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Production and preparation of isotopically labelled human membrane proteins in *Pichia pastoris* for Fast-MAS-NMR analyses

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Running Head

Preparation of MPs from *Pichia pastoris* for Fast-MAS-NMR

Abstract

Membrane proteins (MPs) comprise about one third of the human proteome, playing critical roles in many physiological processes and associated disorders. Consistently, they represent one of the largest classes of targets for the pharmaceutical industry. Their study at the molecular level is however particularly challenging, resulting in a severe lack of structural and dynamic information that is hindering their detailed functional characterization and the identification of novel potent drug candidates.

Magic Angle Spinning (MAS) NMR is a reliable and efficient method for the determination of protein structures and dynamics and for the identification of ligand binding sites and equilibria. MAS-NMR is particularly well suited for MPs since they can be directly analysed in a native-like lipid bilayer environment but used to require aggravating large amounts of isotope enriched material. The frequent toxicity of human MP overexpression in bacterial cultures poses an additional hurdle, resulting in the need for alternative (and often more costly) expression systems. The recent development of very fast (up to 150 kHz) MAS probes has revolutionized the field of biomolecular solid-state NMR enabling higher spectral resolution with significant reduction of the required sample, rendering eukaryotic expression systems cost-effective.

Here is presented a set of accessible procedures validated for the production and preparation of eukaryotic MPs for Fast-MAS ^1H -detected NMR analysis. The methodology is illustrated with the human copper uptake protein hCTR1 recombinantly produced and ^{13}C - ^{15}N uniformly labelled with the versatile and affordable *Pichia pastoris* system. Subsequent purification procedures allow the recovery of mg amounts that are then reconstituted into liposome formulations compatible with solid-state NMR handling and analysis.

Keywords

Pichia pastoris, yeast, membrane protein, isotopic labelling, multilamellar vesicles, MAS-NMR

1 Introduction

In the last two decades, major progress in sample preparation and in biophysical methods specifically developed for Membrane Proteins (MPs) has tremendously

boosted the structural comprehension of vital functions at biological membranes (*1-3*). While a large majority of structural data has been obtained through X-ray diffraction and cryo-EM observation of static objects, complementary information on dynamic has been gathered with dedicated analytical methods such as XFEL (*4*), EPR (*5*), HS-AFM (*6*) and NMR spectroscopy (*7-9*).

Among these techniques, solid-state NMR is gaining an increasing interest with the recent advent of fast Magic Angle Spinning (MAS) probes. This critical technical development has considerably broadened the scope of proton-detected NMR techniques with direct consequences on the structural and dynamic characterization of proteins (*10-12*), including MPs (*13-16*). The increase in MAS speed (probes spinning to 60 kHz and above) correlates not only with a dramatic improvement of ^1H spectral resolution, but also with a significant reduction of sample amount required (0.5 mg or less), which is a major concern for MPs, for which expression yields are usually low. Most importantly, MAS-NMR interrogates conformations of MPs floating in native or native-like lipid bilayers. This technique is thus particularly well adapted to reveal molecular events occurring at the protein-lipid interface (*16-18*), but also to investigate the influence of lipids on MP structures and functions (*19, 20*), which is a key issue in molecular membrane biology (*21, 22*).

In such MAS-NMR studies of MPs, a variety of labelling schemes, experimental sequences and resonance assignment methods have been developed and are reported in very comprehensive reviews (*9, 14, 23*). All of them rely on highly purified proteins selectively or uniformly labelled with ^{13}C and / or ^{15}N isotopes for investigating the structure and conformational dynamics of the whole protein or of particular domains or residues. As a requisite, these methods thus require robust recombinant expression systems compatible with labelling-associated issues and performant enough to produce mg amounts of labelled MPs in a functional form. With various degrees of operability, performance, specificities and limitations,

several cellular and cell-free systems have been developed to this end in the very recent years (see **23-26** and references therein).

One of these robust expression systems emerging for the production of MPs for MAS-NMR is the methylotrophic yeast *Komagataella phaffi*, best known under the name *Pichia pastoris* in applied sciences (**27**). This eukaryotic microorganism already proved highly efficient in producing many eukaryotic MPs representative of various functions (receptors, channels, transporters, enzymes), topologies and assemblies (**28, 29**). A large number of them could be further isolated for molecular investigations, in quantity and quality levels compatible with a wide panel of biochemical and biophysical techniques (see references in **30**).

The power of *P. pastoris* largely relies on its particular methanol utilization metabolism that endows it with promoters tightly regulated upon culturing conditions: fully repressed in presence of preferential carbohydrates (*i.e.*, sugars, glycerol), they are strongly induced when methanol is the sole carbon source (**31**).

With regard to protein production for NMR studies, expression of recombinant genes placed under the control of such promoters is thus particularly well adapted to various labelling strategies, bringing chosen isotopically labelled substrates to the culture during the induction phase (**32**). Accordingly, several studies have reported on the successful implementation of this system to deliver labelled MPs for a number of NMR analyses (**33-35**), including MAS-NMR approaches (**24, 36, 37**). In these studies, uniform ¹⁵N-¹³C-labelling schemes mainly relied on ¹⁵N-ammonium salts and ¹³C-methanol supplementation during induction, combined to the fine tuning of a number of parameters before or during induction (addition of non-inducing ¹³C-carbohydrates -glucose or sorbitol-; cell density, temperature and time scale settings; initial ¹³C-methanol and ¹⁵N-ammonium salts concentrations, supplementation during the process...).

We here present a detailed procedure for the preparation of uniformly ^{15}N - and ^{15}N - ^{13}C -labelled recombinant MPs from *P. pastoris* for their subsequent Fast-MAS-NMR handling and analysis. It includes the description of culturing conditions in isotope-enriched minimal media designed to optimize MP production yields, labelling efficiency and cost concerns. This is achieved by favouring short growth and induction times, thus limiting cell death and degradation events, and by reducing the ^{13}C -methanol substrate to its lowest efficient concentration in order both to limit its toxicity effect and to maximize the cost issue. The procedure also describes each of the following steps leading from MP extraction and purification in detergents, to their reconstitution in multi lamellar vesicles (MLVs) and packing in Fast-MAS rotors for their NMR analysis. The whole process has been validated with two human MPs, a G Protein-Coupled Receptor and the high-affinity copper transporter hCTR1. This homo-trimeric MP is involved in the fine regulation of Cu homeostasis and in the uptake of platinum-based compounds employed in cancer chemotherapy (*38-40*), and is used throughout the chapter to illustrate the whole procedure.

2 Materials

2.1 MP production in labelling conditions

1. A recombinant clone expressing the MP of interest freshly streaked on a YPD agar (1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) dextrose, 2 % (w/v) agar) plate with antibiotic (typically 50 $\mu\text{g}/\text{ml}$ geneticin).
2. Sterile milli-Q water.
3. 1 M KPi phosphate sterile buffer, pH 6: 3.1 % (w/v) dipotassium hydrogen phosphate trihydrate ($\text{K}_2\text{HPO}_4 \cdot 3 \text{H}_2\text{O}$), 11.81 % (w/v) potassium dihydrogen phosphate (KH_2PO_4). Filter sterilize.

4. BMG liquid minimal medium: 700 ml sterile milli-Q water, 100 ml 1 M KPi phosphate buffer pH 6 (10x solution, filter sterilized), 100 ml 13.4 % (w/v) yeast nitrogen base (YNB) without amino acid (10x solution, filter sterilized), 100 ml 10% (v/v) glycerol (10x solution, filter sterilized) (*see Note 1*).
5. ¹⁵N-labelled BMM liquid minimal medium: 800 ml sterile milli-Q water, 100 ml 1 M KPi phosphate buffer pH 6 (10x solution), 2 g YNB without amino acid and ammonium sulfate, 1 g ¹⁵N-ammonium chloride, 100 ml 5 % (v/v) methanol (10x solution). Homogenize and filter sterilize.
6. ¹⁵N-¹³C-labelled BMM liquid minimal medium: 900 ml sterile mQ water, 100 ml 1 M KPi phosphate buffer pH 6 (10x solution), 2 g YNB without amino acid and ammonium sulfate, 1 g ¹⁵N-ammonium chloride, 2 ml 100 % ¹³C-methanol (*see Note 2*). Homogenize and filter sterilize.
7. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7 to 7.4.
8. 2.5 L baffled flasks (e.g., Ultra Yield™ flasks, Thomson).
9. AirOTop™ seals (Thomson) with 0.2 µm re-sealable and sterile membrane barrier.
10. 1 L sterile centrifuge bottles.
11. 50 ml conical disposal tubes.
12. 30 °C and 22 °C shaking incubators.
13. Spectrophotometer (e.g., Biophotometer, Eppendorf).
14. Centrifuge equipped with a rotor suitable for 1 L bottles.

2.2 Yeast cell lysis and Membrane preparation

1. Yeast cell pellets from 4 L culture (four pellets, around 8 g each).

2. TNG buffer: 50 mM Tris-HCl pH 7.4, 0.5 M NaCl, 10 % (v/v) glycerol, 1 mM PMSF (added extemporaneously).
3. TNGE buffer: 50 mM Tris-HCl pH 7.4, 0.5 M NaCl, 10 % (v/v) glycerol, 1 mM EDTA, 1 mM PMSF (added extemporaneously).
4. Acid-washed glass beads (425 – 600 μm diameter, Sigma-Aldrich).
5. High-speed benchtop homogenizer suitable for volumes up to 50 ml (e.g., FastPrep-24, MP biomedical).
6. Centrifuge equipped with a rotor suitable for 50 ml conical tubes.
7. Ultracentrifuge with an appropriate fixed-angle rotor and adapted polycarbonate bottles.
8. 50 ml Potter homogenizer.
9. 14 and 50 ml conical disposable tubes.
10. Protein assay kit for the determination of protein concentration (e.g., Pierce BCA Protein Assay Kit, Thermo Scientific).

2.3 Extraction of MPs from membrane preparation

1. Solubilization buffer: 50 mM Hepes pH 7.4, 300 mM NaCl, 1 % n-Decyl- β -D-Maltopyranoside (DM), inhibitor protease cocktail (added extemporaneously, i.e. cOmpleteTM EDTA-free, Roche).
2. Membrane preparation.
3. Ultracentrifuge with an appropriate fixed-angle rotor and adapted polycarbonate bottles.

2.4 Purification of solubilized MPs

2.4.1 Immobilized Affinity Chromatography (IMAC)

1. Automated protein purification system (ÄKTA Pure or Purifier, Cytiva, or equivalent) equipped with a sample pump.
2. 1 ml prepacked cobalt affinity chromatography column (e.g., Talon Crude 1 ml Cytiva).
3. 0.22 µm filter and filter holder (e.g., reusable bottle top filter from Nalgene).
4. Buffer A: 50 mM Hepes pH 7.4, 300 mM NaCl, 0.1 % DM.
5. Buffer B: 50 mM Hepes pH 7.4, 300 mM NaCl, 300 mM Imidazole, 0.1 % DM.

2.4.2 *Size Exclusion Chromatography (SEC)*

1. Automated protein purification system (e.g., ÄKTA Pure or Purifier, Cytiva, or equivalent).
2. 0.22 µm filter and filter holder (e.g., reusable bottle top filter from Nalgene).
3. SEC running buffer: 50 mM Hepes pH 7.4, 150 mM NaCl, 0.1% DM.
4. SEC column (e.g., Superdex 200 Increase 10/300, Cytiva).

2.5 **SDS-PAGE and Western Blot immunodetection**

2.5.1 *SDS-PAGE*

1. 40 % Acrylamide:Bis-acrylamide 29:1 solution.
2. 3 M Tris-HCl pH 8.45, 0.3 % (v/v) SDS.
3. 80 % (v/v) glycerol.
4. 10 % (w/v) ammonium persulfate (APS).
5. Tetramethylethylenediamine (TEMED).
6. Gel casting stand and electrophoresis chamber (e.g., SureCast system, Invitrogen).
7. MP samples.
8. Protein ladder (e.g., PageRuler prestained protein ladder, Thermo).

9. Tris-Tricine-SDS cathode running buffer: 1 M Tris-HCl pH 8.2, 1 M Tricine, 1 % (v/v) SDS.
10. Tris anode running buffer: 1 M Tris-HCl pH 8.9.
11. 2x Tricine Sample Buffer (SB 2x): 100 mM Tris-HCl pH 6.8, 25 % (v/v) glycerol, 8 % (v/v) SDS, 0.02 % (w/v) Coomassie blue G250, 200 mM DTT.
12. Coomassie blue staining solution (e.g., Quick Coomassie Stain, Neo Biotech).

2.5.2 *Western blotting and immunodetection*

1. Tris-Glycine transfer buffer: 25 mM Tris, 200 mM glycine, 0.02 % (v/v) SDS, 20 % (v/v) ethanol.
2. 0.45 μ m nitrocellulose blotting membrane (e.g., Amersham Protran 0.45 nitrocellulose membrane, Cytiva).
3. Whatman paper.
4. Electroblotting system (e.g., Mini Trans-Blot Cell, Bio-Rad).
5. PBS and PBS containing 0.02 % (v/v) Tween 80 (PBST).
6. Blocking buffer: PBST with 5 % (w/v) non-fat dry milk.
7. Primary anti-protein or anti-tag antibody (here a monoclonal anti-HA antibody from mouse, Sigma).
8. Secondary anti-mouse IgG antibody linked to a reporter system (here an IRDye 800-coupled antibody, Li-Cor).
9. Reagent and detection device adapted to the reporter system selected (here an Odyssey[®] imaging system, Li-Cor).
10. Orbital shaker.

2.6 **Reconstitution in MLVs**

1. 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC) (e.g., 16:0-18:1 PC in chloroform, Avanti).
2. Cholesterol (Avanti).
3. Rotary evaporator (e.g., Rotavapor R-100, Buchi) or Argon stream.
4. Vacuum chamber and pump.
5. Liposome buffer: 50 mM Hepes pH 7.4, 150 mM NaCl.
6. Purified MPs in detergent micelles.
7. Detergent: 10 % (w/v) DM.
8. Dialysis cassettes with appropriate MWCO (e.g., Slide-A-Lyzer™, Thermo Scientific).
9. Ultracentrifuge with an appropriate fixed-angle rotor and adapted polycarbonate bottles.

2.7 Sample packing in Fast-MAS rotors

1. Filling tool for ultracentrifuge (e.g., spiNpack, Giotto Biotech).
2. Ultracentrifuge with swing-out rotor (e.g., SW32, SW28 rotors for Beckman centrifuges).
3. Fast MAS NMR rotor (e.g., Bruker 1.3 or 0.7 mm, JEOL 1 mm or 0.75 mm) with appropriate caps and inserts.

3 Methods

3.1 Production of MPs in isotope-labelling conditions with *Pichia pastoris*

The present procedure starts with an already existing yeast clone expressing the MP of interest. If such a clone is not available, the reader is referred to published extensive protocols ([41](#), [42](#)) detailing the preliminary work required to generate and isolate a *P. pastoris* recombinant clone.

The production of a recombinant MP is based on the overexpression of its corresponding gene placed under the control of the P_{AOX1} promoter (**41**) which is strongly regulated upon a methanol-induced regimen. In a first step, MP expression is fully repressed as yeasts are cultured in a glycerol-containing medium. When an appropriate cell density in a chosen culturing volume is reached in the exponential growth phase, the medium is then replaced by a methanol-containing one that will progressively derepress and then strongly induce the MP gene overexpression. As no expression leakage is detected before the medium exchange, ^{15}N - and/or ^{13}C -labelled substrates are added only during the induction phase to ensure a uniform labelling and MP production at once, and at reduced cost.

Here we describe an optimized protocol for the production of hCTR1 either in ^{15}N -labelling alone or combined with ^{13}C -labelling conditions, using 2.5 L baffled shake-flasks (see **Note 3**).

1. Generously inoculate 500 ml of extemporaneously prepared BMG medium in a 2.5 L baffled flask (see **Note 4**) with a clone freshly streaked on an antibiotic supplemented YPD agar plate (e.g., 50 $\mu\text{g}/\text{ml}$ geneticin). Incubate overnight on a shaker at 220 rpm, 30°C.
2. On the next day, measure OD_{600} of the culture. Dilute the cells in 2 L of fresh BMG to an OD_{600} around 5 (about 2.5×10^8 cells/ml, see **Note 5**). Incubate on a shaker at 220 rpm, 30°C.
3. When the culture reaches an OD_{600} between 10 and 15 (generation time in BMG ≈ 3.5 h, see **Note 1**), pellet the cells by centrifugation in sterile 1 L bottles for 5 min at 5,000 $\times g$, 22 °C.
4. Discard the supernatant and resuspend the cell pellets with 4 L of freshly prepared BMM medium complemented with ^{15}N ammonium chloride only or

combined with ^{13}C methanol. Split into 8 baffled 2.5 L flasks and incubate for an optimal period of time, here 22 hours, in a shaker at 22°C, 220 rpm.

