ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2022, **58**, 4913

Received 23rd December 2021,
Accepted 2nd March 2022

DOI: 10.1039/d1cc07193a

rsc.li/chemcomm

Detergent-free isolation of CYP450-reductase's FMN-binding domain in *E. coli* lipid-nanodiscs using a charge-free polymer†

Bankala Krishnarjuna, Thirupathi Ravula ‡ and Ayyalusamy Ramamoorthy *

The membrane-anchored flavin mononucleotide binding domain (FBD) of CYP450 reductase was extracted in *E. coli* lipid-nanodiscs using charge-free pentyl-inulin polymer. FBD in nanodiscs was found to be conformationally homogenous and enabled high-resolution NMR probing. ^{31}P NMR revealed the polymer's lack of preference for any specific *E. coli* lipids and identified the lipid-types in nanodiscs.

There is significant interest in the high-resolution structures of membrane proteins, which represent ~30% of the proteome in all organisms and perform various physiological functions, and are the target for ~50% of pharmaceutical drugs available on the market.[1] Different types of lipids associated with the transmembrane (TM) domains are essential for the structure, stability, conformational dynamics, and function of membrane proteins.[2,3] Therefore, it is necessary to maintain the native lipid environment for membrane proteins while probing the structure and dynamics towards understanding their physiological function. However, it has not been possible to maintain the native lipids when membrane proteins are extracted using detergent that affect protein–lipid interactions and remove them altogether.[4,5] Although lipid-nanodiscs (composed of a lipid bilayer with the hydrophobic edge surrounded by amphipathic membrane scaffolding protein (MSP)/peptide) provide a stable lipid environment,[6,7] the membrane protein extraction steps (preceding reconstitution into nanodiscs) involving detergents could disrupt native lipid–protein interactions causing protein denaturation/aggregation.[8,9]

The poly(diisobutylene-*alt*-maleic acid) (DIBMA) and poly(styrene-*co*-maleic acid) (SMA)-based polymers are excellent alternatives to detergents and MSP/peptide to study membrane proteins in a lipid environment.[4,5,10] However, these polymers possess one or more limitations due to their high charge density and aromatic nature.[11] The charged polymers can interact with charged lipids in the cell membrane, interfering with lipid–membrane solubilization, affecting different lipid types associated with the target membrane protein and nanodisc formation, and membrane protein conformation/stability.[12,13] In addition, charged polymers can interact with the membrane protein (containing large soluble domains) with a net opposite charge at a given pH, leading to decreased protein yield, altering protein conformation, folding, stability, and function.[13] To avoid these issues, the charge of the polymer used for reconstitution should be the same as that of the target membrane protein that is to be solubilized and extracted/reconstituted.[12]

Ion-exchange chromatography (IEC) is a preferred protein purification method when a membrane protein does not contain affinity tags or if the tags are not desirable/compatible with the function of target membrane protein. Nevertheless, the electrostatic interactions of polymers with the ion-exchange resin render the purification of nanodisc–protein using IEC very difficult. Other limitations include the undesirable interference of the aromatic groups of the polymer with different spectroscopic measurements[14] and low stability in the presence of divalent metal ions and different pH.[15] Although styrene maleimide quaternary ammonium (SMA-QA) was developed to enhance the tolerance against pH variation and divalent metal ions, due to its charge the efficacy of solubilizing cell membranes is low like other reported charged polymers.[12,16,17] These limitations are particularly problematic when these polymers are used to extract low-expressing, intrinsically less-stable membrane proteins such as G-protein-coupled receptors (GPCRs) and mammalian cytochrome P450 (CYP450) enzymes. Additionally, reconstitution of membrane protein complexes and their functional and structural probing can be a great challenge when using polymer-nanodiscs possessing a high charge density.[13]

The newly developed inulin (fructooligosaccharides; n = 2–60)-based polymers contain no ionic groups (Fig. 1), form

Biophysics Program, Department of Chemistry, Macromolecular Science and Engineering, Biomedical Engineering, The University of Michigan, Ann Arbor, MI 48109-1055, USA. E-mail: ramamoor@umich.edu

† Electronic supplementary information (ESI) available. See DOI: 10.1039/d1cc07193a

‡ Present address: T. Ravula, NMRFAM, Department of Biochemistry, University of Wisconsin, Madison, WI 53706-1544, USA.

