

# Chapter 16

## Use of Colloidal Silica-Beads for the Isolation of Cell-Surface Proteins for Mass Spectrometry-Based Proteomics

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### Abstract

Chaney and Jacobson first introduced the colloidal silica-bead protocol for the coating of cellular plasma membranes in the early 1980s. Since then, this method has been successfully incorporated into a wide range of *in vitro* and *in vivo* applications for the isolation of cell-surface proteins. The principle is simple – cationic colloidal silica microbeads are introduced to a suspension or monolayer of cells in culture. Electrostatic interactions between the beads and the negatively charged plasma membrane, followed by cross-linking to the membrane with an anionic polymer, ensure attachment and maintain the native protein conformation. Cells are subsequently ruptured, and segregation of the resulting plasma membrane sheets from the remaining cell constituents is achieved by ultracentrifugation through density gradients. The resulting membrane-bead pellet is treated with various detergents or chaotropic agents (i.e., urea) to elute bound proteins. If proteomic profiling by mass spectrometry is desired, proteins are denatured, carbamidomethylated, and digested into peptides prior to chromatography.

**Key words:** Colloidal silica, Plasma membrane isolation, Cell-surface proteins, Membrane proteomics

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### 1. Introduction

Cell-surface receptors represent an important class of membrane and membrane-associated proteins. Analysis of the membrane proteome is hampered by the heterogeneity and amphiphilic nature of these proteins. The low abundance of this class of proteins – only 2% of all cellular proteins belong to the plasma membrane (1) – further impedes their detection. Although immense scientific interest has propelled the invention and refinement of various methods tackling the aforementioned obstacles, coverage of the membrane proteome remains far from being exhaustive.

Proteomic principles for the isolation of cell-surface proteins encompass centrifugation (2–4) and extraction techniques (5, 6), affinity-based approaches (e.g., lectin- and antibody-based protocols) (7–11), as well as methods exploiting the chemical characteristics of the analytes. The latter have proven to be valuable tools for the mass spectrometry (MS)-based description of cell-surface proteomes and mainly comprise protocols for the chemical capture of glycoproteins, biotinylation, and silica-bead coating (12).

The chemical capture of both glycoproteins and biotinylation relies on the chemical labeling of specific residues in targeted proteins, whereas silica-bead coating exploits the electrostatic forces between the target and reagent. With glycosylation being one of the most abundant posttranslational modifications, a comprehensive profile of cell-surface proteins can be established by the chemical capture of *N*- or both *N*- and *O*-glycosylated proteins using hydrazide chemistry (13, 14). Captured proteins can be subsequently enriched and analyzed. Similarly, selective covalent linking of biotinylation reagents to plasma membrane protein moieties, followed by affinity purification and MS detection, has proven to be a valuable method for cell-surface protein isolation, and biotinylation reagents can be tailored to meet the needs of different experimental conditions (15). *In vivo* applicability of biotinylation has been demonstrated, though extravasation of the reagent into surrounding tissues remains a caveat of this technique (16). The introduction of a chemical modification, which persists on the peptide and will thus be detected in downstream MS analysis, also adds confidence in the identification of bona fide cell-surface proteins. The high specificity conveyed by targeting selected residues (i.e., glycosylation site, residues targeted by the biotinylation reagent used), though, comes at the expense of tagging only surface proteins that contain these moieties. Important *in vitro* (3, 14, 17, 18) and *in vivo* studies (16, 19, 20) have applied all three protocols to the study of diverse scenarios (e.g., cancer, signaling pathways, and stem cell markers), thus validating their feasibility and contributing to our state of knowledge. A continued role of these approaches, possibly implementing improvements to increase efficiency, in future cell membrane research is anticipated.

In this chapter, we focus on the isolation of cell-surface proteins using silica-bead coating. This method, successfully applied to the isolation of membrane proteins in their physiological state for nearly 30 years, is based upon the binding of the negatively charged membrane to a positively charged solid support. It thus represents a further development of earlier approaches exploiting this physical principle, like binding to polylysine or polyethylene-coated beads (21).

