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Reconstitution of integral membrane proteins into isotropic bicelles with improved sample stability and expanded lipid composition profile

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ABSTRACT

Reconstitution of integral membrane proteins into membrane mimetic environments suitable for biophysical and structural studies has long been a challenge. Isotropic bicelles promise the best of both worlds—keeping a membrane protein surrounded by a small patch of bilayer-forming lipids while remaining small enough to tumble isotropically and yield good solution NMR spectra. However, traditional methods for the reconstitution of membrane proteins into isotropic bicelles expose the proteins to potentially destabilizing environments. Reconstituting the protein into liposomes and then adding short-chain lipid to this mixture produces bicelle samples while minimizing protein exposure to unfavorable environments. The result is higher yield of protein reconstituted into bicelles and improved long-term stability, homogeneity, and sample-to-sample reproducibility. This suggests better preservation of protein structure during the reconstitution procedure and leads to decreased cost per sample, production of fewer samples, and reduction of the NMR time needed to collect a high quality spectrum. Furthermore, this approach enabled reconstitution of protein into isotropic bicelles with a wider range of lipid compositions. These results are demonstrated with the small multidrug resistance transporter EmrE, a protein known to be highly sensitive to its environment.

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1. Introduction

Structural and biophysical studies of membrane proteins have traditionally lagged behind those of soluble proteins. One of the major challenges of working with membrane proteins is finding a membrane mimetic environment that is conducive to biophysical studies while still maintaining native structure and function [1]. Techniques such as solution NMR have fast-tumbling requirements that are not fulfilled by conventional lipid vesicles. Detergent micelles, isotropic bicelles, and nanodiscs are some of the media available for the solubilization of integral membrane proteins (IMPs). The high curvature and altered lateral pressure of detergent micelles make them a less than ideal membrane mimetic [1–4]. Detergents must be extensively screened to find a suitable match that preserves native structure and function, and gentler detergents such as alkylglycosides, which may better preserve function, are often not conducive to multidimensional NMR studies [5].

Both isotropic bicelles and nanodiscs provide bilayer environments, and each media has its own merits. Nanodiscs are stable particles that can be separated by physical means such as gel filtration chromatography, unlike isotropic bicelles in which the detergent is in constant equilibrium between monomer and bicelle. This same property of isotropic bicelles allows them to reconstitute at any size by varying the long- to short-chain lipid ratio, whereas nanodiscs can only make discretely sized particles. A new membrane scaffold protein must be expressed and purified for each nanodisc size, and they must be carefully tested to determine the proper ratio of scaffold-to-lipid for each preparation [6]. In addition, NMR spectra of IMPs reconstituted into nanodiscs tend to be broadened [1,2,7].

Use of isotropic bicelles has been limited by their stability and spectral qualities [1,2]. Traditional methods for making bicelle samples require harsh conditions that may not preserve proper protein structure and function. Improved bicelle stability would allow for more widespread use. Previous attempts at increasing bicelle stability and sample lifetime have included using ether-linked lipids and/or adding up to 10% of long- and short-chain lipids with charged headgroups such as PS and PE-DTPA [4,8,9]. However, recent studies have indicated that non-native ether-linked lipids alter the structure and dynamics of antimicrobial peptides [10], making this a less desirable substitution.

Abbreviations: NMR, nuclear magnetic resonance; DM, decylmaltoside; DHPC, 1,2-dihexanoyl-sn-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol); TPP^+, tetraphenylphosphonium$^+$; PS, phosphatidylserine; PE-DTPA, phosphatidylethanolamine-N-diethylenetriaminepentaacetic acid; IMP, integral membrane protein; DLS, dynamic light scattering; TLC, thin layer chromatography.

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Another challenge with isotropic bicelles is the relatively limited lipid compositions currently in use. More varied lipid compositions have been explored with magnetically aligned large bicelles for solid-state NMR [1]. An expanded lipid composition profile would allow for more physiologically relevant isotropic bicelles, similar to nanodiscs, which can be formed with a range of lipids and lipid extracts [2,6].

Here, we present an improved reconstitution of integral membrane proteins into small isotropic bicelles that allows reconstitution into bicelles with a much wider range of lipid compositions. This protocol is easily adaptable to any IMP of interest, with no requirement for organic solvent or specific detergents. It also ensures complete detergent removal, eliminating potential instabilities and inhomogeneities caused by residual detergent and providing an optimal sample for biophysical studies. We have recently demonstrated the utility of these bicelles for solution NMR dynamics measurements of EmrE [11]. These cutting edge NMR measurements would not have been possible without the improved sample stability and homogeneity provided by this method.

2. Materials and methods

2.1. EmrE expression and purification

EmrE was expressed in a pET15b plasmid with an N-terminal 6xHis tag (Geoffrey Chang, Scripps Research Institute) and expressed and purified as previously described [11]. Briefly, isotopically labeled EmrE was grown in ^{15}N- or ^{2}H/^{15}N-labeled M9 minimal media. To purify EmrE, cells were lysed by sonication. The membranes were solubilized in 40 mM DM (Affymetrix Anatrace), purified using Ni-NTA resin (Novagen), and cleaved with thrombin (Sigma-Aldrich). Final purification was performed by gel filtration chromatography on a Superdex 200 column (GE Healthcare) equilibrated with 10 mM DM. The purified EmrE was then reconstituted into isotropic bicelles in deoxygenated buffers.