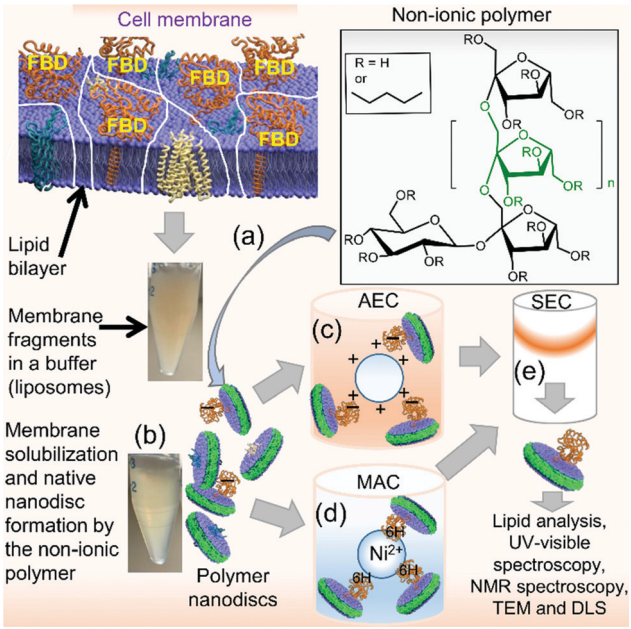

Fig. 1 Schematic of the extraction of the FBD in nanodiscs using inulin (fructooligosaccharides; n = 2–60) functionalized with pentyl groups. (a) The membranes were incubated with pentyl-inulin (boxed) at 4 °C. (b) A transparent solution appeared after adding the pentyl-inulin to cell membranes indicating the formation of soluble nanodiscs (blue-green). The protein was purified using either (c) anion-exchange chromatography (AEC) or (d) metal-ion affinity chromatography (MAC) followed by (e) size-exclusion chromatography (SEC) methods. "+" and "−" indicate the charge on the FBD and resin. 6H indicates the 6His-tag on the FBD.

nanodiscs upon mixing with lipids, and are tolerant to metal ions and a range of pH conditions.[18] Therefore, in this study, for the first time, we chose charge-free inulin functionalized with hydrophobic pentyl groups (pentyl-inulin) to extract membrane proteins in native lipids. The detergent-free membrane protein extraction using pentyl-inulin is demonstrated for the membrane-anchored flavin mononucleotide (FMN)-binding domain (FBD) of NADPH-cytochrome P450 reductase (CPR). The TM domain of CPR is essential to form the CPR-CYP450 functional complex within the lipid-bilayer.[19] The FBD, an immediate electron donor to the CYP450, is a 27.8 kDa protein domain anchored to the lipid bilayer through a single TM domain as confirmed by solid-state NMR experiments.[20] It has a single non-covalently attached FMN co-factor with a net negative charge of −24 at pH 7.4 (Table S1, ESI†). The reported studies used either FBD purified using detergent-based protocols or the truncated-FBD (lacking the TM domain).[7] The contact with detergents during purification could denature a membrane protein and affect its conformational stability. As a result, a functionally active protein may be converted into an inactive protein, as reported for CYP450.[13,21] Furthermore, removing the TM domain, or extracting proteins using detergents, can cause protein dysfunction and structural heterogeneity.[7,22,23] These observations further illustrate the crucial role of the TM domain on the structural folding and function of membrane proteins and also reveal the disadvantages of detergent-based membrane protein purification.

The direct extraction and the characterization of rat FBD in *E. coli* lipid-nanodiscs using pentyl-inulin is depicted in Fig. 1. The detailed protocol is described in the Supplementary Information. Briefly, 3.2 g of wet cells produced ∼1.2 g of membrane fragments. The cell membranes were resuspended and mixed with pentyl-inulin at 1 : 0.5 membrane-to-polymer ratio (w/w) in 20 mM potassium phosphate buffer (pH 7.4) containing 100 mM NaCl and incubated overnight at 4 °C under slow mixing (Fig. 1a and b). Unlike SMA, pentyl-inulin does not require higher salt concentrations (>100 mM) for efficient membrane protein extraction.[11] Then, the protein in the nanodiscs was purified using either diethyl-aminoethyl (DEAE) anion-exchange chromatography (Fig. 1c) or Ni^{2+}–NTA affinity (^{15}N-labelled FBD) chromatography methods (Fig. 1d).

The protein from DEAE beads and Ni^{2+}–NTA beads was eluted using a NaCl gradient and 300 mM imidazole, respectively. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of protein fractions showed a band corresponding to the FBD (Fig. 2a and Fig. S1, ESI†). The fractions containing FBD were pooled together, concentrated, purified and characterized by size-exclusion chromatography (SEC) (Fig. 1e). The chromatogram of a single peak eluted between 8 and 12 mL with a maximum A_{280} at ∼10 mL was observed, indicating the presence of large lipid-nanoparticles containing protein (Fig. 2b and Fig. S1, ESI†). Pentyl-inulin does not contain any aromatic moieties (Fig. 1); hence it did not interfere with protein absorption at ∼280 nm (Fig. S2, ESI†). Additionally, unlike SMA,[4,5] pentyl-inulin was not stained with Coomassie blue; therefore, it did not interfere with the SDS-PAGE analysis of proteins (Fig. S2, ESI†). The SDS-PAGE analysis of SEC fractions revealed the protein band corresponding to FBD (27.8 kDa) with high purity (Fig. 2c and Fig. S1, ESI†). Interestingly, a protein band at ∼56 kDa was also observed on an SDS-PAGE gel (Fig. 2c and Fig. S1, ESI†), which might have

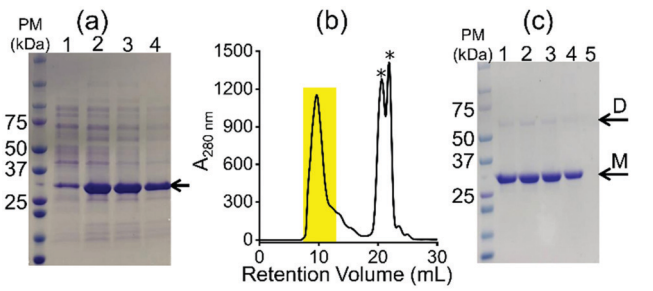

Fig. 2 (a) SDS-PAGE analysis of protein fractions eluted from DEAE-resin using NaCl at 400 (lane 1), 500 (lane 2), 600 (lane 3) and 700 mM (lane 4) concentrations. The arrow indicates the protein band corresponding to the FBD. (b) Purification of FBD by SEC. The absorption peak corresponding to the FBD in nanodiscs is highlighted with a yellow-color. The additional peaks (*) might have appeared from free polymers and other components from cell lysates (see Fig. S5 in ESI†). (c) SDS-PAGE analysis: lanes-1–4 are from the yellow-colored SEC fractions in (b) and lane-5 is from the colorless FBD-free fraction. M denotes protein monomer, and D denotes possibly a small fraction of protein dimer. PM: protein marker.

appeared due to a partial dimerization of FBD in the nanodiscs. The partial dimerization of FBD is similar to that observed for the cytochrome-b5 in nanodiscs.[12] The protein concentration was measured using UV-visible spectroscopy. The measured concentration of purified FBD based on the 451 absorbance peak was ~3.8 mg/1.2 g membranes (Fig. S3, ESI†).

A good dispersion of amide-proton resonances and the resonances in the high-field region (between 0 and −1 ppm) of the ^{1}H NMR spectrum indicate the well-folded conformation of FBD in nanodiscs (Fig. 3a). Based on the reference spectra recorded on pentyl-inulin in the presence and the absence of commercial *E. coli* lipids, the high-intensity resonances in the spectrum of nanodiscs were assigned to pentyl-inulin and *E. coli* lipids (Fig. 3a and Fig. S4, ESI†). The appearance of resonances from the protein, polymer, and lipids in the ^{1}H NMR spectrum confirmed the successful extraction of FBD in the nanodiscs. The stability of nanodiscs was monitored over a week using SEC. Similar SEC elution profiles were observed at different time points, suggesting good stability of the nanodiscs (Fig. S5, ESI†). The elution peak at ~16 mL (in Fig. S5, ESI†) might have appeared from the partial degradation of FBD over time. Nanodiscs were further characterized by recording dynamic light scattering (DLS), which showed a hydrodynamic radius of ~10 nm (Fig. 3b), and by acquiring transmission electron microscopy (TEM) images that revealed nanodiscs with a diameter of 20 ± 2 nm (Fig. 3c). These values are similar to that reported for pentyl-inulin with various synthetic lipids.[18] The results indicate that the pentyl-inulin forms larger nanodiscs as compared to that of SMA and smaller nanodiscs when compared to that of DIBMA.[10]

