Screening Conditions for NMR of Integral Membrane Proteins

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Charles R. Sanders, Vanderbilt University chuck.sanders@vanderbilt.edu phone: 615-833-2586

Background Reading

Solution NMR of membrane proteins: practice and challenges. Sanders CR, Sönnichsen F. Magn Reson Chem. (2006) 44, S24-S40.

Recent Advances in the Application of Solution NMR Spectroscopy to Multi-Span Integral Membrane Proteins. Kim HJ, Howell SC, Van Horn WD, Jeon YH, Sanders CR. Prog Nucl Magn Reson Spectrosc. (2009) 55:335-360

Impact of bilayer lipid composition on the structure and topology of the transmembrane amyloid precursor C99 protein. Song Y, Mittendorf KF, Lu Z, Sanders CR. J Am Chem Soc. (2014)136:4093-4096. (see methods in Supp Info section)

Bicelles at low concentrations. Lu Z, Van Horn WD, Chen J, Mathew S, Zent R, Sanders CR. Mol Pharm. (2012) 9:752-761. (and references therein)

Functional delivery of a membrane protein into oocyte membranes using bicelles. Kang C, Vanoye CG, Welch RC, Van Horn WD, Sanders CR. Biochemistry. 2010 Feb 2;49(4):653-5. (see methods in Supp Info section)

Reconstitution of integral membrane proteins into isotropic bicelles with improved sample stability and expanded lipid composition profile. Morrison EA, Henzler-Wildman KA. Biochim Biophys Acta. (2012) 1818:814-20.

Phase I: Identification of a Model Membrane System Suitable for NMR-Based Structural Studies of a Membrane Protein

Model Membranes to Test:

Detergent Micelles

0.5% DPC

0.5% DM (beta-decylmaltoside)

0.2% CYFOS-7 (Cyclo-Fos-7)

0.2% TDPC (tetradecylphosphocholine)

0.1% LMPC (lyso-myristoylphosphatidylcholine)

0.1% LMPG (lyso-myristoylphosphatidylglycerol)

0.1% LPPG (lyso-palmitoylphosphatidylglycerol)

Mixed Micelles

(listed ratio is mole:mole, listed % is for lipid plus detergent weight)

2:8 SDS:DM 0.5%

1:9 DMPC:DM 0.5%

1:9 DMPG:DM 0.5%

2:8 DMPC:DPC 0.5%

2:8 DMPG:DPC 0.5%

2:8 DMPC:LMPG 0.5%

2:8 CHOBIMALT:LMPC 0.1%

2:8 CHOBIMALT:LMPG 0.1%

(DMPC= dimyristoylphosphatidylcholine DMPG= dimyristyoylphosphatidylglycerol)

Bicelles

Detergent component of the bicelles: always DHPC (D6PC) (dihexanoylphosphatidylcholine)

Lipid Components to try (always at q = 0.3).

DMPC

DMPG:DMPC = 1:4 mol:mol

POPC

POPG:POPC = 1:4 mol:mol

DMPC:cholesterol = 9:1 mol:mol

DMPG:cholesterol= 9:1 mol:mol

POPC:cholesterol = 9:1 mol:mol

(You may also consider testing the sphingomyelin-containing bicelles and bicelles with very long chain lipids described in Song Y, Mittendorf KF, Lu Z, Sanders CR. J Am Chem Soc. (2014)136:4093-4096.

I suggest two stages of testing:

(Test Set 1) Glutaraldehyde Cross-Linking of Each Target in Each Model Membrane System

This quick screen will provide an easy way of testing which model membrane systems FAIL to maintain the test protein in a soluble and monodisperse form.

Method

Purify each protein in DPC. Adjust pH to 6.0. Determine protein concentration by A280. Concentrate to 2 mg/ml. Add EDTA to 0.2 mM and DTT to 10 mM.

Dilute 20 microliters from the 2 mg/ml DPC stock solutions into 400 microliters of each of the 22 model membrane systems chosen for testing (each buffered at pH 6.0 with 25 mM Na-PIPES; 75 mM NaCl, plus 1 mM DTT, 0.2 mM EDTA and room temperature).

FOR THE BICELLE MIXTURES, USE 5% BICELLES

Remove 100 microliter aliquot for use as SDS-PAGE control.

To the remaining 320 microliters add fresh glutaraldehyde to 16 mM.
The glutaraldehyde is from a 25% aqueous stock solution (Sigma, store at -20°C; 25% solution is 2.5 M, add 2.1 microliters per 320 microliters of solution).

Mix for at least two hour at room temperature.

Run SDS-PAGE on both control (non-cross-linked) and cross-linked samples to assess how much non-specific oligomerization (ladder) and/or aggregation (protein disappears or appears a band at very top of gel) takes place in the model membrane system.

Variations of This Test (Optional): Repeat at different temperatures, salt content, or pH.

FOR A GIVEN TARGET PROTEIN, ONLY THOSE MODEL MEMEMBRANES THAT MAINTAIN THE PROTEIN IN A MONODISPERSE STATE WILL BE USED FOR TEST SET 2, BELOW.

(Test Set 2) NMR Screening of Model Membrane Systems

This test set will requires more protein… but not THAT much more.

Each sample will require 220 microliters of 200 micromolar protein. (180 microliters of 0.2 mM protein in a 3 mM NMR tube plus 40 microliters leftover for measurement of A280 and/or other analysis). If the protein is 10 kDa, this means 0.5 mgs of protein for a sample. 1 mg required for a 20 kDa protein.

Each protein will be purified via the established protocol for that protein up through the point where the protein is pure and bound to Ni(II)-agarose resin. The protocol then depends on which model membrane system you are trying to prepare your protein in for NMR testing:

(Route 1) For protein that is destined for testing in micelles or mixed micelles.