2.2. Sample preparation

2.2.1. Reconstitution into isotropic bicelles by detergent exchange

Purified EmrE was concentrated to 0.5 mL and DM reduced to less than 30 mM, assuming monomeric detergent passed through the filter while micelles were concentrated along with the protein. The resulting sample was run over a Superdex 200 column equilibrated with 25 mM DHPC (Avanti Polar Lipids). EmrE in DHPC was concentrated to 250 μL and long-chain lipid added to a final ratio of 3:1 short- to long-chain lipid (at least 75 mM long-chain lipid and 130-fold excess of long-chain lipid:EmrE). Four freeze–thaw cycles were carried out to ensure homogeneous bicelles.

2.2.2. Reconstitution into isotropic bicelles via liposomes

Long-chain lipid (DLPC, DMPC, DPPC, POPC, DOPC, POPE, POPG, and *Escherichia coli* polar lipid extract, Avanti Polar Lipids) was hydrated in buffer above the phase transition temperature for 1–2 hours at 20 mg/mL and then sonicated in a high-power bath sonicator (Laboratory Supplies Company, Inc, Hicksville, NY). The lipid vesicles were then incubated with 0.51% octylglucoside (Affymetrix Anatrace) for 15–30 minutes. Purified EmrE in DM was added at a molar ratio of 1:130, EmrE:long-chain lipid. After incubation for an additional 30–60 minutes, Amberlite (Supelco) was added to remove the detergent. Three aliquots of 30 mg Amberlite per mg detergent were added with incubation at room temperature for 1–2 hours, overnight, and another 1–2 hours. The proteoliposomes were collected via ultracentrifugation (50,000 × *g*, 1 hour, 20 °C) and resuspended in DHPC buffer at a molar ratio of 3:1 short- to long-chain lipid, assuming a 90% recovery of long-chain lipid. The final lipid ratio in the bicelles was confirmed by ^{1}H-NMR using the lipid terminal methyl peaks. The final buffer conditions for all NMR samples were 20 mM potassium phosphate, 30 mM sodium cacodylate, 20 mM NaCl, 0.05% sodium azide, 2 mM TCEP, pH 7, with 2 mM TPP^{+} and EmrE concentrations in the range of 0.5–1.1 mM.

2.3. Transport assay

H^{+}-driven uptake of the substrate dequalinium^{2+} was monitored using fluorescence spectroscopy, following the fluorescence signal of the substrate. EmrE was reconstituted into *E. coli* polar lipid extract as described above with a lipid:EmrE dimer ratio of 640:1. The buffer was 190 mM NH_4Cl, 15 mM Tris pH 7 as in ref. [12]. The sample was extruded through a 200 nm filter to create homogeneous vesicles. The proteoliposomes were diluted ten-fold into either the same buffer (no H^{+} gradient) or a buffer of 140 mM KCl, 10 mM Tricine, 5 mM $MgCl_2$, 10 mM Tris pH 8 (H^{+} gradient). The final protein concentration was 1 μM. Transport was initiated upon the addition of concentrated dequalinium^{2+} to the bulk solution. The experiments were carried out in a Varian Cary Eclipse fluorescence spectrometer, at an excitation wavelength of 350 nm and emission wavelength of 460 nm.

2.4. Dynamic light scattering

DLS experiments were carried out on a DynaPro (model 99-E-50, Protein solutions) with the Dynamics V6 software. The instrument was calibrated with an albumin standard (Thermo Scientific) at varying temperatures and concentrations. The NMR samples were diluted four-fold to decrease secondary scattering effects, while still maintaining sufficient lipid concentration to keep the same effective *q*-value, or ratio of long- to short-chain lipids in the bicelle [13]. Data were collected at 10 °C and 25 °C, for five acquisitions of 5 minutes each.

2.5. Thin layer chromatography

TLC was carried out using Whatman K6 silica gel 60A plates and a 65:24:4 chloroform:methanol:water solvent system. Isotropic bicelle samples were diluted 10-fold in 2:1 chloroform:methanol for spotting onto the plates. After running the plates, they were dried and developed in an iodine vapor chamber overnight.

2.6. Refractive index measurements

A Reichert-Jung Abbe Mark II digital refractometer with water-bath temperature control was used to measure the refractive indices of isotropic bicelles at the pertinent concentrations and temperatures.

2.7. Viscosity measurements

A calibrated Cannon-Ubbelohde semi-micro viscometer, size 50, was used to determine the viscosity of isotropic bicelles. Both refractive index and viscosity were measured under the same conditions (lipid concentrations and temperatures) as the diffusion measurements and used to calculate the hydrodynamic radius.

2.8. NMR spectroscopy

All experiments were carried out on a 700 MHz Varian Inova spectrometer at 45 °C. Lipid ratios were confirmed by integrating the terminal methyl resonances of the short- and long-chain lipids in a ^{1}H spectrum. Standard (^{1}H, ^{15}N)-TROSY HSQC spectra with gradient coherence selection were acquired with 120 and 24 scans per increment, respectively, for non-^{2}H and ^{2}H samples. (^{1}H, ^{15}N)-TRACT [14] was used to measure rotational correlation times. The

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