A native cell membrane nanoparticles system allows for high-quality functional proteoliposome reconstitution

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Abstract

Proteoliposomes mimic the cell membrane environment allowing for structural and functional membrane protein analyses as well as antigen presenting and drug delivery devices. To make proteoliposomes, purified functional membrane proteins are required. Detergents have traditionally been used for the first step in this process. However, they can irreversibly denature or render membrane proteins unstable, and the necessary removal of detergents after reconstitution can decrease proteoliposome yields. The recently developed native cell membrane nanoparticles (NCMN) system has provided a variety of detergent-free alternatives for membrane protein preparation for structural biology research. Here we attempt to employ the MCMN system for the functional reconstitution of channels into proteoliposomes. NCMN polymers NCMNP1-1 and NCMNP7-1, members of a NCMN polymer library that have been successful in extraction and affinity purification of a number of intrinsic membrane proteins, were selected for the purification and subsequent reconstitution of three bacterial channels: KcsA and the mechanosensitive channels of large and small conductance (MscL and MscS). We found that channels in NCMN particles, which appeared to be remarkably stable when stored at 4 °C, can be reconstituted into...
bilayers by simply incubating with lipids. We show that the resulting proteoliposomes can be patched for electrophysiological studies or used for the generation of liposome-based nanodevices. In sum, the findings demonstrate that the NCMN system is a simple and robust membrane protein extraction and reconstitution approach for making high-quality functional proteoliposomes that could significantly impact membrane protein research and the development of nanodevices.

Keywords
KcsA; MscL; MscS; Triggered-release; NCMN; Proteoliposome

Introduction

Integral membrane proteins including channels, transporters, receptors, and enzymes present daunting challenges for biophysical characterization and functional reconstitution. The selection of surfactants that will optimally preserve the structure and functional properties of a given membrane protein can be a formidable prerequisite. Detergents may irreversibly denature the protein, confounding both functional and structural studies. For example, some detergents have been shown to change the stoichiometry of the MscL protein [1–4]. In addition, dodecylphosphocholine-solubilized and membrane-embedded KcsA channels were reported to have structural differences in the N-terminal end of the first transmembrane domain [5], and the pH-induced effects on KcsA and its transmembrane allostery are very different in detergents vs. membranes [6, 7]. The alkyl phosphocholines, popularly used because they often maintain function when other lipids do not, have also been found to be problematic in many instances [8]. Even when detergents maintain native structure, the proteins can exhibit altered thermodynamic and functional characteristics, and have an impractical shelf life. If the membrane protein is to be characterized functionally, this often involves the reconstitution into proteoliposomes.

To overcome the limitations of detergents for structural studies, styrene-maleic acid co-polymer and other membrane active polymers that form native-nanodiscs have recently been used for membrane protein extraction while still interacting with their native membranes [9–11]. The laboratory of Dr. Guo has developed a detergent-free native cell membrane nanoparticles (NCMN) system for membrane protein research that allows for high resolution single particle cryo-EM analysis of both transporters and the associated lipid bilayers [12]; it is distinct from and has advantages over the styrene maleic acid lipid particles (SMALP) system and has also recently been used for the analysis of protein-protein interactions [13]. The NCMN system comprises three linked libraries: 1) an indexed library of polymers that have been demonstrated to extract homogenous native cell membrane nanoparticles for single-particle cryo-EM study from native membranes; 2) the library of preparation and extraction protocols for native cell membrane nanoparticles, tailored for each polymer since each NCMN polymer has unique physical and chemical properties; 3) the library of analysis protocols for potential biological, biochemical and biophysical approaches such as single-particle cryo-EM, Mass Spectrometry, and solid-state NMR. Because the NCMN system is continually growing in the number of high-quality membrane active polymers, many
previously intractable membrane proteins/complexes can now be potentially studied by biochemical and biophysical approaches within native-cell membrane nanoparticles.

Here we report new applications for this NCMN system for channel patch clamp studies as well as triggered-release nanodevice assembly. We focus on the use of NCMN particles for three representative but unrelated integral membrane protein channels: KcsA, a widely studied potassium channel from *Streptomyces lividans* (*S. lividans*), and two mechanosensitive channels from independent families: the mechanosensitive channels of large and small conductance (MscL and MscS, both from *Escherichia coli* (*E. coli*)), which play major roles in rapidly adapting to acute decreases in osmotic environment, and allowing cytosolic components to be rapidly jettisoned from the cell [1, 14–18]. We find that these channels can be functionally reconstituted into liposomes directly from NCMN particles. In addition, we found the system to be advantageous for a nanodevice that uses a MscL-based “triggered nanovalve” whose modality is changed to be actuated by pH [19]. These data suggest that NCMN polymer solubilization and NCMN particle storage may be a preferable method for obtaining stable and reconstitutable functional channel proteins.