5. After induction, harvest the cells by centrifugation in 1 L bottles for 5 min at 5,000 x g, 4 °C.
6. Discard the supernatants and wash each cell pellet (corresponding to 1 L of culture) with 200 ml PBS, pH 7.4. Pool the equivalent of 2 pellets into 1 bottle and centrifuge the suspension for 5 min at 5,000 x g, 4 °C.
7. Discard the supernatant and wash each cell pellet (corresponding to 2 L of culture) with 100 ml PBS, pH 7.4. Split into 4 x 50 ml conical tubes and centrifuge the suspension for 5 min at 5,000 x g, 4 °C.
8. Discard the supernatants and weigh the cell pellets.
9. The yeast pellets can either be directly utilized kept on ice for membrane preparation or snap-frozen in liquid nitrogen and stored at -80 °C.

In this format, each of the four 50 ml conical tube contains about 8 grams of wet cell pellets corresponding to 1 L of culture (i.e., about 32 g of cells for 4 L of culture in labelling conditions).

3.2 Yeast cell lysis and whole membrane preparation

Yeast cells are surrounded by a thick cell wall that protects them from most of the non-mechanical cell disrupting approaches such as enzyme-, heat- or detergent-based techniques. Methods relying on mechanical shear forces (bead- or pressure-based) are thus required to efficiently achieve *P. pastoris* cell lysis. One of these robust and accessible procedures uses glass beads and vigorous mechanical shaking for an efficient lysis, ideally monitored with a programmable device (e.g., the FastPrep-24 homogenizer from MP Biomedicals we use here) for a better reproducibility. To prevent degradation of recombinant proteins by protease during the cell lysis,

addition of protease inhibitors is strongly recommended. After the cell lysis, unbroken cells and large debris are removed by centrifugation and the supernatant is further submitted to ultracentrifugation. A final homogenization of the pellet with a Potter device allows the recovery of the whole membrane fraction. The resulting membrane preparation is further analyzed in order to evaluate its total protein concentration (typically with a BCA or a Bradford assay) and the expression level of the recombinant MP through a western blot immunodetection and via an appropriate activity assay when available.

The following protocol illustrates how we proceed for the whole membrane preparation of cells producing hCTR1 in isotope-labelling conditions (step 3.1.).

1. Thaw the 4 yeast pellets in conical tubes previously obtained (about 8 g each) and resuspend each of them in 25 ml ice-cold TNGE buffer.
2. Add 10 ml acid-washed glass beads.
3. Place the tubes in the cell breaker device (here the FastPrep-24) and proceed to cell lysis by alternating shaking and cooling steps (3 cycles of 40 sec shaking at 6.5 m/sec and 40 sec on ice).
4. Centrifuge for 5 min at 5,000 x g, 4°C, collect the supernatant and store at 4 °C.
5. Resuspend the pellet in up to 25 ml of ice-cold TNGE buffer.
6. Repeat steps 3. to 5. for two additional rounds (or until the supernatant is clear).
7. Centrifuge all the collected supernatants for 5 min at 5,000 x g, 4 °C.
8. Proceed to ultracentrifugation of the supernatants for 30 min at 100,000 x g, 4 °C in appropriate tubes.
9. Discard the supernatant and resuspend the membrane pellet with a Potter homogenizer in 70 ml of ice-cold TNG buffer until the suspension is homogenous. Split the membrane preparation in about 8x 10 ml in 14 ml

conical tubes. These membrane preparations can be used directly for further use and analyses, or can be stored for several weeks at -80 °C.

10. Determine the protein concentration of membrane preparation using a BCA assay kit following the manufacturer's recommendations.

In the experimental conditions presented above, we usually recover about 100 mg of membrane proteins per 10 ml of suspension, i.e., about 200 mg per initial cell pellet corresponding to 1L of culture.

3.3 Extraction of MPs from membrane preparation

A first critical step towards the isolation of MPs in aqueous solutions is to identify the best adapted experimental condition driving their efficient extraction from the lipid bilayer while preserving their structural and functional integrity. This task is principally based on the careful selection of an appropriate amphiphilic molecule, mainly a detergent, used to destabilize the lipid-lipid interactions of biological membranes. It also relies on a number of other parameters, such as detergent and protein concentrations, ionic strength, addition of co-factors and stabilizing compounds and finally temperature and incubation time. The optimal condition is most generally protein-dependent and should ideally be screened and adjusted for each MP of interest (*30, 43-45*). The extraction procedure presented here with DM as a detergent has been optimized for hCTR1.

1. Add 10 ml of membrane preparation (about 100 mg of total membrane proteins) in the solubilization buffer in order to reach a final protein concentration of 2 mg/ml.
2. Incubate for 30 min at RT under gentle agitation.

3. Proceed to ultracentrifugation for 30 min at 100,000 x g at 4 °C in appropriate tubes to separate the non-solubilized (pellet) from the solubilized fractions (supernatant).
4. Filter the solubilized MPs using a 0.22 µm membrane filter. Imidazole can be added at this stage if needed. Keep on ice.
5. The MP solubilization efficiency can be evaluated by immunodetection and activity assay if available.

3.4 Purification of solubilized MPs

Once MPs are solubilized, a panel of strategies and techniques may be considered to purify them to homogeneity, mainly depending on the biochemical properties of the protein itself, on the affinity tags it may present, and on the composition of the buffer it has been solubilized in (*30, 46*). Here we describe a two-step purification protocol specifically designed for hCTR1.

A first immobilized metal affinity chromatography (IMAC) exploits the natural ability of hCTR1 to bind divalent ions, here cobalt grafted on the resin. Besides the invaluable advantage of immobilizing the correctly folded, Co²⁺-binding, homotrimer populations of hCTR1 (*47, 48*), the strategy presents the additional benefit of working with a wild-type protein with no artificial fused tag and with no need to eventually cleave this tag afterwards. It is however very specific to hCTR1 and for other MPs, the IMAC step should rely on tag-based techniques by exploiting a N- or C-terminally fused 10His tag and using nickel or cobalt resins. The IMAC protocol described here is designed for an automated purification on an FPLC instrument (e.g., Äkta protein purification systems) using a pre-packed column, and with a specific flow rate, sequential washing and elution strategy optimized for hCTR1. In

this format, the use of a sample pump is warmly recommended as the volume of the solubilized sample loaded on the column may be significant (one or several hundred ml). Alternative immobilization (such as batch incubation), washing and elution (linear gradient or other fixed concentrations of imidazole) strategies may be applied and optimized depending on the MP of interest.