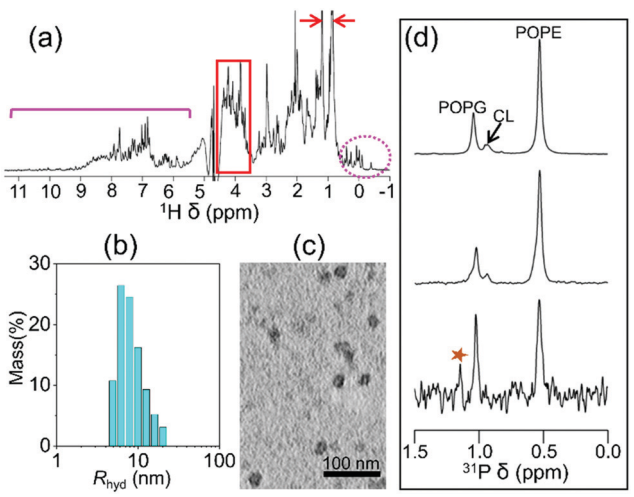

Fig. 3 (a) 1D ^{1}H NMR spectrum of FBD in nanodiscs. A good H^{N} resonance dispersion (magenta bar) and the appearance of resonances in the high-field region (between −1 and 0.5 ppm; circled) indicate the folded conformation of FBD. The resonances from lipids and pentyl-inulin are either boxed or indicated by arrows. (b) DLS profile of a SEC-purified sample (Fig. 2b) showing a large complex with a hydrodynamic radius (R_{hyd}) of ~10 nm. (c) TEM image of a SEC-purified sample (Fig. 2b). The measured particle size is 20 ± 2 nm diameter. (d) ^{31}P NMR spectra of nanodiscs (bottom), commercial *E. coli* lipids in polymer-nanodiscs (middle), and *E. coli* lipids alone (top). The NMR samples were prepared in a buffer containing 50 mM NaCl and 100 mM sodium cholate. * indicates uncharacterized lipids.

The lipids in nanodiscs were characterized using ^{31}P NMR (Fig. 3d).[12] Reference ^{31}P NMR chemical shifts for *E. coli* lipids such as phosphatidylethanolamine (POPE; [zwitterionic]), phosphatidylglycerol (POPG; [anionic]), and cardiolipin (CL) were obtained by recording a ^{31}P NMR spectrum of the commercial *E. coli* lipids (Fig. 3d, top). Polymer-nanodiscs containing commercial *E. coli* lipids were also prepared (Fig. S5, ESI†) and studied by ^{31}P NMR to see the pentyl-inulin effect on the *E. coli* lipid composition. The ^{31}P NMR peaks from all known *E. coli* lipids were observed from the polymer-nanodiscs (Fig. 3d, middle). The measured ratio of the areas of the observed peaks for different *E. coli* lipids in polymer-nanodiscs was similar to that of the polymer-free *E. coli* lipid sample (Table S2, ESI†), indicating that the polymer does not interact with any specific *E. coli* lipids. The ^{31}P NMR peaks observed from nanodiscs were assigned to POPE and POPG (Fig. 3d, bottom). CL was not observed in nanodiscs, because its concentration might be too low to be detected by ^{31}P NMR. The measured POPE : POPG ratio was 1.6 : 1 in the nanodiscs, whereas their ratio was ~3 : 1 in the polymer-nanodisc and polymer-free *E. coli* lipid samples (Tables S2 and S3, ESI†). It is unclear if the observed POPE : POPG ratio in the nanodiscs was due to FBD's preference to interact with them or their abundance in the *E. coli* membrane (Fig. 3d, top trace, and Table S2, ESI†). Therefore, additional studies are needed to understand FBD's selectivity for different types of lipids.

2D [^{15}N–^{1}H]-TROSY-HSQC spectra were acquired at 25 °C on 75 μM of ^{15}N-isotope-labelled FBD. Despite the large size of the nanodiscs (~20 nm diameter), a high-quality TROSY-HSQC spectrum was obtained, displaying well-resolved resonances (Fig. 4), which is in agreement with previous NMR studies on bicelles[20] or 4F peptide-nanodiscs[7,25] containing FBD. The spectral quality improved further when the temperature increased to 32 °C (Fig. 4 and Fig. S6, ESI†) due to the increased mobility of the soluble domain part of FBD at higher temperatures. In addition, the number of low-intensity peaks in the TROSY-HSQC NMR spectrum decreased substantially compared to that reported previously for both truncated-FBD and the detergent-purified FBD in 4F-DMPC nanodiscs, indicating more homogenous conformation for FBD when directly extracted in nanodiscs than that produced by the detergent-based purification methods (Fig. S6, ESI†).[7,22] The measured ^{1}H and ^{15}N line-widths from TROSY-HSQC (@32 °C) were 20–28 Hz and 14–20 Hz, respectively (Fig. S7, ESI†), as previously reported for FBD reconstituted in 4F-DMPC nanodiscs.[7] Due to peak overlap, the line-widths for residues located near the lipid bilayer were not measured. Despite the large size of the nanodiscs (Fig. 3b and c), the high quality NMR spectra obtained in this study indicate the isotropic nature of the soluble domain of FBD and demonstrate the feasibility for further high-resolution structural and dynamics studies by NMR.