**Materials and methods**

**NCMN polymers**

NCMN1–1 and NCMNP7–1 were made based on the previously published protocol [12] using SMA2000 co-polymer powder from Total Cray Valley (Lot # PS18013101) instead of flakes as start materials. For making NCMNP1–1, sodium hydroxide was used for hydrolysis of the maleic anhydrides functional groups. For making NCMNP7–1, instead of using sodium hydroxide, we used 2-aminoethanesulfonic acid to open the maleic anhydride ring and form amide derivatives. Request for NCMN polymers may be directed to Youzhong Guo (yguo4@vcu.edu).

**Preparation of MscS, KcsA and MscL NCMN particles**

The wild-type *E. coli* mechanosensitive channel of small conductance (MscS) was constructed into pRSFDuet-1 with an N-terminal decahistidine tag (pRSF-MscS). The plasmid encoding the pH-gated potassium channel (KcsA) from *S. avidini* with a mutation (T74S) and an N-terminal hexa-histidine tag was cloned into PASK90 vector system (PASK90-T74S-KcsA). The *E. coli* mechanosensitive channel of large conductance (MscL) wild type and mutated at G22/G26C were cloned into pET 21a with a c-terminal hexa-histidine tag (pET-MscL). The mutant MscL was also truncated at its c-terminal end from residue 110 to 136 which has been shown to improve protein expression while maintaining same level of channel function [14, 20, 21] MscS and MscL constructs were separately transformed into *E. coli* BL21 (DE3) pLysS cells whereas, the T74S KcsA construct was transformed into *E. coli* JM83 cells. The transformed cells were grown on LB (Lennox Broth) agar plate containing 25 μg/L Chloramphenicol and 25 μg/L Kanamycin for MscL and MscS, and 100 μg/L ampicillin for T74S KcsA. The plates were incubated overnight at 37 °C. Two single colonies were picked for each plate and transferred in 10 mL of Terrific Broth (TB) with the appropriate antibiotics for each of them. The pre-culture was incubated at 37 °C with 220 rpm shaking overnight. Four mL of pre-culture were then transferred into
two flasks with one liter of TB each and incubated at the same conditions as mentioned before. The optical density at 600 nm (OD 600) of the culture was monitored for ~4 h until it reached a value between ~0.8–1. At this point, the protein expression was induced with 1 mM of IPTG (isopropyl-1-thio-beta-D-galactopyranoside) for MscL and MscS and 200 μg/mL of anhydrotetracycline for KcsA. And the cells were allowed to grow at 20 °C with 220 rpm shaking for 20 h. The cells were harvested by centrifugation at 6,000 g for 15 min at 4 °C. Twenty grams of cell pellets for MscS and MscL were re-suspended in NCMN buffer A (see below). For T74S KcsA, 500 mM KCl was substituted for 500 mM NaCl in in NCMN buffer A. Cells in lysis buffer were lysed by 3 passages through Avestin EmulsiFlex-C3. The cell debris was pelleted by centrifugation at 15,000 g for 30 min at 4 °C. Finally, to get the cell membrane, the supernatant was centrifuged at 250,000 g for 1 hour at 4 °C.

Preparation of native cell membrane nanoparticles was performed following the previously published protocol with slight modification. Briefly, 1.5 to 2 g of pure cell membrane were suspended in NCMN buffer A and homogenized with a Dounce homogenizer in a volume of 25 mL, then 25 mL of 5% stock solution of NCMN polymer (NCMNP1–1 or NCMNP7–1) was added to bring the total volume to 50 mL in a 50 mL conical tube. The ratio of the wet cell membrane and NCMN polymer was approximately 1 g cell membrane per 0.615 g NCMN polymer; in some cases this amount of NCMN polymers could be reduced by half without noticeable decreases in yield. The sample was incubated at room temperature for 2 h. The solution was centrifuged at 15,000 g for 30 min, the supernatant was then further centrifuged at 150,000 g for 1 h. The supernatant was then loaded onto a 5 mL NiNTA (GE Health) at a flow rate of 0.5 ml/min using a syringe pump at room temperature. Then, the NiNTA column was washed with 30 mL NCMN buffer B using an AKTA FPLC (GE Healthcare) and 30 mL NCMN buffer C. Finally, protein sample was eluted with NCMN buffer D. The peak fractions were concentrated for final buffer exchange with NCMN buffer E. The final protein samples concentrated to the desired concentration for single-particle examination using negative stain electron microscopy and proteoliposome reconstitution.

