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Membrane reconstitution of ABC transporters and assays of translocator function

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In this protocol, we describe a procedure for incorporating ATP-binding cassette (ABC) transporters into large unilamellar vesicles (LUVs) and assays to determine ligand binding and solute translocation by these membrane-reconstituted systems. The reconstitution technique as described has been optimized for ABC transporters but can be readily adapted for other types of transport systems. Purified transporters are inserted into detergent-destabilized preformed liposomes and detergent is subsequently removed by adsorption onto polystyrene beads. Next, Mg-ATP or an ATP-regenerating system is incorporated into the vesicle lumen by one or more cycles of freezing-thawing, followed by extrusion through polycarbonate filters to obtain unilamellar vesicles. Binding and translocation of substrates are measured using isotope-labeled ligands and rapid filtration to separate the proteoliposomes from the surrounding medium. Quantitative information is obtained about dissociation constants (K_d) for ligand binding, number of binding-sites, transport affinities (K_m), rates of transport, and the activities of transporter molecules with opposite orientations in the membrane. The full protocol can be completed within 4–5 d.

INTRODUCTION

The key function of many proteins that reside in the lipid membranes of cells is the transport of solutes. Although some functional properties of transport proteins can be determined using detergent-solubilized protein, the study of its translocation activity has an absolute requirement for a two-compartment system. Apart from transport studies, there might be other incentives to analyze membrane proteins in their membrane-inserted state, as the lipid membrane constitutes the native environment of membrane proteins. Although crude cell membranes may suffice for initial characterization of transport proteins, for more detailed analysis an isolated system is required in which parameters can be varied systematically. The first step for the *in vitro* analysis of membrane proteins is the insertion of the purified protein in an artificial lipid membrane, a process called membrane reconstitution.

The mild procedure for membrane reconstitution featured here requires the mixing of detergent-solubilized membrane proteins and detergent-treated lipid vesicles. Subsequently, the detergent molecules associated with the protein and the lipids are removed to insert the protein into the vesicle membrane. This can be performed using (i) dilution, (ii) dialysis, (iii) size-exclusion chromatography or (iv) adsorption onto polystyrene beads^{1,2}. The first three methods are mostly limited to the use of detergents with a high critical micellar concentration (CMC) such as *n*-octyl- β -D-glucoside. Despite the facile methodology involved in their removal, high CMC detergents are often detrimental for the stability of membrane proteins. To be able to use the milder, low CMC detergents such as *n*-dodecyl- β -maltoside (DDM) or Triton X-100, membrane reconstitution is most effectively carried out using polystyrene beads to adsorb the detergent³.

Here, we describe a reconstitution procedure for the incorporation of ATP-binding cassette (ABC) transport proteins into detergent-destabilized preformed-liposomes⁴, using polystyrene beads to adsorb the detergent (outlined in Fig. 1). The method, pioneered by the group of Jean-Louis Rigaud, is a well-controlled process and optimization of the reconstitution procedure is more straightforward

than for the other methods. The process starts with exposing preformed and presized lipid vesicles to increasing concentrations of detergent, resulting in an initial swelling of the liposomes that can be followed by determining the turbidity of the suspension (Fig. 2)⁵. At detergent concentrations below the point where the liposomes are fully saturated with detergent (R_{sat} , indicated in Fig. 2), the efficiency of inserting protein into the membrane is low^{1,6–8}. For most membrane proteins, reconstitution has proven to be most efficient at detergent concentrations in small excess of the point of saturation R_{sat} ^{6,7,9}, and consequently, it is this concentration that we recommend for initial trials (see Fig. 2). Under these conditions, the insertion of many proteins favors one direction. Other proteins may require liposomes exposed to higher detergent concentrations and optimal reconstitution efficiencies are observed between R_{sat} and the point where the liposomes are fully solubilized, R_{sol} . Although some proteins have a tendency to insert into artificial membranes in a particular orientation regardless of the detergent concentration, reconstitution conditions around R_{sol} most often lead to a random orientation^{10,11}. Once the detergent-solubilized membrane protein has been mixed with the detergent-destabilized liposomes, subsequent controlled, slow removal of detergent (using polystyrene beads) leads to the formation of tightly sealed proteoliposomes. The method can be used with several types of detergents. However, as mixing of the transporter complex with the titrated liposomes exposes the protein to the detergent used to destabilize the liposomes, preferably a mild detergent should be used to prevent destabilization of the protein complex in this phase. The low CMC detergent Triton X-100 is generally a good starting point, as it has proven to be most successful in many cases.

ABC transporters are vital to any living system and are involved in the translocation of a wide variety of substrates ranging from ions, sugars, amino acids, vitamins, lipids, antibiotics and drugs to larger molecules such as oligosaccharides, oligopeptides and even high molecular weight proteins^{12,13}. Irrespective of the direction of transport (import or export), the nature of the transported

Figure 1 | Outline of the described procedures for membrane reconstitution and functional analysis of ATP-binding cassette (ABC) transporters.

(a) Membrane reconstitution: liposomes are detergent-destabilized by titration with Triton X-100 and mixed with the detergent-solubilized ABC transporter complex. Subsequent controlled removal of the detergent by polystyrene beads (Bio-Beads) results in the formation of sealed proteoliposomes. (b) Surface-tethering of lipid-anchored substrate-binding proteins (SBPs): This step is optional. Proteoliposomes are mixed with detergent-solubilized lipid-modified SBPs. Bio-Bead-mediated detergent removal anchors the lipid-modified SBPs to the membrane. (c) Inclusion of energy source using freeze-thaw cycles and extrusion, the proteoliposomes are charged with ATP or an ATP-regenerating system (ARS). These proteoliposomes can be used for substrate translocation assays (d). Alternatively, proteoliposomes without ATP/ARS can be used for substrate-binding assays (e).

substrate or its physiological function, all ABC transporters are composed of two hydrophobic transmembrane domains (TMDs) and two water-soluble nucleotide-binding domains (NBDs) bound to the cytosolic face of the TMDs (Fig. 3). In addition to these core domains, hereafter referred to as the translocator or translocation unit, accessory domains or subunits can be part of the ABC transporter¹⁴. ABC import systems use as accessory component a substrate-binding protein (SBP) to capture the substrate and deliver it to the translocator. These SBPs can be either soluble¹⁵ or attached to the membrane through a lipid-anchor¹⁶, a transmembrane peptide¹⁷ or fused to the TMDs and thus an integral part of the translocator¹⁸ (Fig. 3).

Transport of (radio-labeled) substrates is most easily monitored when the substrate is accumulated into the lumen of artificial vesicles. This leads to opposite preferences for the membrane-orientation of ABC import and export systems. Export systems should preferably be inserted inside-out (ISO), allowing them to be energized by supplementing the external medium with ATP. On the contrary, import systems should be incorporated right-side-out (RSO) and ATP should be included in the vesicle lumen. Because ABC transporters operate unidirectionally, a random insertion is not necessarily disadvantageous and can even be beneficial. For instance, for the ABC importer OpuA, the fraction of RSO-oriented complexes was assayed by monitoring solute uptake at the expense of internal ATP. As ATP was gradually depleted and ADP built up inside the vesicle lumen, solute uptake leveled off after some time. Now the fraction of ISO-oriented molecules could be assayed by monitoring solute efflux upon addition of Mg-ATP to the external medium. As the composition of the external medium can be easily modified, this property could be used to determine the ATP/solute stoichiometry¹⁹, ionic strength dependence²⁰ and salt requirement of the transporter²¹.

The protocols presented are based on the successful membrane reconstitution and functional analysis of a range of ABC transporters, including importers with soluble SBPs (BtuCD-F²²),

importers with lipid-anchored SBPs (OppBCDF-A^{23,24}), importers with SBPs fused to the TMDs (OpuA²⁵ and GlnPQ²⁶) and exporters (LmrA²⁷ and BmrA²⁸). The same procedures have also been applied successfully to membrane-reconstitute ion-linked secondary transporters and channel proteins^{6,7,29}. Although we describe here the use of proteoliposomes to monitor transport and binding of radio-labeled substrates, these proteoliposomes can be also used for other types of assays or serve as a basis for the formation of giant unilamellar vesicles (GUVs) that allow the

Figure 2 | Titration of preformed liposomes with Triton X-100.

Liposomes composed of *Escherichia coli* polar lipids and egg PC in a ratio of 3:1 (wt/wt) were extruded through a 400-nm polycarbonate filter, diluted to 4 mg ml⁻¹ and exposed to increasing Triton X-100 concentrations; the physical state of the membrane was probed by measuring the optical density of the suspension. R_{sat} and R_{sol} refer to the detergent concentration where the membrane is saturated with detergent and fully solubilized, respectively. The middle arrow indicates the suggested point in the titration curve at which the liposomes can be combined with the purified membrane protein to commence the reconstitution procedure.

characterization of membrane proteins using confocal microscopy, fluorescence correlation spectroscopy and patch clamp. Although these methods and applications are not described here in detail, we provide relevant references and examples.

Experimental design

The procedure presented here assumes the availability of a stable detergent-solubilized ABC transporter complex and, if required, an SBP in ample amounts (0.1–1 mg). The formation of liposomes of defined size from lipid solutions is described. Starting from these basic components, we detail (i) the membrane insertion of the ABC transporter complex; (ii) the optional membrane-association of lipid-anchored SBPs; (iii) the inclusion of Mg-ATP or an ATP-regenerating system into the lumen of the proteoliposomes; (iv) substrate translocation and (v) binding assays for both ABC import and export systems (outlined in Fig. 1).

Depending on the specific requirements of the ABC transport system or activity assay, the generic membrane reconstitution protocol presented here might require small modifications. To reconstitute a membrane transporter for ligand-binding or translocation assays, a number of parameters should be considered: (i) the stability of protein complex in the detergent-solubilized state; (ii) the specific lipid requirement of the transporter; and (iii) the protein-to-lipid ratios of the proteoliposomes.

Stability of membrane transporters in the detergent-solubilized state. The success of the membrane reconstitution protocol depends to large extent on the quality of the protein preparation used. ABC transporters form oligomeric complexes, often composed of different subunits. Depending on the system, these complexes may dissociate during the purification step. Functional reassembly of an ABC transporter complex from its purified components has been shown³⁰, but this is more the exception than the rule. It is generally recommended to purify the intact complex and use this material for membrane reconstitution.

One critical parameter to consider for the stability of transporter complexes or membrane proteins in general, is the detergent(s) used for solubilization and purification. In most cases, we prefer detergents with an intermediate or low CMC. A good starting point is DDM. The amount of detergent brought into the reconstitution mixture through the protein should be small relative to that needed for destabilization of the liposomes. In this respect, it is important to note that several detergents with a low or medium CMC form relatively large micelles. If concentration of the membrane protein is needed before the reconstitution procedure, this could lead to simultaneous concentration of the protein-free detergent micelles and affect the reconstitution process. However, ABC transporters form large protein–detergent complexes and can be concentrated on membranes with a 100-kDa molecular weight cut-off (MWCO) (e.g., Amicon or Vivaspin 100,000 MWCO concentrators), which allow passage of most detergent micelles^{31–33}. The concentration of protein-free detergent micelles in the concentrated sample can be calculated from the fraction of detergent bound to the protein and the total detergent concentration as determined according to

Figure 3 | Architecture of ATP-binding cassette transport systems. (a) Efflux system; (b) uptake system with periplasmic SBP; (c) uptake system with lipid-anchored SBP; (d) uptake system with transmembrane peptide-anchored SBP; and (e) uptake system with covalently linked substrate-binding domains (SBDs). TMD, NBD and SBP indicate the transmembrane domain, nucleotide-binding domain and substrate binding protein, respectively.

refs. 31,34. Excess of detergent micelles can be removed by careful treatment of the sample with Bio-Beads³⁵.

In addition, the integrity of the transporter complex during purification can be enhanced by the addition of stabilizing agents such as glycerol (10–20%), salt (0.1–1 M of KCl or NaCl), phospholipids (0.05–0.2 mg ml^{−1}) or specific ligands. By taking advantage of the effect of glycerol on the stability, a procedure to generate heterodimers of an ABC transporter has been developed³⁶. Notably, for the ABC transporter Opp, omission of imidazole during the solubilization and purification proved essential to prevent dissociation of the complex²⁴. The recording of a UV spectrum from 240 to 340 nm can be used as a first indication of structural integrity. For fully soluble nonaggregated protein samples, the difference in the absorbance at 320 and 340 nm is 0 and the A_{280}/A_{260} ratio is ~ 1.8 . Furthermore, the stability of a membrane transporter complex in a given condition can be monitored using size-exclusion chromatography. The monodispersity of a preparation usually correlates well with the functional competent state.

Synthetic lipids versus total lipid extracts. We routinely reconstitute ABC transporters into mixtures of dioleoyl-phosphatidylethanolamine (DOPE), dioleoyl-phosphatidylglycerol (DOPG) and dioleoyl-phosphatidylcholine (DOPC)^{20,37}. A 1:1:1 or 2:1:1 molar ratio of DOPE, DOPC and DOPC is recommended, as transporters generally require a significant fraction of nonbilayer (e.g., phosphatidylethanolamine) and anionic lipids (e.g., phosphatidylglycerol or phosphatidylserine). An alternative and cheaper lipid source is the *Escherichia coli* polar lipid extract. We prefer the *E. coli* polar lipid extract obtained by extraction of the *E. coli* total lipid extract from Avanti according to Newman and Wilson³⁸ over the polar lipid extract purified by Avanti. A disadvantage of the Avanti lipid extracts is the batch to batch variation, which might lead to nonreproducible results for membrane proteins that highly depend on the lipid composition.

Protein-to-lipid ratio. For functional studies (ligand binding, solute translocation, confocal microscopy), we routinely incorporate ABC transporter protein complexes into liposomes at protein-to-lipid ratios of 1:20 to 1:200 (wt/wt). For systems, such as OpuA, with a molecular mass of ~ 200 kDa, this corresponds to mol/mol ratios of 1:6,000 to 1:60,000. We have observed significant reductions in the *apparent* specific transport activities if

higher protein-to-lipid ratios (desirable for some biophysical analysis techniques such as attenuated total reflectance–Fourier transform infrared, electron spin resonance and NMR spectroscopy) are

used, for example, see ref. 9. This is most likely caused by a decrease in the efficiency of the reconstitution procedure at these high protein-to-lipid ratios.

MATERIALS

REAGENTS

- Chloroform (Lab-scan, cat. no. 966)
- Diethyl ether (Lab-scan, cat. no. 1550/50)
- Ni²⁺-sepharose resin (Amersham, cat. no. 175318-03)
- Bio-Beads SM-2 polystyrene beads (Bio-Rad, cat. no. 152-3920) (see REAGENT SETUP)
- DDM, anagrade (Anatrace Inc., cat. no. D310)
- Triton X-100 (Anatrace Inc., cat. no. APX-100)
- 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine, DOPE (Avanti Polar Lipids, cat. no. 850725)
- 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylglycerol, DOPG (Avanti Polar Lipids, cat. no. 840475)
- 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine, DOPC (Avanti Polar Lipids, cat. no. 850375)
- *E. coli* total lipid extract (Avanti Polar Lipids, cat. no. 100500) (see REAGENT SETUP)
- Glycerol, 87% (wt/vol) (Merck, cat. no. 1842)
- KH₂PO₄ (Merck, cat. no. 2090)
- K₂HPO₄ (Merck, cat. no. 2091)
- KCl (Merck, cat. no. 2074)
- Na₂-ATP (Roche, cat. no. 10519987001)
- MgSO₄ (H₂O)₇ (Merck, cat. no. 2367/50)
- Creatine kinase (Roche, cat. no. 1073698801)
- Creatine phosphate-disodium salt (Sigma, cat. no. P-7936)
- [Methyl-¹⁴C] choline chloride (50–62 mCi mmol^{−1}) (Amersham, cat. no. CFA 424)
- [Methyl-³H] choline chloride (60–85 Ci mmol^{−1}) (Amersham, cat. no. TRK593)
- Choline oxidase (Sigma, cat. no. C-5896)
- Ammonium sulfate (Merck, cat. no. 240/50)
- PEG 6000 (Merck, cat. no. 8.07491.1000)
- Scintillation liquid (Perkin-Elmer; cat. no. 15325)
- KP_i (see REAGENT SETUP)
- 10× Mg-ATP (see REAGENT SETUP)
- 10× ATP-regenerating system (ARS) (see REAGENT SETUP)
- Buffer A (see REAGENT SETUP)
- Purified ABC transporter (see EQUIPMENT SETUP)
- Purified lipid-anchored SBP (see EQUIPMENT SETUP)
- Purified soluble SBP (see EQUIPMENT SETUP)
- 50% (wt/vol) ammonium sulfate (see EQUIPMENT SETUP)
- Radio-labeled glycine-betaine (see EQUIPMENT SETUP)

EQUIPMENT

- Small-scale extruder (Avestin Inc., cat. no. LF-1)
- Polycarbonate filters, 200- and 400-nm pore size (Avestin Inc., cat. no. LFM-200 and LFM-400)
- Rod sonicator (Vibra Cell; Sonics & Materials)
- Rotary evaporator
- Polyprep columns (Bio-Rad, cat. no. 731-1550)
- Rotary device
- 5-ml PP tubes (Greiner bio-one, cat. no. 115261)
- Table ultracentrifuge (TL-100 and TLA100.4 rotor; Beckman)
- Manifold high-vacuum filtration unit (VWR, cat. no. 89026-69)
- Cellulose nitrate filters, Schleicher & Schuell BA85, 0.45-μm pore size, roll of 30 cm × 3 m (Whatman, cat. no. 10 401 196), filters with a diameter of 25 mm are manually punched from a roll. Alternatively, prepunched filters with a diameter of 25 mm (Whatman, cat. no. 10 402 506) can be used.

- Cellulose acetate filters, Schleicher & Schuell OE66, 0.20 μm pore size, sheets of 30 cm × 60 cm (Whatman, cat. no. 10 404 180), filters with a diameter of 25 mm are manually punched from sheets. Alternatively, prepunched filters with a diameter of 25 mm (Whatman, cat. no. 10 404 106) can be used.
- Dispenser to aliquot 2 ml volumes (Brand, cat. no. 4701131)
- Scintillation counter (Perkin-Elmer Tri-carb 2800TR)
- Cary 100 Bio UV-visible spectrophotometer (Varian)

REAGENT SETUP

Bio-Beads SM-2 beads Wash once with 10 volumes of methanol, once with 10 volumes of ethanol, and finally ten times with 10 volumes of MilliQ. The beads can then be stored in MilliQ at 4 °C for at least 6 months. The amounts of beads used in the procedure correspond to wet weight quantities derived as follows: before use, decant part of the Bio-Beads suspension in a clean Petri dish. Hold the Petri dish at an angle of 45° and place the pipette tip in the Bio-Bead suspension against the edge of the Petri dish. Remove the MilliQ and weigh off the desired quantity of beads. Use these beads immediately. Detergent binding capacities of Bio-Beads can be found in ref. 39.

***E. coli* polar lipid extract** Corresponds to *E. coli* total lipid extract, which has been precipitated with acetone and then extracted with diethyl ether, according to Newman and Wilson³⁸. To prevent oxidation, store lipids under an atmosphere of N₂ or argon and protected from light at −20 °C.

KP_i Prepare by mixing equimolar solutions of KH₂PO₄ and K₂HPO₄ up to the desired pH; pH values given in the protocol are measured at 20 °C. In this protocol, we use KP_i, but functional reconstitution in other buffer systems has been reported^{19,22}.

5× Mg-ATP 50 mM Na₂-ATP plus 50 mM MgSO₄ in 50 mM KP_i, pH 7.0 and adjusted to pH 6.5–7.0 with NaOH (stored in aliquots at −80 °C)

5× ARS 50 mM Na₂-ATP, 50 mM MgSO₄, 120 mM Na₂-creatine phosphate plus 12 mg ml^{−1} creatine kinase in 20 mM KP_i, pH 7.0. Freshly prepared from concentrated stocks of Mg-ATP (125 mM Na₂-ATP plus 125 mM MgSO₄ in 50 mM KP_i, pH 7.0 and adjusted to pH 6.5–7.0 with NaOH; stored in aliquots at −80 °C), creatine phosphate (600 mM Na₂-creatine phosphate in MilliQ, stored in aliquots at −80 °C), and creatine kinase (30 mg ml^{−1} creatine kinase in MilliQ; stored in aliquots at −80 °C)

Buffer A 50 mM KP_i, 20% (wt/vol) glycerol, 200 mM NaCl, 500 mM imidazole, pH 7.8.

Purified ABC transporter At 1 mg ml^{−1} of protein in buffer A plus 0.05% (wt/vol) DDM; examples used are OpuA²⁵, OppBCDF²⁴, GlnPQ²⁶ and LmrA²⁷. In general, protein purity >95% is used, although less pure samples have also been reconstituted successfully. For ABC transporters composed of multiple components, it is recommended to purify the translocator unit as a whole instead of mixing the individually purified components. Owing to the intrinsic instability of detergent-solubilized membrane proteins, we recommend using protein purified immediately before the reconstitution procedure.

Purified lipid-anchored SBP Purified lipid-anchored SBP at 1 mg ml^{−1} of protein in buffer A and 0.05% (wt/vol) DDM; example used is the oligopeptide binding protein OppA²³.

Purified soluble SBP Purified soluble SBP at 5 mg ml^{−1} of protein in 80 mM KP_i, pH 7.0 or 100 mM KP_i, pH 7.0, depending on the system used to energize transport (see Step 32); example used is the vitamin B₁₂ binding protein BtuF⁴⁰.

50% (wt/vol) ammonium sulfate Freshly prepared by dissolving ~50 g of ammonium sulfate in 100 ml (final volume) of dH₂O.

Radio-labeled glycine-betaine Convert choline-chloride to glycine-betaine as described⁴¹. For ³H-choline chloride, evaporate the ethanol and dissolve the remaining powder in 250 μl 50 mM NaPi, pH 8.0. For ¹⁴C-choline chloride, supplement the solution with 1M NaPi, pH 8.0, to a final concentration of 50 mM NaPi. Next, add 16 mg (250 U) choline oxidase and incubate overnight at 37 °C. The conversion can be checked with thin-layer chromatography (TLC).

PROCEDURE

Preparation of preformed liposomes ● TIMING 8 h

1| Mix stock solutions of lipids in chloroform in the desired ratio to a final amount of 20 mg lipids or higher. Dry the lipids in a rotary evaporator at 30 °C for ~30 min.

▲ CRITICAL STEP Use glassware for handling and storing lipids dissolved in chloroform to prevent leaching of polymer from plastics⁴². As chloroform is volatile, transferring exact volumes can be difficult. In their dried state, unsaturated lipids are sensitive to oxidation. Therefore, proceed to the next steps immediately.

2| Add 1 ml of diethyl ether to dissolve the lipids, and subsequently dry the lipids in a rotary evaporator at 30 °C for another 30 min to remove the diethyl ether and traces of chloroform.

3| Suspend the dried lipids at room temperature (~20 °C) in 50 mM KPi, pH 7.0, to a final concentration of 20 mg ml⁻¹. Use a syringe equipped with a long 20-gauge needle that is bent at a 45° angle to reach the sides of the wall of the round-bottomed flask. The suspension should be made homogeneous and without clumps.

4| Sonicate the lipid suspension in six cycles (15 s on/45 s off) at an intensity of 4 μm (peak-to-peak) on ice water under a gentle stream of nitrogen (N₂) gas. In this step, small unilamellar vesicles (SUVs) are generated.

5| Flash-freeze the SUV suspension (aliquots of 0.5–1 ml) in liquid N₂ and subsequently slowly thaw at room temperature (~20 min). By piercing the lid of the cup before this step, violent escape of N₂ gas from the cup is prevented.

6| Repeat Step 5 two times to fuse the SUVs to large multilamellar vesicles (LMVs).

■ **PAUSE POINT** After the third freezing step, LMVs can be stored in liquid N₂ for at least 1 year.

Reconstitution of ABC transporters into detergent destabilized liposomes ● **TIMING 24 h**

7| Extrude the LMVs 11 times through a 400-nm polycarbonate filter to form large unilamellar vesicles (LUVs).

▲ CRITICAL STEP Prewash the extruder with 50 mM KPi, pH 7.0. The extruder device is used for small volumes (0.5–1 ml) of liposomes. Make sure the extruder does not leak and that the polycarbonate filter is not damaged by screwing the device too tightly. The liposome suspension starts off as opaque and becomes translucent after the first extrusion pass. This can be monitored conveniently by holding the syringe up to the light and looking at the syringe graduations through the liposome solution.

8| Dilute the LUVs to 4 mg ml⁻¹ of lipid in 50 mM KPi, pH 7.0.

▲ CRITICAL STEP To stabilize labile detergent-solubilized protein complexes, the LUVs should be diluted with 50 mM KPi, pH 7.0 plus 25% glycerol to reach a final glycerol concentration of 20%. From this point on 20% glycerol should be included in the buffers.

9| Titrate the LUVs with Triton X-100 at room temperature until R_{sat} is reached; use 10 μl aliquots of 10% (wt/vol) Triton X-100 stock solution per 5 ml LUV suspension of 4 mg ml⁻¹. To mix the suspension, invert the tube gently. Allow the Triton X-100/LUV suspensions to equilibrate shortly (~30 s) between each addition (**Box 1**). Monitor the destabilization of the liposomes by measuring the optical density of the suspension at 540 nm (**Fig. 2**). For most lipid compositions, R_{sat} is reached after ~10 additions of Triton X-100.

10| Add another 5 aliquots of 10% (wt/vol) Triton X-100.

11| Combine the purified membrane protein with the detergent-destabilized liposomes. For 1:100 (wt/wt) protein-to-lipid reconstitutions, add 0.2 ml of purified protein (1 mg ml⁻¹ stock solution in buffer A plus 0.05% (wt/vol) DDM) to 5 ml of detergent-destabilized liposomes; incubate for 15 min at room temperature with gentle agitation. The volume of the protein should not exceed 10% of the volume of the liposomes.

▲ CRITICAL STEP To stabilize labile detergent-solubilized protein complexes, Steps 11 and 12 should be performed at 4 °C. Minimize air space (<10%) between solution and cap of tube, as this may lead to dissociation and/or inactivation of protein complexes prior to insertion into the lipid vesicles (as observed for OpuA and GlnPQ).

12| Add 200 mg of Bio-Beads SM-2 per 5 ml of protein–liposome suspension; incubate for 30 min at room temperature with gentle agitation.

13| Add an additional 200 mg of Bio-Beads SM-2 per 5 ml of protein–liposome suspension; incubate for 60 min at 4 °C with gentle agitation.

BOX 1 | TRITON X-100 VERSUS OTHER DETERGENTS FOR MEMBRANE RECONSTITUTION

Although Triton X-100 is not the detergent of choice for protein chemical or biophysical studies (high absorbance in the UV, polydisperse), it has been frequently observed that Triton X-100 is more efficient than other detergents in mediating membrane reconstitution using solute translocation activity as the criterion for reconstitution efficiency. Also, it takes much less time to equilibrate preformed liposomes [large unilamellar vesicles (LUVs)] with Triton X-100 than, for instance, *n*-dodecyl-β-maltoside (DDM)⁴⁵. Purification of the ABC transporters is not performed in Triton X-100, rather DDM or another mild detergent is used in any step before the membrane reconstitution.

BOX 2 | CONVERSION OF LUVS TO GUVS

The large unilamellar vesicles (LUVs) obtained with the protocol (Steps 1–19 or 1–27) can be converted into giant unilamellar vesicles (GUVs)⁴⁶. Briefly, proteoliposomes prepared in volatile buffer (50 mM NH_4HCO_3 , pH 8.0) supplemented with sucrose (see below) are dried overnight under vacuum at 4 °C on UV-ozone cleaned glass or indium-tin oxide (ITO)-coated cover slips. UV-ozone cleaning is not essential for GUV formation but increases the wetting properties of the cover slip surface, making it easier to dry liposomes from aqueous solution. Sucrose is added at 100 mg per g of lipid to stabilize the proteins during dehydration; sucrose most likely interacts through hydrogen bonding with the proteins and prohibits lipid phase transitions from liquid crystalline to gel phase (during dehydration) and gel to liquid crystalline phase (during subsequent rehydration). Rehydration is done by adding 0.5 ml 10 mM KPi , pH 7.0, at room temperature. Optionally, 10 mM MgCl_2 is added or electroformation (using ITO-coated cover slips) is performed when using neutral lipids only⁴⁷. GUV formation is monitored by fluorescence microscopy. The GUVs are used in confocal microscopy and fluorescence correlation spectroscopy (lipid and protein mobility, protein–protein interactions) and patch clamp (electrical recording of ion translocation) studies.

- 14| Add an additional 200 mg of Bio-Beads SM-2 per 5 ml of protein–liposome suspension; incubate overnight at 4 °C with gentle agitation.
  - 15| Add an additional 200 mg of Bio-Beads SM-2 per 5 ml of protein–liposome suspension; incubate for 120 min at 4 °C with gentle agitation.
  - 16| Remove Bio-Beads SM-2 by filtration over sintered glass in disposable polyprep columns; the proteoliposomes end up in the eluate.
  - 17| Wash the Bio-Beads SM-2 with 50 mM KPi , pH 7.0, and, if glycerol was included during the reconstitution, dilute the proteoliposomes tenfold to lower the glycerol concentration to 2%.
  - 18| Collect the proteoliposomes by centrifugation at 4 °C (20 min at 267,000g at r_{av}).
  - 19| Suspend the proteoliposomes to 20 mg ml^{-1} of lipid in 50 mM KPi , pH 7.0 and flash-freeze in aliquots of 0.1–0.5 ml. If applicable, the proteoliposomes can now be supplemented with lipid-anchored SBPs. The procedure used for surface-tethering of lipid-anchored SBPs is described in Steps 20–27. For translocation experiments with ABC import systems, first Mg-ATP or an ARS needs to be included in the vesicle lumen as described in Steps 28–30. For translocation experiments with ABC export systems, continue with Steps 47–49. For ligand-binding assays, continue with Steps 50–59. LUVs can be converted to GUVs as described in Box 2.
- **PAUSE POINT** The proteoliposomes can be stored in liquid N_2 for several months.
- Surface-tethering of lipid-anchored SBPs** ● **TIMING 4 h**
- 20| Slowly thaw the multilamellar liposomes (product of Step 6) or proteoliposomes (product of Step 19) at room temperature and extrude 11 times through a 400-nm polycarbonate filter.
  - ▲ **CRITICAL STEP** See Step 7.
  - 21| Dilute the (proteo)liposomes to 4 mg ml^{-1} of lipid in 50 mM KPi , pH 7.0.
  - 22| Combine the purified lipid-anchored SBP with the (proteo)liposomes. For a 1:50 (wt/wt) protein-to-lipid ratio, add 0.4 ml of purified protein [1 mg ml^{-1} stock solution in buffer A plus 0.05% (wt/vol) DDM] to 5 ml of (proteo)liposomes. Incubate for 20 min at room temperature with gentle agitation.
  - 23| Add 400 mg of Bio-Beads SM-2 per 5 ml of protein–liposome suspension; incubate for 60 min at 4 °C with gentle agitation.

BOX 3 | DIAMETER AND INTERNAL VOLUME OF (PROTEO)LIPOSONES

Apart from the pore diameter of the polycarbonate filters used for the extrusion procedure, the size of the resulting liposomes depends on other factors such as the number of filter passes⁴⁸, the lipid composition⁴⁹ and the buffer used⁵⁰. Analysis of liposome size distribution can be performed by dynamic light scattering. In general, liposomes produced by extrusion through 200 filters yield a more homogeneous size distribution and a low amount of multilamellar vesicles compared to filters with 400 nm pores. Approximate vesicle sizes after extrusion through 400- and 200-nm filters are 350 ± 100 nm and 170 ± 20 nm, respectively (J. Knol and B.P., unpublished data). The specific internal volume of liposomes can be determined according to ref. 51, but approximate values are $0.90 \pm 0.05 \mu\text{l mg}^{-1}$ of lipid and $0.73 \pm 0.04 \mu\text{l mg}^{-1}$ lipid for liposomes after extrusion through 400 and 200 nm, respectively (J. Knol and B.P., unpublished data).

24| Add an additional 400 mg of Bio-Beads SM-2 per 5 ml of protein–liposome suspension; incubate for 60 min at 4 °C with gentle agitation.

25| Remove Bio-Beads SM-2 by filtration over sintered glass in polypropylene columns; the (proteo)liposomes with lipid-anchored SBP end up in the eluate.

26| Collect the proteoliposomes by centrifugation at 4 °C (20 min at 267,000*g* at r_{av}).

27| Suspend the proteoliposomes to 20 mg ml^{−1} of lipid in 50 mM KP_i, pH 7.0 and flash-freeze in aliquots of 0.5–1 mL.

■ **PAUSE POINT** The proteoliposomes with lipid-anchored SBP can be stored in liquid N₂ for several months.

Inclusion of Mg-ATP or ATP-regenerating system ● **TIMING 2 h**

28| Slowly thaw proteoliposomes (20 mg ml^{−1} of lipid) from Steps 19 or 27 and mix with 0.2 volume of 5× Mg-ATP or 5× ARS.

29| Flash-freeze the proteoliposome suspension (aliquots of 0.5 mL) in liquid N₂ and subsequently slowly thaw at room temperature (~20 min).

30| Repeat Step 29. Two cycles of freeze-thaw are generally sufficient to equilibrate the Mg-ATP or ARS. Five cycles of freeze-thaw are needed to scramble the orientation of the transporter complex fully in the membrane.

■ **PAUSE POINT** After flash-freezing in liquid N₂, the proteoliposomes can be stored in liquid N₂ for several months.

Solute uptake assay for ABC import systems ● **TIMING 4 h**

31| Slowly thaw the multilamellar proteoliposomes from Step 30 and extrude 11 times through a 200-nm polycarbonate filter to form unilamellar proteoliposomes (**Box 3**).

▲ **CRITICAL STEP** It is essential to prewash the extruder with 50 mM KP_i, pH 7.0 plus 1× Mg-ATP or 1× ARS (see Step 7 for use of the extruder device).

32| Dilute the proteoliposomes with medium isotonic to the luminal content. For a luminal composition of 50 mM KP_i, pH 7.0 plus 10 mM Mg-ATP, an external medium of 80 mM KP_i, pH 7.0 is used. For 50 mM KP_i, pH 7.0 plus 1× ARS, an external buffer of 100 mM KP_i, pH 7.0 is used.

33| Collect the proteoliposomes by centrifugation at 4 °C (20 min at 267,000*g* at r_{av}) and suspend the pellet in isotonic media (see Step 32).

34| Remove external Mg-ATP/ARS by centrifuging the suspension at 4 °C (20 min at 267,000*g* at r_{av}) and suspend the proteoliposomes to 80 mg lipid ml^{−1} in isotonic media (keep the concentrated suspension on ice).

35| Dilute the proteoliposomes to 5 mg lipid ml^{−1} in isotonic media (see Step 32). For ABC transporters that are osmotically activated, additional salt or sugar should be added to the medium. For ABC import systems that require a soluble SBP, the latter should be added to the medium.

36| Preincubate the diluted proteoliposomes at 30 °C in a water bath for 2 min. Use a gently rotating magnetic stirrer bar to mix the suspension throughout the assay.

37| Add isotopically labeled substrate to a final concentration of 0.1–100 μM; typically ~100,000 counts per minute per 40 μL sample. Unlabeled substrate can be mixed with radio-labeled substrate if higher substrate concentrations are required.

38| At given time intervals (e.g., 15, 30, 60, 120 and 240 s), take a 40 μL sample and dilute with 2 mL of ice-cold medium, in composition and molarity equal to the assay medium (Steps 32 and 35).

39| Immediately filter the samples by pouring the solution on top of a prewetted 0.45 μm pore size cellulose nitrate filter under high vacuum, using a Manifold vacuum filtration device. Filtration should be completed within 5 s.

▲ **CRITICAL STEP** Most likely due to electrostatic interactions, proteoliposomes containing charged lipids are retained quantitatively (>95%) on cellulose nitrate filters, even when the vesicles are smaller than the pore size of the filters (0.45 μm for BA85 filters). Cationic ligands such as bradykinin and other peptides, on the other hand, strongly bind to cellulose nitrate and neutral cellulose acetate filters (OE66; pore diameter of 0.2 μm) should be used instead. Retention of small proteoliposomes on cellulose acetate filters is not quantitative; it can be enhanced by adding the appropriate antibody (Ab) before the dilution with ice-cold medium supplemented with 8% (wt/vol) PEG 6000 (ref. 23), presumably through Ab-mediated crosslinking of vesicles.

40| Wash the filter with 2 ml ice-cold buffer.

41| Dry the filters for 2 h at 37 °C.

42| Dissolve the filters in 2 ml of scintillation liquid.

43| Determine the radioactivity on the filters by liquid scintillation counting. For solute export assays with ABC import systems, continue with Steps 44–46. For ligand-binding assays, continue with Steps 50–59.

▲ **CRITICAL STEP** Take zero time points and/or blank samples (liposomes without protein) and subtract the ‘zero’ values from the apparent uptake values. Determine the specific radioactivity of the medium (counts per minute per volume; Step 37) to relate the radioactivity count to the substrate concentration.

? TROUBLESHOOTING

Solute export assay for ABC import systems ● TIMING 4 h

44| The activity of the ABC import systems reconstituted in the inside-out orientation can be assayed by monitoring the efflux of pre-accumulated substrate, using proteoliposomes with Mg-ATP in the lumen. Follow Steps 31–37 and incubate the proteoliposomes with the externally added radio-labeled ligand until the uptake of the substrate has leveled off (as determined in separate measurements, Steps 31–43).

45| Initiate export of the radio-labeled ligand by the addition of 10 mM Mg-ATP, take 40 µl samples at given time intervals and dilute with 2 ml ice-cold isotonic medium without Mg-ATP (Step 38).

46| Filter the sample and determine the radioactivity (Steps 39–43).

? TROUBLESHOOTING

Solute translocation assay for ABC export systems ● TIMING 4 h

47| Slowly thaw the proteoliposomes from Step 19 and extrude 11 times through a 200-nm polycarbonate filter to form unilamellar vesicles. Keep the suspension on ice.

▲ **CRITICAL STEP** Prewash the extruder with 50 mM KPi, pH 7.0 (see Step 7 for use of the extruder device).

48| Dilute the proteoliposomes to 5 mg lipid ml⁻¹ in 50 mM KPi, pH 7.0 plus 10 mM Mg-ATP.

49| Measure transport of radio-labeled substrate into the lumen of the proteoliposomes as described in Steps 36–43.

? TROUBLESHOOTING

Ligand-binding assay ● TIMING 4 h

50| Slowly thaw proteoliposomes (20 mg ml⁻¹ of lipid) from Steps 19 or 27 and extrude 11 times through a 200-nm polycarbonate filter to form unilamellar proteoliposomes. Keep the concentrated suspension on ice.

51| Dilute the proteoliposomes to 5 mg ml⁻¹ of lipid in 50 mM KPi, pH 7.0.

52| Preincubate at 30 °C for 2 min.

53| Add radio-labeled substrate to a final concentration of 0.1–40 µM. Keep the concentration radio-labeled substrate constant and increase the fraction nonlabeled substrate.

▲ **CRITICAL STEP** For ligand-binding studies, we generally use [³H]-labeled substrates with a high specific activity (60–85 Ci mol⁻¹).

54| After 1 min of incubation, take two or more 40 µl samples and dilute each with 2 ml of ice-cold 50% (wt/vol) ammonium sulfate.

▲ **CRITICAL STEP** To prevent co-precipitation of ligands like peptides, for example, the nonapeptide [³H]-bradykinin, ice-cold 8% (wt/vol) PEG 6000 is used instead of ammonium sulfate (Steps 54 and 56).

55| Immediately filter the samples by pouring the solution on top of a prewetted 0.45 µm pore size cellulose nitrate filter under high vacuum, using a Manifold vacuum filtration device.

▲ **CRITICAL STEP** See Step 39 for the type of filter to be used.

56| Wash the filters with 2 ml of ice-cold 50% (wt/vol) ammonium sulfate.

57| Dry the filters for 2 h at 37 °C.

58| Dissolve the filters in 2 ml scintillation liquid.

59| Determine the radioactivity on the filters by liquid scintillation counting.

▲ CRITICAL STEP Include blank samples (liposomes without protein or binding-incompetent mutants) and subtract the 'blank' values from the apparent binding values. Determine the specific radioactivity of the medium (counts per minute per volume) to relate the radioactivity count to the substrate concentration. It is important to treat the filters (wetting with the same buffer; subsequent drying) for the blank and total count samples in the same manner as those for the ligand-binding (and transport) measurements; liquid scintillation counting of [³H]-labels is sensitive to quenching by medium components.

? TROUBLESHOOTING

? TROUBLESHOOTING

Problem: If little or no solute translocation is observed in Steps 43, 46 or 49, consider the following options:

Possible reasons and solutions:

1. The purified ABC transporter complex is not sufficiently stable in the detergent-solubilized state. The stability and monodispersity of a membrane transporter preparation can be determined using size-exclusion chromatography. The stability of the complex throughout the purification procedure might be improved by switching to a different detergent and/or the inclusion of glycerol (10–20%), salt (0.1–1 M KCl/NaCl) or lipids (0.05–0.2 mg ml⁻¹).
2. The ABC transporter complex has dissociated during the reconstitution procedure. This can be verified by analyzing the proteoliposomes by SDS-PAGE; for electrophoresis of membrane reconstituted protein, the SDS concentration in the sample buffer needs to be increased to 4% (wt/vol). Critical steps to prevent complex dissociation are mentioned throughout the protocol.
3. The ABC transporter complex has inserted unidirectionally, in either the inside-out or right-side-out orientation, which might not be compatible with the transport assay. The orientation of the complex can be scrambled by performing five freeze-thaw cycles.
4. The lipid composition of the liposomes does not meet the requirement of the ABC transporter complex. Use a less-defined mixture, for example, *E. coli* polar lipid extract, but preferably a lipid extract from the native organism.
5. The removal of the detergent was incomplete, resulting in permeable, leaky vesicles in which (substrate) gradients cannot be stably maintained. Although the total quantity of Bio-Beads suggested in the protocol presents a large overcapacity for binding of most, if not all, detergents, the capacity or rate of detergent binding of the Bio-Beads might be compromised. Prepare a fresh batch of Bio-Beads and/or increase the incubation time and size of the final Bio-Beads addition.
6. No or few ABC transporter complexes have been incorporated into the liposomes. Analysis of the proteoliposomes by SDS-PAGE might provide an indication whether this is the case, but this method does not discriminate between protein complexes peripherally associated with or inserted into the lipid membrane. Freeze-fracture electron microscopy is the method of choice to determine whether the proteins are fully bilayer-embedded. Parameters that should be systematically varied to improve the reconstitution efficiency are the protein-to-lipid ratio, the type of detergent used to titrate the liposomes, the stage of solubilization of the liposomes before addition of the protein, the rate of detergent removal during the reconstitution procedure (can be manipulated by varying the size of the Bio-Beads additions). Ultimately, detergent removal by dilution (fast), dialysis or gel-filtration should be considered^{1,2}.

Problem: If little or no solute translocation is observed in Step 43, consider the following option:

Possible reasons and solutions: The ABC transporter complex has a high basal ATPase activity, resulting in depletion of ATP in the lumen of the vesicles. This might be verified by performing an ATPase assay using the ATP-Lite-M system (Packard; see ref. 19). Depletion of ATP in the vesicles lumen might be prevented using the ATP-regenerating system and performing all steps starting from the inclusion of the ARS at 4 °C.

Problem: If a high radioactivity count is measured that does not vary in time at Steps 43, 46 or 49, consider the following options:

Reasons and solutions:

1. The radio-labeled substrate might bind strongly to the filter. This might be prevented by soaking the filters before use in high concentrations of the unlabeled substrate or replacing the cellulose nitrate filters by cellulose acetate filters.
2. The radio-labeled substrate might bind strongly to the membrane-associated SBP. When binding is tight and high levels of SBP are used, this may mask the uptake reaction. Radio-labeled substrate bound to the SBP can be chased by including a large excess (1–5 mM, final concentration) of unlabeled substrate in the ice-cold stop buffer. Because ABC transporters operate unidirectionally, accumulated substrate will not be chased.

Problem: If no substrate binding is observed at Step 59, consider the following option:

Possible reasons and solutions: The ammonium sulfate precipitated SBP may not sufficiently trap the substrate and dissociation may occur during the washing steps. The protein is not suited for this type of assay and the binding of substrates should be assayed using other methodologies, such as equilibrium dialysis or spectroscopic measurements^{4,43,44}.

Figure 4 | Glycine-betaine binding and transport by OpuA. The proteoliposomes were suspended in media as specified under ANTICIPATED RESULTS and the experiments were performed at 30 °C. (a) [^3H]-Glycine betaine binding: the decrease in bound radioactivity with increasing concentration of glycine betaine is shown for proteoliposomes with OpuA (open circles) and empty liposomes (closed circles). The processed binding data are presented in **Supplementary Figure 1a** online. (b) [^{14}C]-glycine-betaine uptake: the time course of glycine-betaine (radioactivity) accumulation is shown. The inset shows the initial uptake rate. Uptake in proteoliposomes with (open squares, open circles) and without (closed circles) ATP-regenerating system was assayed in 100 mM KPi , pH 7.0, without (open circles; isotonic) or with 250 mM KCl (open squares, closed circles; hyperosmotic). The processed uptake data are presented in **Supplementary Figure 1b** online.

ANTICIPATED RESULTS

We used the osmoregulatory ABC transporter OpuA from *Lactococcus lactis* to test the reconstitution procedure (Steps 1–19 and 28–30) and translocation (Steps 31–43 and 44–46) and ligand-binding (Steps 50–59) assays. OpuA proteoliposomes were formed by reconstituting purified OpuA in buffer A plus 0.05% (wt/vol) DDM at a protein-to-lipid 1:50 (wt/wt) into preformed liposomes composed of 50 mol% DOPG and 50 mol% DOPE (Steps 1–19). For [^{14}C]-Glycine betaine uptake studies (Steps 31–43), the ATP-regenerating system (ARS) was incorporated (Steps 28–30), and the proteoliposomes were suspended in 100 mM KPi , pH 7.0. For [^3H]-Glycine betaine binding studies (Steps 50–59), the proteoliposomes were prepared in 50 mM KPi , pH 7.0.

A typical [^3H]-Glycine betaine binding curve is shown in **Figure 4a** (open circles). In these experiments, a constant amount of [^3H]-Glycine betaine [4.88 pmol; 381 nCi (14.1 kBq) per 40 μl , yielding a total radioactivity count of $\sim 300,000$ c.p.m.] was used. The Glycine betaine concentration was varied by mixing the [^3H]-Glycine betaine with varying amounts of unlabeled Glycine betaine. Blank samples were taken by omitting the proteoliposomes from the incubation medium and using empty liposomes instead (**Fig. 4a**; closed circles). The processed data, subtraction of blank values and expression of bound Glycine betaine in pmol mg^{-1} of OpuA, are shown in **Supplementary Figure 1a** online.

Typical [^{14}C]-Glycine betaine uptake curves are shown in **Figure 4b**. Transport of Glycine betaine by OpuA is stimulated when the internal ionic strength reaches threshold levels, which can be elicited by an osmotic upshift²⁰. Uptake under iso-osmotic (open circles) and hyperosmotic conditions (plus 250 mM KCl; open squares) are shown (the inset shows the linearity of uptake in the first 90 s). Also depicted in the figure are data from blank samples, using the proteoliposomes without ARS inside and prepared for the ligand-binding studies (closed circles). In these experiments, [^{14}C]-Glycine betaine was added to a final concentration of 42.5 μM and activities of 100 nCi (3,70 kBq) per 40 μl sample, corresponding to a total radioactivity count of $\sim 200,000$ c.p.m.). The processed data, subtraction of blank values and expression of transported Glycine betaine in nmol mg^{-1} of OpuA, are shown in **Supplementary Figure 1b** online.

ABC transporters of the OTCN and PAO family most often have the SBP fused to the TMD^{14,18} and the substrate-binding domains are thus co-reconstituted in Steps 1–6. For OpuA, we observed that five cycles of flash-freezing in liquid N_2 and slow thawing at room temperature, followed by extrusion of the vesicles through 200-nm polycarbonate filters, yields proteoliposomes in which approximately half of the molecules have the RSO and the other half the ISO orientation²¹. If 10 mM of Mg-ATP is incorporated in the vesicle lumen, then uptake through RSO-inserted OpuA halts after ~ 10 min owing to depletion of ATP and build-up of ADP (strong competitive inhibitor of the ATPase of ABC transporters). If Mg-ATP is now added to the external medium, one observes efflux of pre-accumulated [^{14}C]-Glycine betaine as a result of activation of ISO-inserted OpuA (**Supplementary Fig. 2** online). The analysis of oppositely oriented OpuA molecules in one and the same vesicle preparation has allowed us to dissect for instance the sidedness of ionic regulation and lipid dependence²¹ and to determine the ATP/solute stoichiometry¹⁹, which would be difficult otherwise. In principle, this approach is also possible for ABC importers with soluble and lipid-anchored SBPs and ABC exporters but may require some fine-tuning of the reconstitution steps.

Note: Supplementary information is available via the HTML version of this article.

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1. Rigaud, J.L., Pitard, B. & Levy, D. Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins. *Biochim. Biophys. Acta* **1231**, 223–246 (1995).
2. Rigaud, J.L. & Lévy, D. Reconstitution of membrane proteins into liposomes. *Methods Enzymol.* **372**, 65–86 (2003).
3. Holloway, P.W. A simple procedure for removal of Triton X-100 from protein samples. *Anal. Biochem.* **53**, 304–308 (1973).
4. Poolman, B. *et al.* Functional analysis of detergent-solubilized and membrane-reconstituted ATP-binding cassette transporters. *Methods Enzymol.* **400**, 429–459 (2005).
5. Almog, S. *et al.* States of aggregation and phase transformations in mixtures of phosphatidylcholine and octyl glucoside. *Biochemistry* **29**, 4582–4592 (1990).

6. Knol, J. *et al.* Unidirectional reconstitution into detergent-stabilized liposomes of the purified lactose transport system of *Streptococcus thermophilus*. *J. Biol. Chem.* **271**, 15358–15366 (1996).
7. Knol, J., Sjollem, K. & Poolman, B. Detergent-mediated reconstitution of membrane proteins. *Biochemistry* **37**, 16410–16415 (1998).
8. Lévy, D., Gulik, A., Bluzat, A. & Rigaud, J.L. Reconstitution of the sarcoplasmic reticulum Ca(2+)-ATPase: mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. *Biochim. Biophys. Acta* **1107**, 283–298 (1992).
9. Fang, G. *et al.* Manipulation of activity and orientation of membrane-reconstituted di-tripeptide transport protein DtpT of *Lactococcus lactis*. *Mol. Membr. Biol.* **16**, 297–304 (1999).
10. Rigaud, J.L., Paternostre, M.T. & Bluzat, A. Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 2. Incorporation of the light-driven proton pump bacteriorhodopsin. *Biochemistry* **27**, 2677–2688 (1988).
11. Eytan, G.D. Use of liposomes for reconstitution of biological functions. *Biochim. Biophys. Acta* **694**, 185–202 (1982).
12. Higgins, C.F. Multiple molecular mechanisms for multidrug resistance transporters. *Nature* **446**, 749–757 (2007).
13. Davidson, A.L. & Chen, J. ATP-binding cassette transporters in bacteria. *Annu. Rev. Biochem.* **73**, 241–268 (2004).
14. Biemans-Oldehinkel, E., Doeven, M.K. & Poolman, B. ABC transporter architecture and regulatory roles of accessory domains. *FEBS Lett.* **580**, 1023–1035 (2006).
15. Neu, H.C. & Heppel, L.A. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J. Biol. Chem.* **240**, 3685–3692 (1965).
16. Sutcliffe, I.C. & Russell, R.R. Lipoproteins of gram-positive bacteria. *J. Bacteriol.* **177**, 1123–1128 (1995).
17. Albers, S.V. *et al.* Glucose transport in the extremely thermoacidophilic *Sulfolobus solfataricus* involves a high-affinity membrane-integrated binding protein. *J. Bacteriol.* **181**, 4285–4291 (1999).
18. van der Heide, T. & Poolman, B. ABC transporters: one, two or four extracytoplasmic substrate-binding sites? *EMBO Rep.* **3**, 938–943 (2002).
19. Patzlaff, J.S., van der Heide, T. & Poolman, B. The ATP/substrate stoichiometry of the ATP-binding cassette (ABC) transporter OpuA. *J. Biol. Chem.* **278**, 29546–29551 (2003).
20. Biemans-Oldehinkel, E., Mahmood, N.A. & Poolman, B. A sensor for intracellular ionic strength. *Proc. Natl. Acad. Sci. USA* **103**, 10624–10629 (2006).
21. Mahmood, N.A., Biemans-Oldehinkel, E., Patzlaff, J.S., Schuurman-Wolters, G.K. & Poolman, B. Ion specificity and ionic strength dependence of the osmoregulatory ABC transporter OpuA. *J. Biol. Chem.* **281**, 29830–29839 (2006).
22. Borths, E.L., Poolman, B., Hvorum, R.N., Locher, K.P. & Rees, D.C. *In vitro* functional characterization of BtuCD-F, the *Escherichia coli* ABC transporter for vitamin B12 uptake. *Biochemistry* **44**, 16301–16309 (2005).
23. Detmers, F.J. *et al.* Combinatorial peptide libraries reveal the ligand-binding mechanism of the oligopeptide receptor OppA of *Lactococcus lactis*. *Proc. Natl. Acad. Sci. USA* **97**, 12487–12492 (2000).
24. Doeven, M.K., Abele, R., Tampé, R. & Poolman, B. The binding specificity of OppA determines the selectivity of the oligopeptide ATP-binding cassette transporter. *J. Biol. Chem.* **279**, 32301–32307 (2004).
25. van der Heide, T. & Poolman, B. Osmoregulated ABC-transport system of *Lactococcus lactis* senses water stress via changes in the physical state of the membrane. *Proc. Natl. Acad. Sci. USA* **97**, 7102–7106 (2000).
26. Schuurman-Wolters, G.K. & Poolman, B. Substrate specificity and ionic regulation of GlnPQ from *Lactococcus lactis*. An ATP-binding cassette transporter with four extracytoplasmic substrate-binding domains. *J. Biol. Chem.* **280**, 23785–23790 (2005).
27. Margolles, A., Putman, M., van Veen, H.W. & Konings, W.N. The purified and functionally reconstituted multidrug transporter LmrA of *Lactococcus lactis* mediates the transbilayer movement of specific fluorescent phospholipids. *Biochemistry* **38**, 16298–16306 (1999).
28. Orelle, C., Dalmas, O., Gros, P., Di Pietro, A. & Jault, J. The conserved glutamate residue adjacent to the Walker-B motif is the catalytic base for ATP hydrolysis in the ATP-binding cassette transporter BmrA. *J. Biol. Chem.* **278**, 47002–47008 (2003).
29. Jung, H., Tebbe, S., Schmid, R. & Jung, K. Unidirectional reconstitution and characterization of purified Na⁺/proline transporter of *Escherichia coli*. *Biochemistry* **37**, 11083–11088 (1998).
30. Horn, C., Bremer, E. & Schmitt, L. Functional overexpression and *in vitro* re-association of OpuA, an osmotically regulated ABC-transport complex from *Bacillus subtilis*. *FEBS Lett.* **579**, 5765–5768 (2005).
31. Urbani, A. & Warne, T. A colorimetric determination for glycosidic and bile salt-based detergents: applications in membrane protein research. *Anal. Biochem.* **336**, 117–124 (2005).
32. Strop, P. & Brunger, A.T. Refractive index-based determination of detergent concentration and its application to the study of membrane proteins. *Protein. Sci.* **14**, 2207–2211 (2005).
33. daCosta, C.J. & Baenziger, J.E. A rapid method for assessing lipid:protein and detergent:protein ratios in membrane-protein crystallization. *Acta Crystallogr. D Biol. Crystallogr.* **59**, 77–83 (2003).
34. Møller, J.V. & le Maire, M. Detergent binding as a measure of hydrophobic surface area of integral membrane proteins. *J. Biol. Chem.* **268**, 18659–18672 (1993).
35. Pebay-Peyroula, E. *et al.* Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside. *Nature* **426**, 39–44 (2003).
36. Biemans-Oldehinkel, E. & Poolman, B. On the role of the two extracytoplasmic substrate-binding domains in the ABC transporter OpuA. *EMBO J.* **22**, 5983–5993 (2003).
37. van der Heide, T., Stuart, M.C. & Poolman, B. On the osmotic signal and osmosensing mechanism of an ABC transport system for glycine betaine. *EMBO J.* **20**, 7022–7032 (2001).
38. Newman, M.J. & Wilson, T.H. Solubilization and reconstitution of the lactose transport system from *Escherichia coli*. *J. Biol. Chem.* **255**, 10583–10586 (1980).
39. Rigaud, J., Levy, D., Mosser, G. & Lambert, O. Detergent removal by non-polar polystyrene beads. Applications to membrane protein reconstitution and two-dimensional crystallization. *Eur. Biophys. J.* **27**, 305–319 (1998).
40. Borths, E., Locher, K., Lee, A. & Rees, D. The structure of *Escherichia coli* BtuF and binding to its cognate ATP binding cassette transporter. *Proc. Natl. Acad. Sci. USA* **99**, 16642–16647 (2002).
41. Boch, J., Kempf, B. & Bremer, E. Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant glycine betaine from exogenously provided choline. *J. Bacteriol.* **176**, 5364–5371 (1994).
42. Pidgeon, C., Apostol, G. & Markovich, R. Fourier transform infrared assay of liposomal lipids. *Anal. Biochem.* **181**, 28–32 (1989).
43. Silhavy, T.J., Szmecman, S., Boos, W. & Schwartz, M. On the significance of the retention of ligand by protein. *Proc. Natl. Acad. Sci. USA* **72**, 2120–2124 (1975).
44. Miller, D.M. 3rd, Olson, J.S., Pflugrath, J.W. & Quiocho, F.A. Rates of ligand binding to periplasmic proteins involved in bacterial transport and chemotaxis. *J. Biol. Chem.* **258**, 13665–13672 (1983).
45. Kragh-Hansen, U., le Maire, M. & Møller, J.V. The mechanism of detergent solubilization of liposomes and protein-containing membranes. *Biophys. J.* **75**, 2932–2946 (1998).
46. Doeven, M.K. *et al.* Distribution, lateral mobility and function of membrane proteins incorporated into giant unilamellar vesicles. *Biophys. J.* **88**, 1134–1142 (2005).
47. Angelova, M.I., Soléau, S., Méléard, P., Faucon, J.F. & Bothorel, P. Preparation of giant vesicles by external AC electric fields. Kinetics and applications. *Prog. Colloid Polym. Sci.* **89**, 127–131 (1992).
48. Mayer, L.D., Hope, M.J. & Cullis, P.R. Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta* **858**, 161–168 (1986).
49. MacDonald, R.C. *et al.* Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta* **1061**, 297–303 (1991).
50. White, G.F., Racher, K.I., Lipski, A., Hallett, F.R. & Wood, J.M. Physical properties of liposomes and proteoliposomes prepared from *Escherichia coli* polar lipids. *Biochim. Biophys. Acta* **1468**, 175–186 (2000).
51. Oku, N., Kendall, D.A. & MacDonald, R.C. A simple procedure for the determination of the trapped volume of liposomes. *Biochim. Biophys. Acta* **691**, 332–340 (1982).