In this study, we have demonstrated that charge-free pentyl-inulin is an excellent alternative to detergents to extract and study membrane proteins in a lipid environment. Additionally,

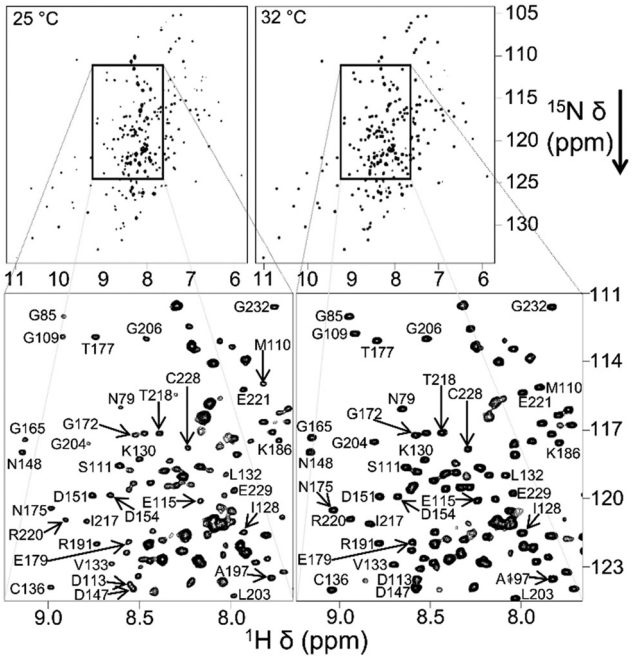

Fig. 4 2D [^{1}H–^{15}N]-TROSY-HSQC NMR spectra of 75 μM ^{15}N-labelled FBD in nanodiscs recorded at 800 MHz NMR spectrometer. For easy reading, expanded regions are shown below, highlighting few peaks with a substantial signal improvement at higher temperature (32 °C). The spectra were processed using identical parameters. The sample was prepared in 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl.

pentyl-inulin-based cell membrane extraction affords nanodiscs that contain membrane proteins in a stable conformation. Importantly, it is feasible to identify the lipid types associated with the extracted membrane proteins in nanodiscs by ^{31}P NMR and other biophysical methods.[5,24] The information on lipid composition can be useful to study membrane proteins prepared using detergent-based methods. Furthermore, due to its non-ionic nature, pentyl-inulin is compatible with ion-exchange and affinity chromatography purification resins. Despite the large size of extracted *E. coli* lipid-nanodiscs (~20 nm diameter), the high quality NMR spectra obtained for FBD demonstrates the suitability of inulin-based polymer nanodiscs to study membrane proteins by NMR spectroscopy. Additionally, it is shown that the pentyl-inulin does not interfere with UV-based spectroscopic measurements of proteins. We believe that the directly extracted membrane proteins are highly suitable for functional assays, cryo-EM studies, NMR studies under solution/aligned conditions, and solid-state NMR studies to characterize the TM domains.[18,20] We foresee that the non-ionic inulin-based polymer will be useful in the functional reconstitution and structural studies of complexes composed of oppositely charged proteins or protein-RNA.

This study was supported by NIH (R35 GM139572 to A. R.).

Conflicts of interest

The inulin-based polymers are filed for a US patent.

