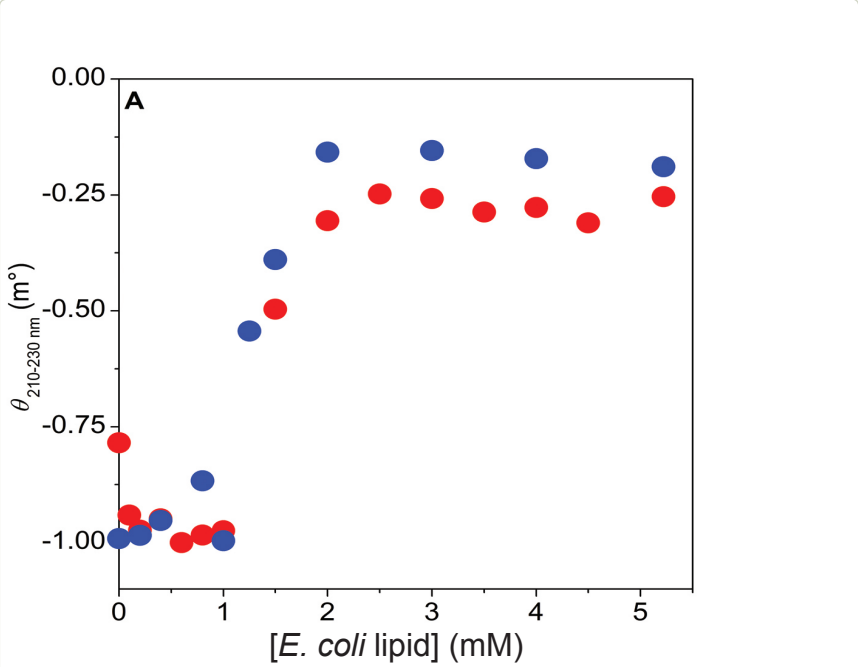

Figure 2A. Reconstitution of 0.5 µM KcsA solubilised in 30 mM OG into *E. coli* lipid extract probed by ACD Ellipticity averaged over the wavelength range 210–230 nm versus *E. coli* lipid concentration. Two independent experiments are shown (red and blue).

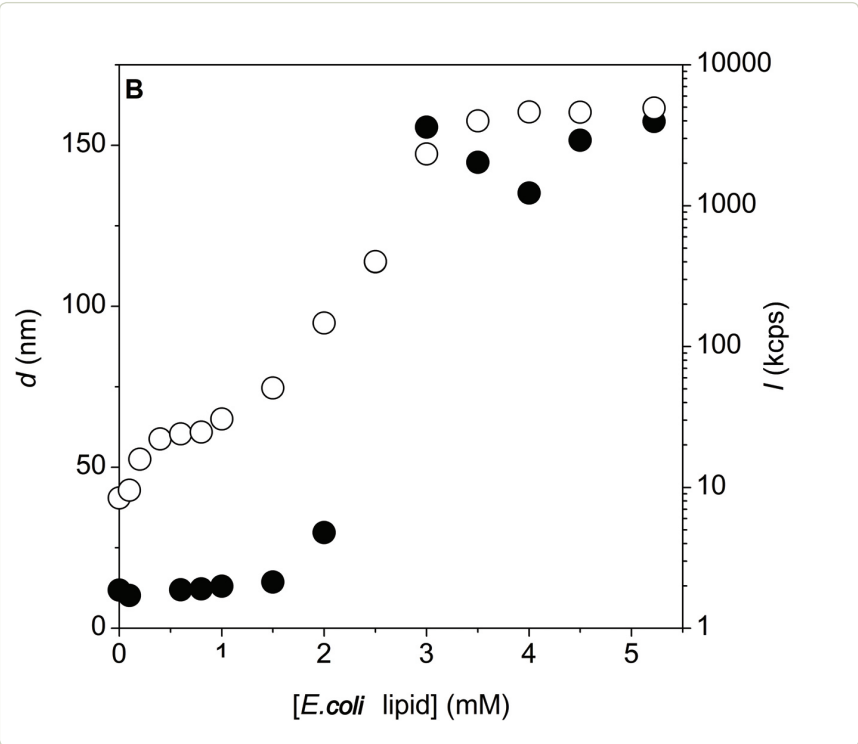

Figure 2B Mean particle diameter, *d*, (closed) and light scattering intensity, *I*, (open) measured by DLS as a function of *E. coli* lipid concentration.