The second purification step of the procedure consists in a classical Size Exclusion Chromatography (SEC) using a Superdex 200 Increase column (Cytiva). This step is also designed for an automated purification using a FPLC instrument, which is a crucial point as the flow rate and pressure need to be tightly controlled.

3.4.1 IMAC

1. Equilibrate a 1 ml prepacked cobalt column with at least 10 column volumes (CVs) buffer A at 1 ml/min.
2. Inject the solubilized MPs onto the column with a sample pump at 0.5 ml/min.
3. Proceed to a washing step with 10 CVs of buffer A at 1 ml/min (same flow rate in the following steps).
4. Proceed to a second washing step with 5 CVs of 1.6 % of buffer B (8 mM imidazole) and collect 500 µl fractions.
5. Proceed to the elution step with 10 CVs of 100 % buffer B (300 mM imidazole) and collect 500 µl fractions.
6. Spare 20 µl of every fractions of interest and proceed to SDS-PAGE analysis with Coomassie blue staining and immunoblotting).

3.4.2 SEC

1. Equilibrate the SEC column (e.g., Superdex 200 Increase 10/300, Cytiva) with 2 CVs of SEC running buffer at 0.5 ml/min.
2. Depending on the results of the SDS-PAGE analysis from the IMAC purification, inject the appropriate fraction onto the SEC column at 0.3 ml/min and collect 500 μ l fractions (*see Note 6*).
3. Store 20 μ l of each fractions of interest for SDS-PAGE analysis.
4. The purified MPs obtained can be used directly for reconstitution in MLVs or can be snap frozen and stored at -80 °C.

In the format presented here, 100 mg of initial membrane preparation yields about 0.4 mg of DM-purified hCTR1 on average, i.e., about 0.8 mg per liter of culture. **Figure 1** illustrates a typical SEC chromatography and the corresponding SDS-PAGE (see section 3.5 below) profiles obtained in these conditions.

3.5 SDS-PAGE and Western Blot immunodetection

The following protocol has been originally published in (49) for the immunodetection of tagged MPs in membrane preparation samples. It is here updated with minor adjustments and focused on the analysis of purified MPs samples, either in detergent micelles or in proteoliposomes. The proportions are given for casting two gels of 1 mm thickness in a SureCast gel system from Invitrogen.

3.5.1 10 % SDS Tricine Polyacrylamide Gel Preparation

1. Prepare the separating gel by mixing 3.75 ml of acrylamide solution (40 % 29:1), 3.95 ml of Tris-HCl SDS buffer, 1.9 ml of 80 % (v/v) glycerol, 4.35 ml

H₂O, 135 µl of APS and 9 µl of TEMED. Mix and immediately cast the gel, allowing space for the stacking gel.

2. Prepare the stacking gel by mixing 0.6 ml of acrylamide solution (40 % 29:1), 1.6 ml of Tris-HCl SDS buffer, 4.1 ml H₂O, 90 µl of APS and 6 µl of TEMED. Mix and cast carefully with a pipet over the separating gel (no need to wait for the polymerization of the separating gel which density is increased by the presence of glycerol, thereby permitting the stacking gel to layer on top of it without mixing). Insert a gel comb immediately without introducing air bubbles.
3. Let the gel polymerize for about 30 min.

3.5.2 *Gel electrophoresis and Coomassie Staining*

1. Preincubate 20 µl of the fractions of interest with 20 µl of SB 2x for about 10 min at room temperature (*see Note 9*).
2. Load in parallel two 10 % SDS-polyacrylamide gels respectively with 20 µl and 5 µl of each sample. Add also one well with 4 µl of protein ladder in each gel. The first gel will be used for total protein staining with Coomassie blue, the second one will be transferred onto a nitrocellulose membrane for the immunodetection of the MP of interest.
3. Proceed to electrophoresis using Tris-Tricine-SDS cathode running buffer and Tris-anode running buffer in a tank unit. Run for about 1 h 30 at 100 V.
4. Stain the first gel with Coomassie blue following manufacturer's instructions.

3.5.3 *Protein Transfer and Western Blot immunodetection*

1. Transfer the proteins from the second gel onto a nitrocellulose membrane by electroblotting in Tris-glycine transfer buffer for about 1 h 30 at 100 V. An ice

pack can be added into the electroblotting device to mitigate the heat produced.

2. Incubate the membrane in 30 ml blocking buffer for 1 h at room temperature on an orbital shaker. Alternatively, incubate the membrane overnight at 4 °C.
3. Remove the blocking solution and incubate the membrane with the selected antibody diluted in blocking buffer (for hCTR1 we use a monoclonal anti-HA antibody at 0.4 µg/ml final concentration) for 1 h at room temperature on an orbital shaker, or alternatively overnight at 4 °C.
4. Wash the membrane three times with 50 ml of PBST on an orbital shaker for 5 min at room temperature.
5. Remove the PBST and incubate the membrane with the adapted anti-IgG antibody diluted in blocking buffer (here an IRDye 800-coupled anti-mouse antibody at 0.4 µg/ml final concentration) for 1 h at room temperature on an orbital shaker, or alternatively overnight at 4 °C.
6. Wash the membrane three times 50 ml of PBST on an orbital shaker for 5 min at room temperature.
7. Remove the PBST and wash the membrane with 50 ml of PBS on an orbital shaker for 5 min at room temperature.
8. Store the membrane in PBS until revelation.
9. Proceed to the membrane revelation according to the reporter system selected and following the manufacturer's recommendations (here the membrane is scanned and analyzed with an Odyssey Infrared Imager from Licor).