Notes and references

1 A. Krogh, B. Larsson, G. von Heijne and E. L. L. Sonnhammer, *J. Mol. Biol.*, 2001, **305**, 567–580.
2 R. Dawaliby, C. Trubbia, C. Delporte, M. Masureel, P. Van Antwerpen, B. K. Kobilka and C. Govaerts, *Nat. Chem. Biol.*, 2016, **12**, 35–39.
3 C. Martens, R. A. Stein, M. Masureel, A. Roth, S. Mishra, R. Dawaliby, A. Konijnenberg, F. Sobott, C. Govaerts and H. S. McHaourab, *Nat. Struct. Mol. Biol.*, 2016, **23**, 744–751.
4 S. C. Lee, T. J. Knowles, V. L. Postis, M. Jamshad, R. A. Parslow, Y. P. Lin, A. Goldman, P. Sridhar, M. Overduin, S. P. Muench and T. R. Dafforn, *Nat. Protoc.*, 2016, **11**, 1149–1162.
5 J. M. Dörr, M. C. Koorengevel, M. Schäfer, A. V. Prokofyev, S. Scheidelaar, E. A. W. van der Cruijsen, T. R. Dafforn, M. Baldus and J. A. Killian, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 18607.
6 S. G. Sligar and I. G. Denisov, *Protein Sci.*, 2021, **30**, 297–315.
7 B. Krishnarjuna, T. Yamazaki, G. M. Anantharamaiah and A. Ramamoorthy, *Chem. Commun.*, 2021, **57**, 4819–4822.
8 H. Yin and A. D. Flynn, *Annu. Rev. Biomed. Eng.*, 2016, **18**, 51–76.
9 J. U. Bowie, *Curr. Opin. Struct. Biol.*, 2001, **11**, 397–402.
10 A. A. Gulamhussein, R. Uddin, B. J. Tighe, D. R. Poyner and A. J. Rothnie, *Biochim. Biophys. Acta, Biomembr.*, 2020, **1862**, 183281.
11 A. H. Kopf, J. M. Dörr, M. C. Koorengevel, F. Antoniciello, H. Jahn and J. A. Killian, *Biochim. Biophys. Acta, Biomembr.*, 2020, **1862**, 183125.
12 B. Krishnarjuna, T. Ravula and A. Ramamoorthy, *Chem. Commun.*, 2020, **56**, 6511–6514.
13 T. Ravula, N. Z. Hardin, J. Bai, S. C. Im, L. Waskell and A. Ramamoorthy, *Chem. Commun.*, 2018, **54**, 9615–9618.
14 A. O. Oluwole, B. Danielczak, A. Meister, J. O. Babalola, C. Vargas and S. Keller, *Angew. Chem., Int. Ed.*, 2017, **56**, 1919–1924.
15 K. S. Simon, N. L. Pollock and S. C. Lee, *Biochem. Soc. Trans.*, 2018, **46**, 1495–1504.
16 T. Ravula, N. Z. Hardin, S. K. Ramadugu, S. J. Cox and A. Ramamoorthy, *Angew. Chem., Int. Ed.*, 2018, **57**, 1342–1345.
17 M. Barniol-Xicota and S. H. L. Verhelst, *Commun. Biol.*, 2021, **4**, 218.
18 T. Ravula and A. Ramamoorthy, *Angew. Chem., Int. Ed.*, 2021, **60**, 16885–16888.
19 A. Nath, Y. V. Grinkova, S. G. Sligar and W. M. Atkins, *J. Biol. Chem.*, 2007, **282**, 28309–28320.
20 R. Huang, K. Yamamoto, M. Zhang, N. Popovych, I. Hung, S.-C. Im, Z. Gan, L. Waskell and A. Ramamoorthy, *Biophys. J.*, 2014, **106**, 2126–2133.
21 A. Luthra, M. Gregory, Y. V. Grinkova, I. G. Denisov and S. G. Sligar, *Methods Mol. Biol.*, 2013, **987**, 115–127.
22 I. Barsukov, S. Modi, L. Y. Lian, K. H. Sze, M. J. Paine, C. R. Wolf and G. C. Roberts, *J. Biomol. NMR*, 1997, **10**, 63–75.
23 M. Wang, D. L. Roberts, R. Paschke, T. M. Shea, B. S. S. Masters and J.-J. P. Kim, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 8411–8416.
24 C. Barnaba, T. Ravula, I. G. Medina-Meza, S.-C. Im, G. M. Anantharamaiah, L. Waskell and A. Ramamoorthy, *Chem. Commun.*, 2018, **54**, 6336–6339.
25 E. Prade, M. Mahajan, S.-C. Im, M. Zhang, K. A. Gentry, G. M. Anantharamaiah, L. Waskell and A. Ramamoorthy, *Angew. Chem., Int. Ed.*, 2018, **57**, 8458–8462.