In summary, the spectral changes observed upon increasing the concentration of *E. coli* lipid do not represent changes in protein concentration or conformation. Rather, they are hallmarks of differential light scattering and differential absorption flattening by vesicles appearing above a certain lipid concentration threshold. These phenomena are generally considered a nuisance that may cause severe artefacts in CD and other kinds of optical spectroscopy. However, in the particular case of membrane-protein reconstitution, one can exploit the spectral changes caused by the presence of lipid vesicles to observe the transfer of KcsA from small, well-dispersed protein–detergent complexes into large, densely packed proteoliposomes.

CONCLUSION

We demonstrate how the reconstitution of an integral membrane protein can be monitored with the aid of ACD and validate our results by DLS, a method traditionally used to follow membrane-protein reconstitution. Although spectral distortions caused by differential light scattering and differential absoption flattening light scattering at high lipid concentrations are obvious, the use of a short optical path length of 0.2 mm reduces their adverse effects to such an extent that structural information can still be obtained and may, in fact, be used to follow the reconstitution process step by step. Building on these proof-of-principle experiments, automation should enable systematic screens for reconstitution conditions on a structural basis, including the nature and concentration of the detergent used for solubilisation as well as the composition and concentration of the lipid mixture employed for reconstitution.

REFERENCES

[1] S. Fiedler, J. Broecker, S. Keller, Protein folding in membranes, Cell. Mol. Life Sci. **67** (2010) 1779–1798.

[2] J. L. Rigaud and D. Lévy, Reconstitution of membrane proteins into liposomes, Methods Enzymol. **372** (2003) 65–86.

[3] E. Serebrany, G. A. Zhu, E. C. Yan, Artificial membrane-like environments for *in vitro* studies of purified G-protein coupled receptors, Biochim. Biophys. Acta **1818** (2012) 225–233.

[4] D. Lichtenberg, R. J. Robson, E. A. Dennis, Solubilisation of phospholipids by detergents. Structural and kinetic aspects, Biochim. Biophys. Acta **737** (1983) 285–304.

[5] M. L. Jackson, C. F. Schmidt, D. Lichtenberg, B. J. Litman, A. D. Albert, Solubilization of phosphatidylcholine bilayers by octyl glucoside, Biochemistry **21** (1982) 4576–4582.

[6] M. Paternostre, O. Meyer, C. Grabielle-Madelmont, S Lesieur, M. Ghanam, M. Ollivon, Partition coefficient of a surfactant between aggregates and solution to the micelle–vesicle transition of egg phosphatidylcholine and octyl glucopyranoside, Biophys. J. **69** (1995) 2476–2488.

[7] M. T. Paternostre, M. Roux, J. L. Rigaud, Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 1. Solubilization of large unilamellar liposomes (prepared by reverse-phase evaporation) by triton X-100, octyl glucoside, and sodium cholate, Biochemistry, **27** (1988) 2668–2677.

[8] O. Lambert, D. Levy, J. L. Rank, G. Leblanc, J. L. Rigaud, A new “gel-like” phase in dodecyl maltoside-lipid mixtures: implications in solubilisation and reconstitution studies, Biophys. J. **74** (1998) 918–930.

[9] H. Heerklotz, A. D. Tsamaloukas, S. Keller, Monitoring detergent-mediated solubilization and reconstitution of lipid membranes by isothermal titration calorimetry, Nat. Protoc. **4** (2009) 686–697.

[10] O. O. Krylova, N. Jahnke, S. Keller, Membrane solubilisation and reconstitution by octylglucoside: comparison of synthetic lipid and natural lipid extract by isothermal titration calorimetry, Biophys. Chem. **150** (2010) 105–111.

[11] H. Schrempf, O. Schmidt, R. Kümmerlen, S. Hinnah, D. Müller, M. Betzler, T. Steinkamp and R. Wagner, A prokaryotic potassium ion channel with two predicted transmembrane segments from *Streptomyces lividans*, EMBO J. **14** (1995) 5170–5178.

[12] L. R. Eriks, J. A. Mayor, R. S. Kaplan, A strategy for identification and quantification of detergents frequently used in the purification of membrane proteins, Anal. Biochem. **323** (2003) 234–241.

[13] G. D. Fasman, Differentiation between transmembrane helices and peripheral helices by the deconvolution of circular dichroism spectra of membrane proteins, Circular Dichroism and the Conformational Analysis of Biomolecules; G. D. Fasman, Ed.; Plenum Press, New York (1996) 381–412.

[14] P. K. Vinson, Y. Talmon, A. Walter, Vesicle–micelle transition of phosphatidylcholine and octyl glucoside elucidated by cryo-transmission electron microscopy, Biophys. J. **56** (1989) 669–681.

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