Morrissey Lab Protocol for Reconstituting Purified Tissue Factor into Phospholipid Vesicles (Tissue Factor Relipidation) Using Octylglucoside Dialysis

Protocol from James H. Morrissey , Dept. of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

Background

Tissue factor (TF; sometimes called thromboplastin) is the protein responsible for triggering blood clotting. It is an integral membrane protein which must be incorporated into phospholipid vesicles for optimal activity, and furthermore, the vesicles must include some phospholipids whose head groups have a net negative charge. The result -- TF incorporated into phospholipid vesicles -- functions by binding and allosterically activating the plasma serine protease, coagulation factor VIIa. The TF:VIIa complex triggers blood clotting by proteolytically activating coagulation factors IX and X, converting them into active serine proteases.

The technique described here for incorporating TF into phospholipid vesicles—sometimes termed "relipidation"—uses the dialyzable, non-ionic detergent,*n*-octyl-beta-D-glucopyranoside (octylglucoside). In this adaptation of the general method of Mimms et al. [1] , phospholipids and TF are both dissolved in octylglucoside, forming mixed micelles. Since octylglucoside has a high critical micelle concentration (CMC = 20 to 25 mM), it can readily be removed from solutions by dialysis. As octylglucoside dialyses out, the phospholipids organize into unilamellar vesicles. TF becomes embedded in these vesicles by virtue of its single membrane-spanning domain, located near the C-terminus of the protein. Typically, about 50 to 80% of the TF molecules face outward in these vesicles. (The remaining TF molecules face inward and are therefore unable to interact with factor VII/VIIa.) For a published example of the the use of this method to relipidate tissue factor (TF), and for considerations of how to quantify the amount of TF facing outward in the vesicles, see Neuenschwander et al. [2] .

Since TF is an integral membrane protein, it is often solubilized in buffers containing a non-ionic detergent like Triton X-100 . TF dissolved in Triton can be used in this relipidation protocol with good results, but be aware that the final product will contain small amounts of Triton X-100. This is because Triton X-100 has such a low critical micelle concentration that it cannot readily be removed by dialysis. To obtain relipidated TF that is not contaminated with detergent, it is preferable to use TF stock solutions that contain a readily dialyzable detergent like CHAPS or octylglucoside, rather than Triton.

Phospholipids in aqueous solution are subject to oxidation. For this reason, once TF has been relipidated it should typically be used within about 2 or 3 weeks. (For some applications, older TF preparations can still be used with good results. Be aware,

though, that such preparations may contain oxidized phospholipids.) If you freeze and thaw relipidated TF, you run the risk of causing vesicle fusion and the formation of multilamellar vesicles. (Again, for some applications, this might not be problem.) Finally, do not use phospholipid stock solutions that are old (for the same reason—risk of oxidation). The Avanti Polar Lipids catalog gives some guidelines for how long various phospholipid stock solutions can be stored at -20 degrees C.

Head groups of phospholipid molecules (especially the negatively charged phospholipid, phosphatidylserine) interact with calcium ions. In some cases, addition of calcium ions to phosphatidylserine-containing vesicles promotes vesicle aggregation and fusion. This can be minimized by using vesicles containing no more than 20 mol% phosphatidylserine. For most applications, TF activity is maximal when vesicles contain 20 mol% phosphatidylserine or less, so there is normally no reason to exceed this level. Note that soluble tissue factor (sTF; consisting of amino acids 1-219) cannot be incorporated into phospholipids; you must use TF in which the membrane spanning domain is intact.

You can make "blank" vesicles (containing phospholipid but no TF) simply by leaving out the TF in the above protocol. This results in a preparation of large unilamellar vesicles (LUV). Alternative procedures for making blank phospholipid vesicles include sonication and membrane extrusion .

Method

Preparation of phospholipid solution in octylglucoside

1. For each sample, dispense 2.6 micromole total phospholipids in a glass test tube, using the desired molar ratio of phospholipids (e.g., 80 mol% PC, 20 mol% PS). If you plan to use the vesicles in high sensitivity spectroscopy work, avoid contact of the chloroform solutions with plastic pipet tips (to avoid extracting plasticizers and other components of the plastic). This is less of a problem with activity or clotting assays, as the levels of these contaminants are too low to cause any problems.

Examples*—amounts of phospholipids to use in making PCPS or PCPSPE vesicles:*

For PC:PS vesicles (80:20 molar ratio)		
63 µ**L PC** (at 25 mg/ml) (or **158** µ**L** at 10 mg/ml)	= 1.58 mg	= 2.08 µmole
42 µ**L PS** (at 10 mg/ml)	= 0.42 mg	= 0.52 µmole

For PC:PE:PS vesicles (40:40:20 molar ratio)		
32 µ**L PC** (at 25 mg/ml) (or **79** µ**L** at 10 mg/ml)	= 0.79 mg	= 1.04 µmole
80 µ**L PE** (at 10 mg/ml)	= 0.80 mg	= 1.04 µmole
42 µ**L PS** (at 10 mg/ml)	= 0.42 mg	= 0.52 µmole

---

2. Dry the phospholipid mixture down under a gentle stream of argon or nitrogen. If possible, set the tube at an angle so the phospholipids form a thin film on the side of the tube. Make sure that the gas stream doesn't blow any of the solution out of the tube!

3. When the tube appears dry, speed-vac for an additional 60 minutes under high vacuum. This ensures that residual chloroform is removed.

4. To the tube of dried-down phospholipids, add 400 µL of freshly prepared OG/HBS solution (room temperature). Vortex to completely dissolve the dried-down phospholipids. You may need to use a pipet to dislodge any pellet at the bottom of the tube. Once dissolved, keep at room temperature until next step.

Relipidation procedure

5. To the tube containing 400 µL of phospholipid/octylglucoside solution, add the desired amount of membrane TF (preferably, dissolved in CHAPS or octylglucoside, not Triton X-100) and enough HBSA to make the final volume 1 ml. A typical molar ratio of phospholipid to TF is 8700:1, although we have used ratios as high as 50,000:1 and as low as 3,000:1 [2] . The final volume will be 1 ml (see example below). Do this step at room temperature.

Relipidation example

In this example we will relipidate dcTF (recombinant, membrane-anchored human TF lacking most of the cytoplasmic domain) using a PL:TF ratio of 8700:1. Therefore, we will need a final concentration of 0.3 µmolar dcTF. The Mr of dcTF is 28,989, so we will need to add 8.7 micrograms dcTF (0.3 nmole) per ml of phospholipid/OG solution. To use a different ratio of phospholipid to TF, change the amount of TF added at this step; do not change the amount of phospholipid.

Mix together (to give 1 ml total):	
400	µL phospholipid/octylglucoside solution
x	µL dcTF stock (to give 0.3 nmole TF)
600 - *x*	µL HBSA
Final product: 300 nM dcTF + 2.6 mM phospholipid (8700:1 molar ratio of PL:TF)	

---

6. Mix well and incubate the sample for 30 min at room temperature.

7. Dialyze the sample at room temperature against three changes of HBS (24 hr each, for a total of 72 hr). Store the final product at 4 degrees C.

Final product:

The final product is about 1 ml of relipidated TF containing approximately 2.6 mM phospholipid. Because the recovery from dialysis may not be 100%, these amounts are only approximate. If you need precise concentrations of available TF and total phospholipid, perform an analysis of exposed TF (titrate with factor VIIa by measuring the TF-induced increase in VIIa amidolytic activity), and an analysis of phospholipid content (measure total phosphorus content). See Neuenschwander et al. [2] . for more details.

Buffers and stock solutions

Reagents

Octylglucoside (*n*-octyl-beta-D-glucopyranoside) is available from Calbiochem and other suppliers. It is expensive, so don't make up more of the octylglucoside solution (below) than you will use at one time.

Phospholipid Stock Solutions*				
	Phospholipid name	*concentration*	*MW*	*cat. #*
PC	L-alpha-Phosphatidylcholine, egg	10 or 25 mg/ml	761	840051
PS	L-alpha-Phosphatidylserine, bovine brain-Na salt	10 mg/ml	810	840032
PE	L-alpha-Phosphatidylethanolamine, bovine liver	10 mg/ml	768	840026

*We buy our phospholipids from Avanti Polar Lipids , dissolved in chloroform. The stock solutions should be stored at -20 degrees C under argon. Each time you remove an aliquot of phospholipid from the stock bottle, be sure to overlay the solution inside with argon gas before capping the vial and returning it to the freezer. Do not store these stock phospholipid solutions more than 3 months (6 months for PC).

Buffers

HBS *(store at room temperature)*
100 mM NaCl 20 mM Hepes/NaOH buffer, pH 7.5 0.02% (w/v) sodium azide

HBSA *(store at 4 degrees C)*
0.1% (w/v) bovine serum albumin in HBS

OG/HBS solution *(make fresh)*
100 mM *n*-octyl-beta-D-glucopyranoside in HBS (= 29.2 mg octylglucoside per ml HBS)

References

(NOTE: If you want to cite a reference for this protocol, use publication 2, below)

1. Mimms LT, Zampighi G, Nozaki Y, Tanford C, and Reynolds JA. Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside.*Biochemistry* 20:833-840, 1981. [Abstract]

2. Neuenschwander PF, Fiore MM, and Morrissey JH. Factor VII autoactivation proceeds via interaction of distinct protease-cofactor and zymogen-cofactor complexes: Implications of a two-dimensional enzyme kinetic mechanism. *J Biol Chem* 268:21489-21492, 1993. [Abstract] [Reprint]