NCMN buffer A: 50 mM HEPES, pH 8.4, 500 mM NaCl, 5% Glycerol, 20 mM Imidazole, 0.1 mM TCEP.

NCMN buffer B: 25 mM HEPES, pH 7.8, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole, 0.1 mM TCEP.

NCMN buffer C: 25 mM HEPES, pH 7.8, 500 mM NaCl, 5% Glycerol, 75 mM Imidazole, 0.1 mM TCEP.

NCMN buffer D: 25 mM HEPES, pH 7.8, 500 mM NaCl, 5% Glycerol, 300 mM Imidazole, 0.1 mM TCEP.

NCMN buffer E: 40 mM HEPES, pH 7.8, 200 mM NaCl, 0.1 mM TCEP.

**Negative stain electron microscopy (EM)**

The following protocol for negative stain was used. An aliquot of 0.1 mg/mL MscS, MscL and T74S-KcsA (3 μL) sample was applied to a glow-discharged copper grid (CF300-CU-
TH; Electron Microscopy Sciences, Hatfield, PA, USA), left to be absorbed by the grid surface for 1 min and then gently dried with a filter paper. The grids were washed with three drops of ultrapure water and three drops of uranyl acetate of 2% (w/v), with the last drop held for 1 min. After each step, the grids were gently blotted with filter paper touching the grid's edge. Finally, the grids were left to air dry for 1 or 2 min. The negative stain images were collected at the University of Virginia Advanced Microscopy Facility at a nominal magnification of 62,000 x on a Tecnai F20 Twin transmission electron microscope (FEI, Hillsboro, OR, USA) operating at 120 kV. Digital micrographs were recorded on a Gatan US4000 chargedcoupled device camera (Gatan, Warrendale, PA, USA).

**Expression, extraction and purification of KcsA by n-decyl-β-D-maltopyranoside (DM)**

As described previously [22], a single colony of JM83 cells containing T74S KcsA construct in PASK 90 was inoculated in 10 mL LB media containing ampicillin (100 μg/mL) at 37 °C with shaking at 250 cycles per minute. When OD 600 reached to about 0.5, this pre-culture was transferred into 1 L LB and incubated in the same condition until its OD 600 was about 0.9. The induction of protein expression with anhydrotetracycline (Sigma-Aldrich, Co., St Louis, MO, USA) of final concentration 200 μg/mL was performed in the same cultural condition overnight and cells were then harvested by centrifugation (4993 g, 20 min) and the pellets were stored at ~80 °C. For T74S KcsA protein extraction the cell pellet was re-suspend into 30 ml of buffer containing 50 mM Tris and 150 mM KCl with pH adjusted to 7.5, which was supplemented with deoxyribonuclease I (DNAse, 0.5 μg/mL) (Sigma-Aldrich, Co., St Louis, MO, USA), protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and lysozyme (1 mg/mL, Sigma-Aldrich, Co., St Louis, MO, USA). The suspension was incubated at room temperature for 30 min before being passed through French press twice at 16 K PSI in a cold room. Then DM was added to a final concentration of 40 mM and the sample was rotated at 4 °C overnight. After ultracentrifugation at 70,000 g for 30 min to remove membrane components, the clear supernatant was added with imidazole of 10 mM final concentration and incubated with Ni-NTA beads (Thermo Scientific, Rockford, IL, USA) at room temperature for 1 h to allow the binding of the histidine-tagged T74S KcsA protein to the beads. The Ni-NTA beads were then transferred to a column and washed 3 times with the protein buffer with 10 mM imidazole and 4 mM DM to remove non-specifically bound proteins and T74S KcsA protein was then eluted with 200 mM imidazole in the protein buffer with 4 mM DM. Imidazole was removed by buffer exchange and meanwhile the protein was concentrated using a 50 kDa centrifugal filter tube (Merck Millipore Ltd., IRL). The protein was then aliquoted and flash-frozen in liquid nitrogen before stored at ~80 °C freezer.

**Chemical labeling of MscL in NCMM particles**

Sulphhydryl dimethylglycine was synthesized according to Koçer A, et al. [23]. Note that while some NCMM polymers are not charted, the polymers used in this work are; NCMNP1–1 is sensitive to pH and divalent ions, but NCMNP7–1 is not. Thus, care was taken, especially for the former, with pH and divalent cations during the process. Because the addition of pH sensor decreases the pH of the solution, the pH of the protein buffer (40 mM HEPES, 200 mM NaCl, pH to 8) containing pH sensor was readjusted to about 7.4 before labeling to avoid precipitation of NCMM polymers by acidic pH [24]. The pH sensor:
protein:lipid ratio is 20:1 in mg/mg. Labeling was performed at room temperature for 1 h followed by buffer exchange to remove free sensor and NCMN polymer molecules by using a 50 kDa centrifugation unit. Each time about 250 μL of protein sample was brought up to 10 mL in protein buffer, centrifuged at 3011 g for about 5 min to reach a volume of about 250 μL (Thermo Scientific Sorvall ST 16R centrifuge). This step was to prevent excessive free membrane active polymers from lysing the liposomes.