In brief, the method relies on electrostatic attachment of the negatively charged cell-surface to cationic silica microbeads,

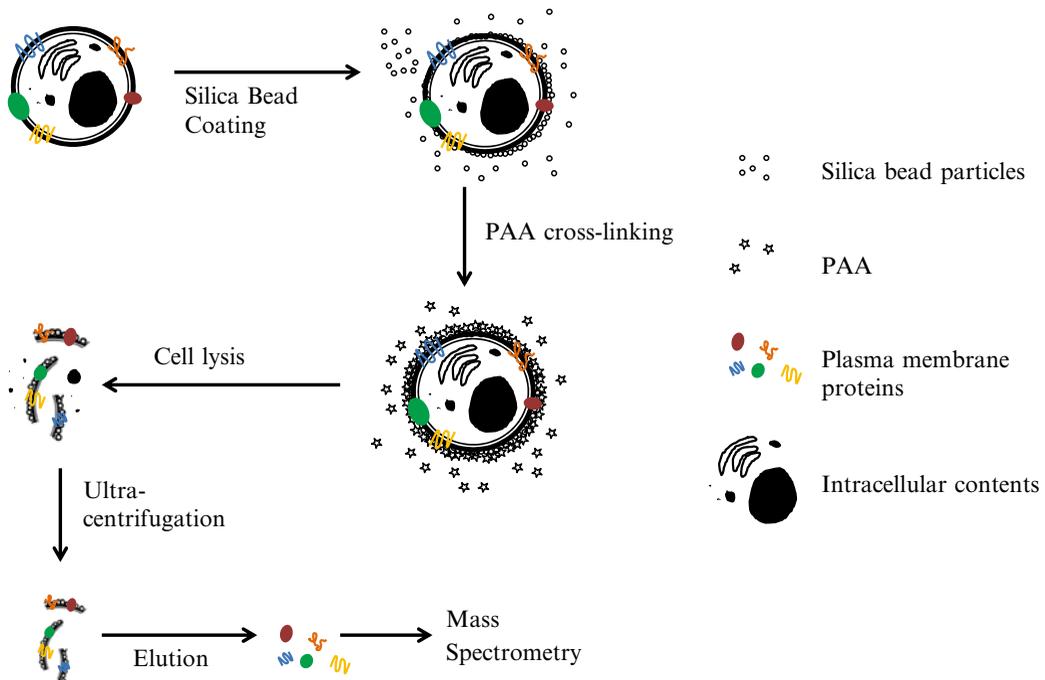


Fig. 1. Silica-beads are attached to the positively charged cell-surface. Subsequently, attached beads are cross-linked and the remaining free positive charges are blocked using polyacrylic acid (PAA). Cell lysis and ultracentrifugation yield purified, silica-bead-bound membrane proteins. These are eluted from the beads and can subsequently be prepared for mass spectrometric analysis.

followed by cross-linking of the beads by an anionic polymer (see Fig. 1). After cell rupture, the procedure yields large open sheets of high-density plasma membranes, which can be easily separated from cell debris and lysate by centrifugation. Furthermore, the rapid coating of surface proteins and subsequent cross-linking guarantee the preservation of native protein orientation in the lipid bilayer, prevent the formation of membrane vesicles upon cell lysis by stabilizing an open sheet structure, and shield the targeted proteins from chemical or enzymatic manipulation. Co-isolation of intracellular material is kept at a minimum by immediate blockage of silica-beads through cross-linking. Thus, in their first description of the technique, Chaney and Jacobson report a 15- to 17-fold enrichment of probed plasma membrane markers (21). Furthermore, the method is capable of distinction between different plasma membrane domains (e.g., the apical and basolateral sides of the plasma membrane) (22–24).

An early study by Jacobson's group reports the successful application of the method to selectively describe the topology of three distinct domains of HeLa cell plasma membranes (22). