OptiPrep™ Application Sheet S27

Fractionation of plasma membrane and plasma membrane domains using cationic colloidal silica

- OptiPrep™ is a 60% (w/v) solution of iodixanol in water, density = 1.32 g/ml
- To access other Application Sheets referred to in the text return to the Subcellular Membranes Index; key Ctrl "F" and type the S-Number in the Find Box.
- Important technical notes, information regarding alternative methodologies and membrane analysis are contained in the "Technical Notes and Review" section (Section 4)

1. Background

1a. Colloidal silica strategy

There have been many attempts to stabilize the plasma membrane (PM) of cultured cells against fragmentation during homogenization; thus retain it as a large sheet and so facilitate its separation from all the other subcellular membranes. In 1968 the use of chemical "hardening" agents such as Zn^{2+} and fluoroscein mercuric acetate [1] was introduced. While undoubtedly effective, the toxic nature of these agents meant that functional studies were compromised and the method never gained a wide popularity. Then in 1977, the adsorption of the PM on to a positively charged polylysine (or polyethylenimine) coated bead, followed by shearing away of the rest of the cell, was developed by Jacobson and Branton [2]. The remainder of (unbound) PM was however lost in the shearing process.

More recently this problem of selective isolation of only part of the PM was avoided by coating the cells (in suspension) sequentially with cationic silica (approx 500Å diameter) and an anionic polymer (polyacrylic acid), thus forming a dense coat (pellicle) around the cell [3]. The polyacrylic acid cross-links and stabilizes the silica colloid and also blocks the residual positive charge on the surface of the colloidal particles. Repeating the binding of the cationic silica and anionic polymer can increase the thickness and the density of the pellicle [3]. The procedure has also been adapted to the isolation of basolateral and apical domains of the PM from polarized cells by coating the exposed (apical) surface of a bovine aortic endothelial cell monolayer, then lysing the cells by liquid shear in a jet of hypotonic solution, to leave the basolateral domain remaining on the substratum (e.g. ref 4).

1b. Separation of colloidal silica-bound membranes using Nycodenz®

The colloidal silica membranes are usually separated from the native membranes by a high-density solution of Nycodenz® (usually 70%, w/v) upon which the total lysate (adjusted to approx 50% Nycodenz®) is layered [5-8]. The Si-bound membranes are then allowed to pellet at approx 20,000 *g* for 20 min [5-7] or at 60,000 *g* for 40 min [8]. Sometimes the lysate is not density-adjusted [9,10]. Occasionally the density-adjusted lysate is layered over a continuous gradient of 55-70% (w/v) Nycodenz® [11-13] or discontinuous gradient of 55%, 60%, 65% and 70% (w/v) Nycodenz® [14]. The relative efficacy of a continuous or discontinuous gradient is not clear.

1c. Separation of colloidal silica-bound membranes using Optiprep™

The rapidly-sedimenting PM-pellicle complexes can be separated from other organelles either on a simple density barrier of OptiPrep™ (60%, w/v iodixanol) [15-17] or on a discontinuous iodixanol gradient [18]. The lower concentrations of iodixanol (compared to Nycodenz®) may reflect the lower density of native organelles in this medium and the simplicity of use of OptiPrep™ certainly makes this the medium of choice.

- The following protocols describe the techniques as applied to monolayers of cultured cells.

- Section 2 describes the binding of colloidal silica to the plasma membrane and homogenization.
- In Section 2b monolayer cells are detached from the culture dish and coated with silica to provide a total PM fraction.
- In Section 2c the cell monolayer is coated with silica before detachment to provide the apical and basolateral domains of the PM.
- Section 3 describes the gradient purification using either OptiPrep™ or Nycodenz®.

2. Treatment of cell monolayers with colloidal silica (adapted from refs 3 and 4)

2a. Solutions required (see Section 4.1)

A. Hank's Balanced Salt Solution (HBSS)

B. Coating buffer: 135 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 mM MES-NaOH, pH 5.5

C. Cationic silica colloid diluted to 1% (w/v) with Solution B

D. Polyacrylic acid: 1 mg/ml in Solution B, adjusted to pH 5.0 with NaOH

E. Lysis buffer: (LB): 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2.5 mM imidazole-HCl, pH 7.0

Keep the following stock solutions at 4°C:
1 M MES; purchase as a ready-made solution
1 M NaCl: 5.84 g per 100 ml water
100 mM $MgCl_2 \bullet 6H_2O$: 2.03 g per 100 ml water
100 mM $CaCl_2 \bullet 2H_2O$: 1.47 g per 100 ml water
250 mM imidazole: 1.7 g per 100 ml water

Solution B: To 50 ml water add 2 ml of MES, 13.5 ml of NaCl, 1 ml of $MgCl_2$ and 0.5 ml of $CaCl_2$ stock solutions; adjust to pH 5.5 with 1 M NaOH and make up to 100 ml.

Solution E: To 50 ml water, add 1 ml of imidazole, 1 ml of $MgCl_2$ and 0.5 ml of $CaCl_2$ stock solutions; adjust to pH 7.0 with 1 M HCl and make up to 100 ml.

2b. Coating and homogenization of detached cells

Carry out all operations, except Step 1, at 0-4°C.

1. Detach the cells from the substratum using the usual regime of EDTA and/or trypsin or collagenase and wash the cells twice in Solution A.

2. Wash the cell three times in Solution B.

3. Suspend the cells (e.g. 50 µl packed cell volume) in 0.2 ml of Solution B (see Section 4.2).

4. Rapidly mix 1.25 ml of the diluted cationic silica colloid (Solution C) is with the cell suspension (see Section 4.2).

5. Immediately after mixing dilute with approx. 8 vol. of Solution B and centrifuge the suspension at 700 *g* for 5 min to pellet the cells.

6. Aspirate and discard the colloidal silica supernatant.

7. Suspend the cells in the original volume of Solution B and mix with 5 vol. of Solution D (see Section 4.3).

8. Immediately after mixing dilute with approx. 8 vol. of Solution B and centrifuge the suspension at 700 *g* for 5 min. to pellet the cells.

9. Suspend the cell pellet in Solution E and leave for 30 min.

10. Homogenize the cells using a Dounce homogenizer (see Section 4.4).

11. Centrifuge the homogenate at 900 *g* for 10 min.

12. Resuspend the pellet in 2 ml of E, by sonication for 1 sec at a low setting (see Section 4.5).

2c. Coating and lysis of cell monolayers (see Section 4.6)
Carry out all operations, except Step 1 at 0-4°C.

1. Wash the monolayer twice in Solution A.

2. Wash the monolayer twice in Solution B.

3. Coat the cells with Solution C and then wash the monolayer with Solution B.

4. Coat the cells with Solution D and wash the monolayer twice with Solution B.

5. Wash the monolayer very quickly with a small volume of Solution E and aspirate to make sure that all residual amounts of Solution B are removed.

6. Pour 2-3 ml of Solution E on to the monolayer and allow to stand on ice for approx 30 min to lyse the cells and monitor lysis using light microscopy.

7. Assist lysis if necessary by repeatedly expelling Solution E from a syringe attached to a short narrow metal cannula (approx 18G), but be careful not to cause detachment of the cells.

8. When approx 90% of the cells have been lysed aspirate and retain the suspension, which contains the apical PM domain plus organelles released from the lysed cells.

9. Carefully wash the residual material (basolateral PM) with Solution E and add the washes to the original aspirate. Residual material may be solubilized for analysis by SDS-PAGE and Western blotting.

10. Centrifuge the aspirate (plus washes) at 900 *g* for 10 min.

11. Resuspend the pellet in 2 ml of E, by sonication for 1 sec at a low setting (see Section 4.5).

3. Gradient purification of the total PM or apical PM domain
3a. Ultracentrifuge rotor requirements
Any swinging-bucket rotor capable of approx 100,000 *g* with a tube volume of approx. 5 ml (e.g. Beckman SW55Ti)

3b. Using OptiPrep™
Separate the total PM or the apical PM domain from the sonicate by layering upon 3 ml of OptiPrep™ **OR** 1 ml each of 5%, 43% (w/v) iodixanol and undiluted OptiPrep™ in tubes for the chosen rotor. Prepare the two lower density solutions by diluting OptiPrep™ with Solution E (see Section 2a) 1:11 and 43:17 v/v, respectively. Centrifuge at 30,000 *g* for 30 min at 4°C (see Section 4.7) and resuspend the pellets of PM or apical PM in a suitable solution for analysis.

3c. Using Nycodenz®
To make a 100% (w/v) Nycodenz® stock solution: place 55 ml of Solution E (see Section 2a) in a 150 ml beaker on a heated magnetic stirrer set at approx. 50°C and add 100 g of Nycodenz® in small amounts until dissolved. Allow the solution to cool to room temperature and if necessary make up to 100 ml with Solution E. Dilute this stock solution with Solution E to make up any lower density solutions. It is easier to make up a stock solution of a lower Nycodenz® concentration of say 70% or 75% (w/v), but larger volumes will be required for the adjustment of the sample to 50% (w/v). See Section 1b for gradient and centrifugation conditions and see Section 4.7. For more information on the preparation of both continuous and discontinuous gradients see **Application Sheet S03**.

4. Technical Notes and Review

4.1 Coating and Homogenization Solutions

The solutions used for coating the cells with the pellicle of cationic silica and polyacrylic acid are all buffered with MES and are marginally acidic (pH 5-5.5) but otherwise quite variable in composition. The same solution is normally used for initially suspending the cells and the vehicle for the two coating reagents. Chaney and Jacobson [3] used 140 mM sorbitol, 20 mM MES, pH 5.0 for *Dictyostelium*, while for mouse embryo fibroblasts Cain et al [15] used a hyperosmotic buffer containing 280 mM sorbitol, 150 mM NaCl, 20 mM MES. The buffer used for CHO cells [16] and for bovine aortic endothelial cells [4] however was more similar to a routine buffered saline solution (supplemented with low levels of divalent cations) and approx. isoosmotic and is the one that is described in this OptiPrep™ Application Sheet. What effect the osmolality and composition of the coating solution has on the coating process is not clear.

It is necessary to have the silica colloid in excess and in the original method published by Chaney and Jacobson [3], the concentration of cationic silica colloid used in the coating process was higher (3-4% w/v, final concentration) than the approx 1% (w/v) more widely used in later studies. In the earlier study however much larger numbers of non-mammalian (*Dictyostelium*) cells were used (see Section 4.3) Cationic silica colloid can either be prepared according to the method of Chaney and Jacobson [3] or purchased commercially from Sigma-Aldrich or EKA Chemicals.

Lower concentrations of polyacrylic acid (approx 0.2 mg/ml) have also been used [15]; and while this is normally in the same coating solution as is the silica colloid, for *Dictyostelium*, the polyacrylic acid was dissolved in 70 mM NaCl, 20 mM MES-NaOH, pH 6.5 [3].

The lysis buffer may be any suitable buffer that will permit efficient disruption of the silica pellicle coated cells. Although **Application Sheet S06** describes a variety of buffers that are routinely used for the homogenization of cultured cells, they may be unsuitable for coated cells. Protease inhibitors may be included in Solution E at the operator's discretion.

4.2 Preparation of cells for coating

For larger numbers of cells, Chaney and Jacobson [3] used a different approach. *Dictyostelium* (2 ml packed cell volume) in 140 mM sorbitol, 20 mM MES, pH 5.0 (4 ml final volume) was added slowly to 4 ml of the cationic silica colloid (diluted to 6-8%, w/v with buffered sorbitol) while vortexing very gently. It may be necessary to optimize the coating conditions for different cell types and different numbers of cells.

4.3 Reaction with polyacrylic acid

Chaney and Jacobson [3] used the same method of slow addition of the cell suspension to polyacrylic acid on a vortex mixer as described for the silica colloid in Section 4.2.

4.4 Homogenization of coated cells

The homogenization protocol should be tailored to the cell (or tissue) type. The coat may render the cells less susceptible to disruption by routine liquid shear techniques, although the use of hypoosmotic lysis buffer appears to obviate any such problems and Cain et al. [15] were then subsequently able to homogenize mouse embryo fibroblasts in a Dounce homogenizer. Other standard procedures such as 12-20 passages through a syringe needle (Gauge Number (G) varies from 21 to 25), sometimes preceded by Dounce homogenization, or the ball-bearing homogenizer ("cell cracker") may be applicable, but which is the most effective for coated cells can only be determined experimentally. Pellicle-coated *Dictyostelium* was most effectively homogenized by nitrogen cavitation [3].

Ideally the procedure should be as gentle and reproducible as possible, the aim being to cause at least 95% cell disruption without damage to the major organelles, particularly the nuclei and lysosomes. The type and severity of the homogenization process will have consequences for the integrity of the organelles and the size of the vesicles produced from tubular structures in the cytoplasm. Some hints on homogenization of "native" cells are given in **Application Sheet S06**.

4.5 Resuspension of coated PM fractions

Using a Branson Sonifier 185, fitted with a microprobe, setting 2 was used by Stolz and Jacobsen [4]. It is important to that any coated material is applied to gradients as an homogeneous suspension. Sonication is the recommended procedure but since the coated PM pellet will also contain nuclei, this needs to be carried out as gently as possible otherwise any DNA released from the nuclei may cause serious aggregation.

4.6 Alternative cell handling

Note that the cells may also be grown on micro-carrier beads [4].

4.7 Gradient centrifugation of coated PM fractions

For the isolation of the coated fraction it is not clear if continuous gradients [11-13] or discontinuous gradients [e.g. refs 14 and 16] have any practical advantages over the simple density barrier format [5-8,15]. *g*-Forces range from 20,000-60,000*g* and centrifugation times from 20-45 min.

5. References

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