Detergent free protein reconstitution assisted by NCMN polymers: Lipids dissolved in chloroform were dried under argon while rotating in a glass tube to form a thin lipid film on glass wall, which was further dried under argon for 30 min. Liposomes were formed by either rehydration into appropriate buffers [25, 26] or in the presence of sucrose solution [27–29]. Proteins in the format of NCMN particles were then added for reconstitution as described for each specific method.

Dehydration/rehydration after protein reconstitution for patch-clamp studies

A lipid film of azolectin (20% soy PC, Avanti Polar Lipids Inc, Alabaster, AL, USA) was rehydrated into buffer A (10 mM Tris, 1 mM Ethylenediaminetetraacetic acid (EDTA), and 1 mM (ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′′-tetraacetic acid), pH 8.0) at 20 mg/mL and the suspension was incubated at 45 °C for at least 2 h. The formed liposomes were sonicated until semi-clear in a bath sonicator (FS6, Fisher Scientific), which normally took around 10 to 15 min, to reduce their size before the addition of proteins. Proteins in either NCMN particles or DM were added with a protein/lipid ratio from 1/250 to 1/500 (mg/mg) and the mixture was incubated at room temperature with gentle shaking for 1 h to enable protein reconstitution. To remove excessive NCMN polymers or DM, overnight dialysis was performed with 3 buffer exchanges, each with 1000 mL of buffer B (10 mM Tris, 100 mM NaCl, 0.2 mM EDTA, and 0.002% NaN₃, pH 8.0) and the molecular cutoff of the dialysis tube is 6–8 kDa (note that the NCMN polymers are around 7.5 kDa but are not of globular shape, they thus pass through these dialysis membranes). The proteoliposomes were then pelleted by a 20 min air-driven ultracentrifugation at 30 psi (AirfugeTM, Beckman Instruments Inc, Palo Alto, CA, USA), re-suspended into buffer C (10 mM MOPS, 5% ethylene glycerol, pH 8.0) and desiccated overnight under vacuum at 4 °C and used for patch-clamp experiments within one week. The desiccated liposomes were rehydrated in buffer D (5 mM Hepes, 10 mM KCl, and 2 mM MgCl₂ plus 320 mM sucrose, pH 8.0) at a lipid concentration of 90 mg/mL for at least 2 h before experiments in the same day. Then 1 μL of liposomal suspension was put into a recording chamber of a patch-clamp set up to form giant unilaminar proteoliposomes for patch-clamp study.

Protein reconstitution in sucrose solution for patch-clamp study: Sucrose reconstitution was performed similar to previously described [28]. Briefly, azolectin lipid film was formed as described above followed by prehydration with 5 μL H₂O at 45 °C for 25 min. Then 1 mL sucrose solution of 0.4 M was slowly poured into the glass tubes containing the lipid film, which was then incubated at 45 °C for 3 h to enable small lipidosome formation. For reconstitution of proteins from either NCMN polymers or DM, mucelle samples were added into liposome suspension with a protein/lipid ratio (1/250 to 1/500 mg/mg) followed by 1 h incubation at room temperature with gentle shaking and then
overnight incubation at 4 °C [28]. Liposomes tend to aggregate at the bottom of the tubes and 1 μL of cloudy liposomal suspension at the bottom of the glass tube was put into the recording chamber of a patch-clamp set up to form giant unilaminar proteoliposomes for patch-clamp study.

**Protein reconstitution for triggered release:** The lipid component of liposomes for triggered release of calcein is 70% 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 20% cholesterol and 10% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) in molar ratio. After dehydration, the lipid film is rehydrated into 50 mM calcein disodium (Sigma Aldrich, St. Louis, MO, USA) in the buffer (10 mM Tris-HCl with pH adjusted to 8.0) to form calcein containing liposomes. The protein NCMN particles were then added to calcein containing liposomes for reconstitution in the presence of free calcein in the liposome suspension with a protein/lipid ratio of about 1/1000 (mg/mg) and the final calcein-concentration is calculated to be 35 mM.

**Electrophysiological analysis:** All patch-clamp studies were performed at room temperature. For the recording of T74S KcsA, patchclamp of proteoliposomes were performed as those described in the literature [30–32] with modifications. Patch buffer contains 10 mM MOPS (3-(N-morpholino)propanesulfonic acid), 200 mM KCl and 40 mM MgCl₂ with pH adjusted to 7.4 for bath solution and 3.0 for pipette solution. Data were acquired at 80 mV (pipette voltage) unless indicated. For the recording of MscL and MscS, patch-clamp of liposomes was performed as described previously [25]. Patch buffer contains 5 mM HEPES, 200 mM KCl, 90 mM MgCl₂, and 10 mM CaCl₂ with pH adjusted to 8.0. Data were acquired at 20 mV (pipette voltage). A sampling rate of 33 kHz and a 5 KHz low pass filter was used for the recordings using an AxoPatch 200B amplifier and Digidata 1440A from Molecular Devices (Sunnyvale, CA, USA) and data were analyzed using pCLAMP10 software (Molecular Devices, Sunnyvale, CA, USA). At least 2 batches of proteoliposomal preparations were patched for each protein channel. In the reconstitution procedures described above, different concentrations of NCMN particles were added into the reconstitution system; at these levels no changes in membrane leakage or patch-clamp compatibility was observed. There were also no differences observed in patch-clamp properties or quality of the membrane relative to proteoliposomes and liposomes without NCMN.

**Calcein efflux assay:** The calcein efflux assay was performed as previously described [23, 33]. Calcein was added to the buffer to hydrate the lipid film to form liposomes as described above. Following addition of NCMN particles for reconstitution, free calcein and residual NCMN polymers were removed by passage through a G-50 fine Sephadex column (GE Healthcare Inc., Piscataway, NJ, USA) with vesicle buffer (10 mM Tris-HCl, 350 mM NaCl, 1 mM EDTA, pH 8.0). The eluted vesicle suspension was placed into clear 96-well plates and their fluorescence was recorded at 538 nm with the excitation at 485 nm using a fluorescent micro plate reader (Fluoroskan Ascent, Thermo Scientific Inc., Waltham, MA, USA). The baseline signal of samples was recorded for 5 min followed by 30 min recording after the pH of the suspension was changed to 6. Vesicles were finally lysed with 0.5% Triton X-100 to determine the total fluorescence levels of calcein encapsulated in vesicles.
Results

General strategy

Procedures used for the NCMN polymer-assisted channel protein extraction, purification and reconstitution into liposomes are somewhat analogous to those used with detergents, as illustrated in Fig. 1. The NCMN polymer is amphipathic and so can efficiently intercalate into the membrane and form NCMN particles. Channel proteins of interest that are contained in NCMN particles can then be enriched by affinity purification using affinity tags. The purified protein can be either used directly for liposomal reconstitution for functional analysis or chemically modified at designated locations in the channel for the purpose of triggered-release of a liposomal cargo by specific stimuli (Fig. 1 B).

Preparation of native cell membrane nanoparticles

Membrane channels from plasmid-directed overexpression in E. coli, KcsA, MscS and MscL, were extracted from cell membranes directly using active membrane polymers selected from the NCMN polymer library developed in the laboratory of Dr. Guo and processed to form native cell membrane nanoparticles using a modification of the previously published protocol [12]. The purity, homogeneity and the sizes of the nanoparticles were examined using negative stain electron microscope analysis; micrographs such as that shown in Fig. 2 illustrate that the particles are homogeneous, consistent with the expected size for the proteins, with little impurity.

Functional reconstitution for patch-clamp analyses

KcsA has been an ideal protein for method development, including for the preparation of proteins in styrene-maleic acid lipid particles (SMALP) and nanoscale apolipoprotein-bound bilayers (NABBs) particles [34, 35]. However, whether NCMN particles could be used for reconstitution and whether they would be compatible with normal functional activity in conventional patch-clamp analysis was not known. To increase the probability that its activity could be detected with greater sensitivity, we selected a T74S KcsA mutant protein that, like other mutants such as E71A, has been reported to have dramatically reduced inactivation [22]. There are currently two popular reconstitution methods used to generate giant unilaminar proteoliposomes amendable for patchclamp recording. The traditional dehydration/rehydration method [26] dries and subsequently rehydrates proteoliposomes, resulting in giant unilaminar liposome formation. A more recently developed method [28] forms giant unilaminar liposomes in the presence of a sucrose solution, potentially streamlining the preparation and better preserving function. Reconstitution of functional channels via the dehydration/rehydration method was successful with NCMNP7–1, but not the more commonly used NCMNP1–1 polymer, presumably because of the improved stability of NCMNP7–1 in the presence of divalent ions and a wide range of pH. As shown in Fig. 3, NCMNP7–1 extracted T74S KcsA proteins exhibited similar sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) migration patterns as those extracted in the detergent ndecyl-beta-D-maltopyranoside (DM), namely that both appear mainly as tetramers migrating at about 45 kDa, consistent with previous findings [36, 37]. Upon functional reconstitution using the sucrose method, T74S KcsA in NCMNP7–1 particles vs in DM showed indistinguishable conductances of $84.88 \pm 6.13 \text{ pS}$ ($n = 7$) and
83.38 ± 13.50 pS (n = 6), respectively. For both reconstitution conditions, rectification at negative pipette voltages was observed with conductances about half of those observed at positive voltages: 41.63 ± 3.61 pS for both NCMNP7–1 (n = 3) and DM (n = 3); these data are consistent with previous electrophysiological studies of the channel [30, 34]. The fraction of positive patches was 6.2% and 21% for NCMNP7–1 and DM extracted T74S KcsA proteins, respectively. When the activity was observed, we saw single-channel activity in the recordings of T74S KcsA protein from NCMNP7–1 particles, while by contrast 5 out 6 recordings of T74S KcsA protein from DM extraction show 2 or more simultaneous channel activities suggesting clustering. The observation that T74S KcsA channel activities are observed in standard patch-clamp recordings after reconstitution demonstrates a proof-of-concept that NCMN particles can be used in functional reconstitution studies for channels.

MscS and MscL mechanosensitive channels from E. coli [1] were also successfully reconstituted from NCMN particles using the dehydration/rehydration method. MscS and MscL proteins that were solubilized and purified by NCMNP1–1 were characterized by SDS-PAGE gel (Fig. 4 A and B). Their monomers run at 25 kDa and 15 kDa, respectively, as expected. Four MscS channel activities were observed by electrophysiology to the patch pipette (Fig. 4 A) at negative pressure (in a pressure-dependent fashion). Similarly, four MscL channels were seen to be activated in a pressure-dependent manner (shown in Fig. 4 B). The conductance, kinetics and sensitivity of the channels were indistinguishable from channels recorded in native membranes and TritonX-100 solubilized and reconstitution systems in our lab and others [1, 25, 38, 39]. The fraction of patches that showed channel activities were 65% and 47% for MscS and MscL, respectively. The channels were stable in the particle form at 4 °C; excellent activity was observed for samples reconstituted after 3 months storage for MscL, and 8 months for MscS, in contrast with channel proteins processed by detergent extraction, which are routinely found to be viable for functional studies only up to 7 days after purification if stored at 4 °C. Thus, NCMN particles present an attractive alternative for isolation, storage, and functional reconstitution of channel proteins.

**Functional reconstitution of modified channels into a nanodevice**

To determine whether NCMN particles could preserve a nuanced function during reconstitution consistent with broader applications, we attempted assembly of functional liposomal nanodevices [19] from NCMN particles of chemically manipulated MscL. To perform this, we first overexpress MscL with two cysteine mutations located at pore constriction sites: G22C/G26C. Without endogenous cysteine residues in MscL, these cysteines can be specifically labeled with a thiol reagent containing a dimethylglycine molecule, which has a pKa around 7.4 and can be protonated in acidic pH. After acquiring proton charges, the MscL channel can be gated by electrostatic repulsion, thus making a pH-sensitive triggered nanovalve, as previously described [19]. To preserve the viability of such a device, the nanovalves are reconstituted into stealth liposomal vesicles, which contain PEGylated lipids to avoid clearance when used in *in vivo* animal experiments [40–43]. The labeling and reconstitution of MscL allows for the assembly of a nanodevice theoretically capable of delivering cargo in specific low-pH environments, such as inflamed or malignant...
tissues [19]. Cysteine-mutated MscL channels were modified with the thiol reagent while in the NCMN particles. To test payload release by low pH, we encapsulated calcein dye inside liposomes. Calcein is self-quenching at the high concentrations inside the liposomes, but gives a measurable fluorescence signal after being released from liposomes through gated MscL pH-triggered nanovalves. Free calcein dye was present in the solution during the reconstitution of the proteins in the NCMN particles to keep the “load” within the liposomes. Reconstitution was performed just by mixing liposomes with the NCMN particles containing the modified MscL channels. The liposomes were then purified using size exclusion chromatography to remove residual free NCMN polymers and un-encapsulated payload (Fig. 5 A). This streamlined protocol is much more complicated for reconstitution of MscL in detergent, such as Triton X-100, which requires detergent titration for optimal reconstitution as well as complete removal of detergent after reconstitution in order to avoid leakage of payload during the size exclusion procedure. As noted with previous studies [19], the proteoliposomes are stable for at least several hours, suggesting that membrane integrity is maintained after protein insertion. As shown in Fig. 5 B, decreasing the pH of the solution containing the liposomal sample leads to an 80% increase in fluorescent signal of calcein, indicating the release of dye.

Discussion

Implications and perspectives

Traditionally, detergent optimization is a protein-dependent careful balance between stripping the lipids from the protein, yet not upsetting critical interactions required for channel function [44–46]. Once in detergent, many proteins are labile and the functional protein concentration decreases over the course of hours or a few days, presumably because of denaturation and/or aggregation. Finally, upon reconstitution, detergents can be difficult to remove completely, the use of Bio-Beads SM2 can decrease yields, and residual detergent can be problematic in functional studies, especially for mechanosensitive channels and liposomal-based cargo-loaded nanodevices. As an emerging alternative, amphipol derivatives have recently been shown to allow direct extraction of membrane proteins (https://doi.org/10.21203/rs.3.rs131488/v1), and it is for the future to determine if and how well the lipids may be preserved in this system, and if it is appropriate for reconstitution and functional studies.

The use of more established membrane-active-polymers has an obvious advantage: it unambiguously extracts integral membrane proteins from cells in a water-soluble form while conserving a patch of native membrane around them [24], and it has become evident that channels may have intimate, functional and stabilizing interactions with lipids [47, 48] that detergents may not easily reproduce. The properties and diversity of NCMN polymers have increased, allowing for tailored use for membrane proteins of different properties and sizes. Thus far, this approach has been primarily used in structural studies [11, 49] with a few notable exceptions in which function was measured. One study demonstrated that bacterial reaction centers purified using membrane-active-polymers do not display the modified functional properties that typically result from detergent solubilization, and direct comparisons show that reaction centers are more stable in this co-polymer/lipid environment.
than in a detergent micelle or even in the native membrane [50]. In this case, the protein was studied in its solubilized form and was not reconstituted into alternative lipid environments. A second study functionally reconstituted cytochrome c oxidase from *S. cerevisiae* mitochondria into liposomes [51]. Finally, to our knowledge, the only study of channel reconstitution thus far was KcsA, which was fused to planar lipid bilayers [35]. However, planar lipid bilayers are not appropriate for all channels. The conventional patch-clamp techniques offer more ways of intervention during channel recording including applying pressure/tension to activate mechanosensitive channels. The activities of the mechanosensitive channels used in this study, MscS and MscL, have not been observed in planar lipid bilayers, presumably because appropriate membrane tensions cannot be achieved – liposome reconstitution and patch-clamp have been necessary.

Here we present membrane-active-polymer assisted channel purification and reconstitution into liposomes without the presence of detergent using three independent channels: T74S KcsA, MscS and MscL. NCMNP1–1 worked well for MscS and MscL functional reconstitution into proteoliposomes. However, NCMNP1–1 did not allow for the reconstitution of functional T74S KcsA into proteoliposomes despite many trials. We needed to use a different membrane active polymer, NCMNP7–1 and the sucrose reconstitution technique to see activity. NCMNP1–1 is the active membrane form of SMA2000 (Total Cray Valley) hydrolyzed with sodium hydroxide and is the most popular used styrene-maleic acid co-polymer. The failure of NCMNP1–1 in the reconstitution of T74S KcsA may be a reflection of the limitations of popular styrene-maleic acid co-polymers. Indeed, we have often found that styrene-maleic acid copolymers work for some membrane proteins but not others for single-particle cryo-EM analysis. In many cases, the particles prepared with styrene maleic acid copolymers are highly heterogeneous; thus, styrene maleic acid copolymers appear to have limited applications. Because of the complexity of the native cell lipid environment, it seems improbable to have a universal polymer that works equally well for all membrane proteins. Hence, there is a distinct advantage of generating a library of polymers to accommodate the unique properties of the array of membrane proteins.

**Conclusions**

Using three structurally distinct membrane protein channels, we have demonstrated that the NCMN system yields itself to functional reconstitution in proteoliposomes; the approach is straightforward and has many advantages. It allows for the generation of quality proteoliposomes amenable for either patch-clamp analyses or liposomal efflux assays. It works for azolectin-based blisters subsequent to a standard dehydration/rehydration protocol, as well as the sucrose method for reconstitution (described in [25] and [28]). It also works very well for modifying and reconstituting channels into stealth liposomes loaded with cargo. Although we only tested membrane protein channels, the technique could also be applicable to membrane-associated transporters, receptors and enzymes. Normally, when reconstituting channels for efflux studies, the detergent needs to be titrated to achieve optimal reconstitution [19]. Moreover, there is risk of the detergents denaturing a proportion of the proteins, and additional steps must be included to remove detergent after the reconstitution for example using Bio-Beads SM2, to ensure that payload does not leak out during the subsequent size exclusion fractionation. These extra steps lead to decreased yield.
of cargo-containing liposomes. These issues are conveniently avoided when using NCMN polymer isolated material. Finally, the NCMN polymer solubilized and purified channels are quite stable at 4 °C, viable in their native-lipid environment for months, as contrasted with typical stability of a few hours or days when stored in detergent. This would improve efficiency and yield, and allow for industrial-scale storage of modified channels and subsequent assembly to form tailored or commercial nanodevices.

Without the over delipidation and potential denaturation issues of detergents, in principle any membrane protein/complex can be stabilized in the form of NCMN particles and reconstituted into functional proteoliposomes. This unique combination of the NCMN system and reconstitution into proteoliposomes provides a powerful nanobiotechnology approach for making high-quality functional proteoliposomes that could significantly impact basic membrane protein research as well as the development of nanodevices including those for small-molecule drug delivery and membrane-protein-related vaccine development.

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Abbreviations:

- **NCMN**: native cell membrane nanoparticles
- **SMALP**: styrene maleic acid lipid particles

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Fig. 1.
Schematic illustration of NCMN polymer assisted protein purification and liposomal reconstitution. A: Average repeat-unit of NCMN polymer, where $R$ represents a hydrophobic group, $R'$ and $R''$ represent hydrophilic groups (e.g. carboxyl, phosphate, or sulfite groups) and m/n ratios are unique for each of NCMN polymers. B: NCMN polymer assisted protein purification and reconstitution. Amphipathic NCMN polymers (red lines) insert into E. Coli cytoplasmic membrane (green rectangles) to remove patches of membranes (NCMN particles) with embedded membrane proteins (dark and light blue ovals). Wrapped by
NCMN polymers, the patches of membrane with protein are stable in solution. NCMN particles containing exogenous expressed channel proteins with His-tag (purple stars) are then extracted by affinity purification. Purified membrane proteins are directly incubated with preformed liposomes for patch-clamp analysis of channel function (left). A similar reconstitution can be used for modified channels allowing triggered-cargo-release from “stealth” vesicles that contain PEGylated lipids that allow for evasion of normal clearing mechanisms in the body (right).
Fig. 2.
Representative negative stain electron microscopy micrographs. All micrographs were taken at a magnification of 62,000 × with an FEI Tecnai F20 EM microscope at UVA. A. KcsA NCMN particles show an average size of 5–7 nm. B. MscS NCMN particles show an average size of 8–13 nm. C. MscL NCMN particles show an average size of 5–8 nm, and some evidence of aggregation. All scale bars represent 50 nm.
Fig. 3.
Electrophysiological analysis of T74S KcsA channel function. T74S KcsA channel activity after DM mediated reconstitution (top) or after NCMNP7–1 mediated reconstitution (bottom). The inserts (left of the traces) are SDS-PAGE of the proteins solubilized and purified with DM or NCMNP7–1. “C” and “O” represent the closed, and opening state of the channels. Numbers reflect opening of multiple channels; Channels are activated by the low pH (pH = 3) of the pipette solution and are recorded under an 80 mV pipette potential. Unexpected multiple channel activities of DM extracted T74S KcsA are seen in 5 out of 6 recordings. Meanwhile, all patch recordings of NCMNP7–1 extracted T74S KcsA show only a homogeneous single channel activity.
Fig. 4.
Electrophysiological analysis of channel function of MscS and MscL. A: MscS channel activity; B: MscL channel activity. The inserts are SDS-PAGE of the proteins solubilized and purified with NCMN polymers. “C” and “O” represent the closed and opening states of the channels; numbers reflect openings of multiple channels. Upper trace of each panel shows current traces at holding potential of −20 mV while the lower trace shows pressure applied. Note that both MscS and MscL open after pressure application.
pH sensitive calcein efflux through chemically modified MscL channels. A: Schematic illustration of calcein efflux assay. After MscL protein (blue open ovals) reconstitution into liposomes (blue circles), NCMN polymers (red lines) are removed via elution through size exclusion column. Chemical modification introduced pH sensitive molecules (yellow dots) to the pore constriction sites of MscL channel, which thereby gain charges (red dots) at acidic pH and gate the channel through electric repulsion. Therefore, acidic pH applied leads to efflux of calcein (black triangles) through open MscL channels, which result in appearance of green fluorescence (which before efflux was quenched at high concentration giving an orange color). B: Calcein efflux assay (representative of three independent experiments). The fluorescent signal of proteoliposomal suspension was monitored for 5 min before the pH of the solution was changed from 8 to 6. The efflux of calcein in pH 6 was then monitored for 30 min before Triton X-100 was added to lyse the liposomes for 5 min to obtain the control or 100% value. The fluorescent signal at each time point was normalized to the total calcein signal, expressed as a percentage of total, representing the percentage of calcein release.