Re-equilibrate the protein in 12 X 1 columns volumes of a solution of 25 mM NaPi, 100 mM NaCl, pH 7.5 plus the micelles or mixed micelles of interest.

Elute the protein with 250 mM imidazole, pH 7.8 plus the micelles or mixed micelles of interest.

Adjust pH to 6.0 with acetic acid.

Measure A280 to determine protein concentration and also weigh the solution to determine total volume.

Use concentrator to do 3X exchange with 25 mM PIPES, plus 75 mM NaCl, plus 0.2 mM EDTA, plus 1 mM DTT, pH 6.0.

Prior to final exchange (after adding the third batch of PIPES buffer) add D2O to 8 %. Then concentrate to 0.2 mM.

You are now ready for NMR at both 25 and 45 deg. C.

(Route 2) For protein that is destined for testing in bicelles.

Purify the membrane protein of interest using metal ion affinity chromatography using standard methods until you reach the stage where you have a few mgs of pure protein bound to ca. 2 ml of resin in a gravity column, bathed in a detergent solution. Then, re-equilibrate the column with 5 X 1 resin bed volumes of a 1% bicelle solution in 20 mM imidazole, 100 mM NaCl (and 0.5 mM DTT, if needed) pH 7.8. Then, elute the protein using a 2% bicelle solution buffered with 250 mM imidazole at pH 7.8, preferably monitoring elution at A280 with a UV detector. It typically takes about 3 resin bed volumes of solution to elute the protein. Measure the volume and A280 of the eluted pool of bicellar protein to determine protein concentration and total mgs (blank the UV spectrometer with the elution buffer with 2% bicelles).

Concentrate 5-10X (typically from about 5 ml to 0.5-1.0 mL) using an Amicon Ultra Centrifugal filter with a MWCO of 10 kDa. The concentrated C99 sample (0.5 mL) in 250 mM imidazole is then diluted back to its starting volume volume of 15 mM DHPC (the CMC of DHPC is 15 mM) solution in 25 mM PIPES plus 75 mM NaCl plus 0.2 mM EDTA, 1 mM DTT, pH 6.0 and concentrated 5-10X again. Repeat the dilution/re-concentration step one more time. D2O is then added to the concentrated sample to 8%. Transfer 200 microliters to a 3 mm diameter NMR tube.

Run NMR at 25C and 45C.

An alternative way of preparing bicellar protein samples is based on first reconstituting the protein into lipid vesicles and then using a detergent such as DHPC to solubilize the vesicles to form bicelles. See the Henzler-Wildman reference listed at the beginning of this document for a robust protocol. A protocol for this alternative approach is presented as an appendix at the end of this document, but we don't swear by it.

Phase 2: Optimization of Final Conditions

From the phase I tests, it is hoped that you will find one (or possibly) more type of model membrane that yields promising NMR data at pH 6.0 and 45C. In phase 2 you will start with that model membrane and optimize further.

Part A: Measure TROSY spectrum for samples that contain the preferred model membrane system (from Phase I) plus one of the following:

(i) 5 mol% cholesterol
(ii) 5 mol% sphingomyelin (egg)
(iii) 5 mol% cholesterol + 5mol% sphingomyelin

Part B: Measure TROSY spectrum for samples for the best system so far (based on Phase I results plus the results of Part A) as you vary the pH: 5.0, 6.0, 6.5 *(you will already have pH 6.0 data from the phase I tests).*

Part C: For the very best composition found based on all of the above, acquire TROSY NMR spectra at 30 and 50ºC and compare with the data you already have for 45ºC.

Appendix: Alternative method for preparing bicellar protein samples for NMR.
This protocol is not yet fully optimized.

Purify protein in SDS or DPC using standard methods and then reduce pH to 6.0. Then measure A280 and determine protein concentration and volume.

Mix an aliquot of the protein solution containing 2X enough protein for an NMR sample with mixed micelles composed of 250 mM SDS(or DPC) plus 50 mM lipid mixed micelles so that protein:lipid mol:mol ratio is 200:1.

Dialyze out SDS or use Bio-Beads to remove the SDS; upon complete removal of SDS vesicles will form spontaneously. NOTE: IF USING DIALYSIS THE TEMPERATURE DURING DIALYSIS MUST BE GREATER THAN 25 DEG C TO REMAIN ABOVE THE PHASE TRANSITION TEMPERATURE OF DMPC OR DMPG. IF POPC OR POPG IS BEING USED THEN TEMPERATURE DOESN'T MATTER SINCE PHASE TRANSITION TEMPERATURE IS BELOW 0.
Bio-Beads are a quick and easy way to remove detergent, but must be used with care because they sometimes inactivate and/or absorb membrane proteins… it depends on the protein.

Concentrate the protein-containing vesicles to a concentration of 0.3 mM protein (you may not be able to use a centrifugal concentrator for this; you can try, but see CS if it does not work).

If using a centrifugal concentrator you can also adjust the composition of the buffer solution. Change to: 25 mM PIPES plus 76 mM NaCl plus 0.2 mM EDTA, 1 mM DTT, pH 6.0.

Remove the protein/vesicles from the concentrator and determine volume and calculate the moles of lipid and protein that must be present (assuming no loss during reconstitution and concentration). Add DHPC from a 30% w/v stock solution to bring q to 0.3 (0.3 moles of lipid per 1 mole detergent). Mix thoroughly using cooling and mild warming. You can also bath sonicate to help achieve a clear solution.

Add D2O to 8%.

Run NMR at 25C and 45C.