3.6 Reconstitution in MLVs

As underlined in the introduction section, one of the major advantages of the MAS-NMR approach resides in its invaluable capacity to study MPs within a lipid bilayer and to evaluate how this membrane environment may influence its structure, conformation and function. Although detergent micelles are essential to solubilize MPs, their biochemical and structural properties are very far from those of biological membranes, and their liquid-phase state is obviously not compatible with solid-state NMR. Necessarily, detergent-purified MPs have thus to be reconstituted in membrane mimicking systems for MAS-NMR studies, among which liposomes are the primary model of choice (*9, 50*).

Liposomes or lipid vesicles are spherical lipid bilayer structures. They form spontaneously upon lipids hydration, constituting large multilamellar vesicles (MLVs) of up to 1 μm of diameter and a dozen of stacked bilayers (*50, 51*). Thanks to their low curvature close to local constraints of the cell, their size and bilayer organization, they are considered as good membrane mimics. They also offer the great advantage to be easily tunable in term of lipid composition, and they can also be supplemented with other components such as MP cofactors or protein interactants. Depending on well-defined preparation protocols (*52*), MLVs can further be processed into unilamellar vesicles of different sizes, ranging from giant (GUVs), to large (LUVs) and small unilamellar vesicles (SUVs), sizing respectively around 10 μm , 100 nm and 20 nm.

This section presents a protocol optimized for the reconstitution of hCTR1 in MLVs, with a formulation adapted to sample filling into fast-MAS rotors (see section 3.6) and compatible with solid-state NMR analyses.

The procedure basically consists in mixing hCTR1 in DM micelles with preformed liposomes saturated in detergent beforehand. The detergent is subsequently removed by a dialysis step (*see Note 7*), upon which the MPs progressively and spontaneously

integrate into the lipid vesicles. Here again, the experimental conditions are protein-dependent with a number of parameters to be adjusted, including the choice of lipid(s) and the lipid-to-protein ratio, as well as the dialysis format, device and duration.

1. Using glass pipettes and vessels, mix lipids from stock solutions in chloroform, here POPC and cholesterol with a 10:1 (mol:mol) ratio.
2. Remove the chloroform by processing the solution in a rotary evaporator. Alternatively, chloroform may also be vaporized by blowing a gentle stream of nitrogen or argon over the solution. A visible dry lipid film is formed on the wall of the glass vessel.
3. Traces of chloroform are further removed by placing the container on a vacuum system overnight, especially if chloroform has been evaporated under a gas stream.
4. Hydrate the lipid mixture in the liposome buffer to get a concentration of 10 mg/ml by pipetting up and down, and at a temperature above the phase transition of the lipids (here 4 °C or higher). Multilamellar vesicles form spontaneously.
5. Add the detergent to saturate the MLVs, here 1% DM. Mix gently by pipetting up and down. Incubate 1h at RT.
6. Add the purified MPs (here hCTR1 in DM) to the detergent saturated MLVs at the desired lipid-to-protein ratio (LPR). Here a LPR of 2:1 (w/w) is used. Mix gently by pipetting up and down. Incubate 1h at RT.
7. Start dialysis by transferring the sample in a dialysis system (cassette or bag) with the appropriate molecular weight cut off (10 kDa MWCO for hCTR1). Plunge the dialysis tool in an adapted volume of the liposome buffer, here a ratio of 1 ml of sample for 250 ml of liposome buffer for hCTR1.

8. Run dialysis at 10 °C with a gentle agitation (beware to keep the mixture above the transition phase temperature of the lipids) and change the dialysis buffer every 24 h.
9. When the solution become milky (after about 24h for hCTR1), transfer the mixture in appropriate tubes for ultracentrifugation.
10. Ultracentrifuge at 100,000 × g, 4 °C for 30 min and remove the supernatant.
11. Resuspend the pellet in 1 ml of liposome buffer by gently pipetting up and down. Spare 20 µl of this resuspended pellet and 20 µl of the supernatant for further analyses (*see Note 8*).
12. The proteoliposome mixture obtained (**Figure 2**) can be directly used for Fast-MAS-NMR analysis.

3.7 Sample packing in Fast-MAS NMR rotors

Since NMR spectroscopy is generally a rather insensitive method, efficient sample packing is crucial to maximize the detectable signal. MLV preparations are viscous and cannot be handled and packed like a dry powder. Traditional protocols involve ultracentrifugation in a tube and subsequent transfer of the compressed sample into 4 or 3.2 mm NMR rotors using a spatula or a tabletop microcentrifuge. Rotors of smaller size (e.g., 1.3 mm) for Fast-MAS render this methodology impractical and we highly recommend using a designated filling tool for direct ultracentrifugation of the MLV sample into the rotor (*53, 54*) (*see Figure 3a*). As the time and force required vary slightly between samples, we generally start with 1 h at 100,000 × g and only increase the centrifugal force and time if found necessary.

1. Assemble the filling tool according to the user instructions and load it with an appropriate amount of sample to achieve complete filling

2. Fill a regular ultracentrifuge tube with water and balance out the filling tool.
3. Centrifuge for 1 hour at 100.000xg and 10 deg C

Remove the supernatant and access sample, use inserts if desired, close rotor with the appropriate caps

Figure 3b exemplifies 1D NMR spectra obtained in few minutes at 60 kHz MAS on an 800 MHz spectrometer. Spectra filtered through ^{13}C (grey line) or ^{15}N (black line) report respectively on aliphatic and amide 1Hs, demonstrating efficient isotope incorporation. By sampling of the heteronuclear chemical shift evolution, 2D fingerprints are acquired in ~0.5 - 1 h (**Figure 3c**) and can be used to evaluate the impact of different reconstitution conditions and the overall homogeneity of the preparation.

4 Notes

1. In non-labelling conditions, *Pichia pastoris* is classically cultured in rich media supplemented with yeast extract and meat peptone *i.e.*, BMGY for the growth phase and BMMY for the induction phase. In the corresponding defined BMG and BMM media described here, the generation time is significantly increased, from about 2 h to 3.5 h in the case of BMGY *vs* BMG.
2. 0.5 % (v/v) methanol is usually added during the induction phase. Following an optimization study, we found that a concentration reduced to 0.2 % methanol was yielding similar amounts of purified MPs, thereby substantially diminishing the cost issue for labeling with ^{13}C -methanol.
3. The culturing conditions presented here were specifically adjusted for an optimal production of hCTR1. A number of parameters may indeed influence the production yields of functional MPs in a protein-dependent manner and their optimization is often beneficial. Those notably include the adjustment of

the induction temperature and time, the addition of stabilizing compounds or the modulation of cell density and pH among others (55, 56).

4. It is recommended to maintain a 1 to 5 ratio between the volume of the yeast culture and the total volume of the baffled flask for an optimal aeration of the culture.
5. It is important to recall that the linear correlation between OD₆₀₀ measurement and cell density is limited and instrument dependent. After a standard calibration of the Eppendorf Biophotometer we use, we determined that one OD₆₀₀ unit corresponds to approximately 5×10^7 *P. pastoris* cells/ml with this instrument. We also identified 0.3 OD₆₀₀ as the linearity limit and we dilute our samples accordingly.
6. A maximum of 500 µl can be injected onto a Superdex 200 10/300 column. If the fractions of interest for the IMAC have a greater volume, they can be injected successively. Alternatively, they may be concentrated to the final desired injection volume, but we do not recommend this option with hCTR1 as this leads to oligomerization / aggregation events.
7. The dialysis efficacy is strongly depending on the properties of the detergent to be removed, and on its critical micelle concentration (CMC) in particular: the higher the CMC, the easier the detergent is removed during the reconstitution step. When working with detergent with low CMC, they may require a longer time for dialysis, which may be detrimental to the MP structure and activity. To overcome this issue, the use or addition of polystyrene beads adsorbing the detergent may be a helpful option (57, 58).
8. A rough estimate of the reconstitution efficiency can be achieved with an SDS-PAGE analysis of the supernatant and the pellet after ultracentrifugation. If the detergent removal is not complete and proteins are still in detergent

micelles, they are detected in the supernatant. Ideally, an activity evaluation may be performed if an adapted assay is available.

9. We do not recommend boiling MP-containing samples prior to electrophoresis. Boiling often leads to the aggregation of MPs that eventually get stuck in the concentrating gel.

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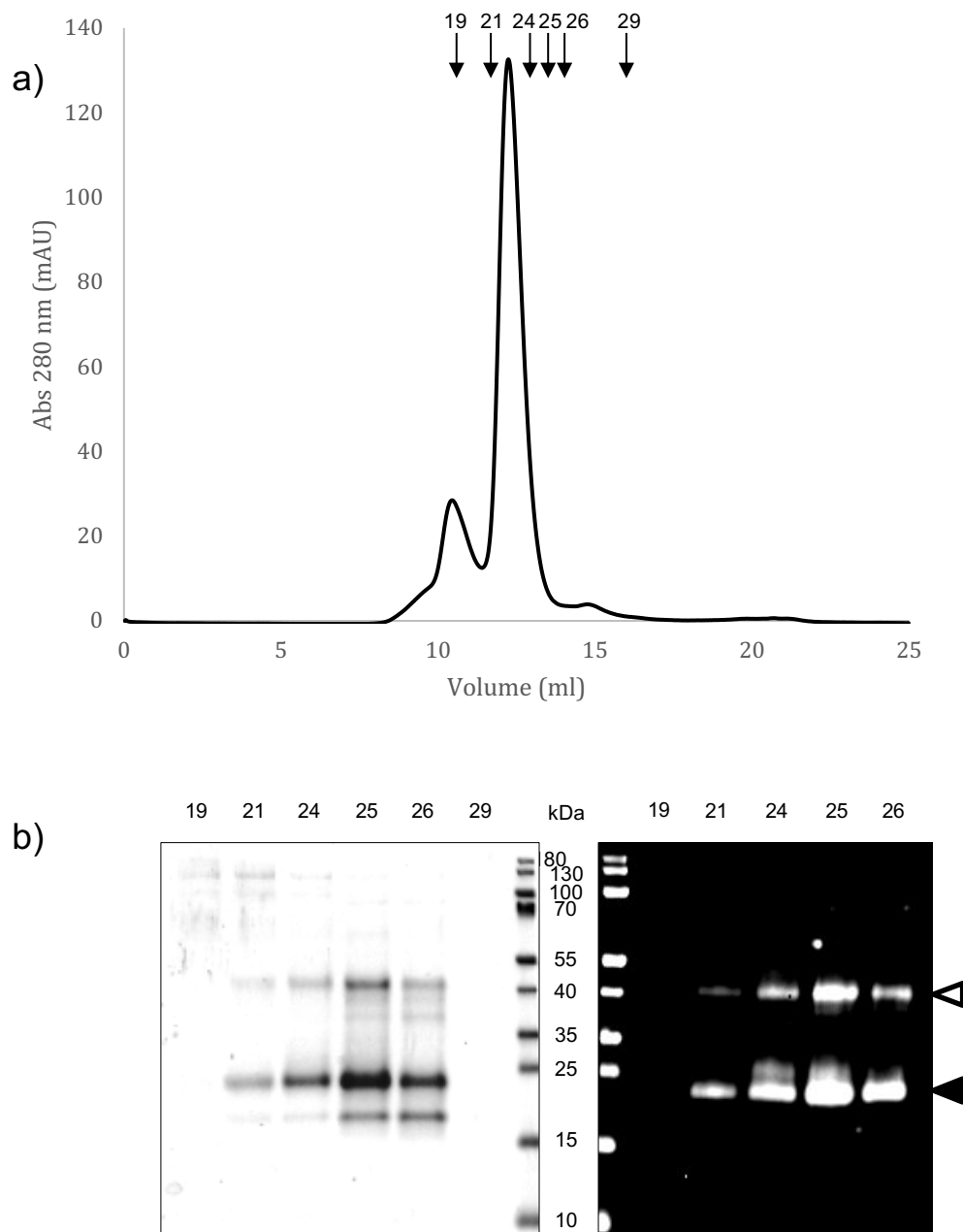


Figure 1: Purification profile of hCTR1 obtained with the presented procedure.

a) SEC elution profile obtained on a Superdex 200 10-300 column (Cytiva).

Representative fractions are indicated with black arrows. b) SDS-PAGE (10% (w/v) polyacrylamide Tris-Tricine gels) of the SEC representative fractions analyzed by Coomassie Blue staining (left panel) and Western Blot (right panel). Purified hCTR1 appears in fractions 24 to 26 as a major band corresponding to its monomeric form (22 kDa, black triangle) and as an SDS-resistant dimer (44 kDa, white triangle). An additional band of lower molecular weight is also detected on the Coomassie blue stained gel. Immunoblotting with a monoclonal anti-HA antibody from mouse

(Sigma, $0.4 \mu\text{g}/\text{ml}$). Molecular weight markers are PageRuler from Thermo, with masses (kDa) indicated in between.

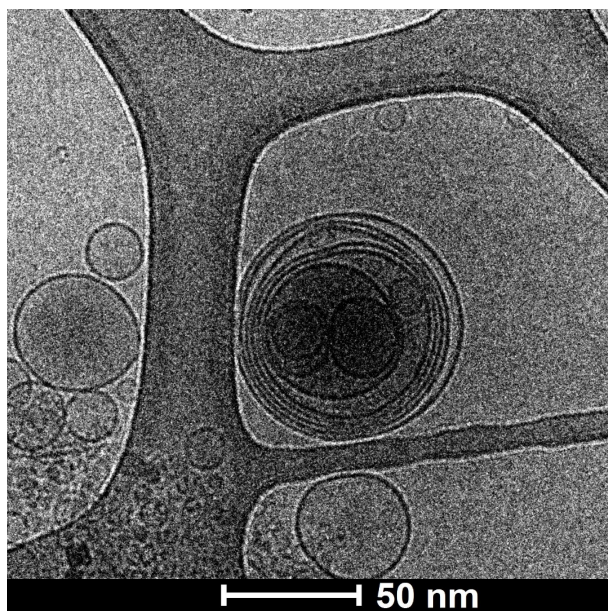


Figure 2: Visualization of hCTR1 reconstituted in MLVs by cryo-TEM.

MLV sample was blotted on a Lacey carbon film grid with a Vitrobot MARK IV instrument (FEI). Image (28,000X magnification) was acquired at 200 KV on a Glacios microscope (ThermoFisher).

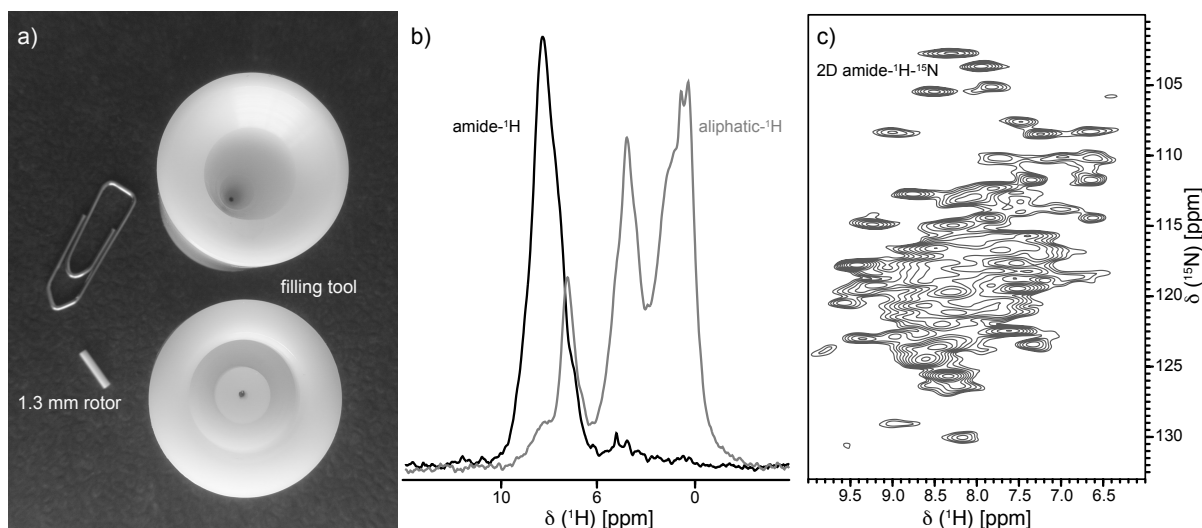


Figure 3: Fast-MAS-NMR analysis of hCTR1 in MLVs. a) 1.3 mm solid-state NMR rotor and filling tool. b) one dimensional amide-1H (1D 1H-15N CP-HSQC) and aliphatic-1H (1D 1H-13C CP-HSQC) spectra. c) two dimensional 1H-15N CP-HSQC spectrum of $^{13}\text{C},^{15}\text{N}$ labeled hCTR1 in POPC-Cholesterol MLVs recorded on an 800 MHz spectrometer with 60 kHz MAS.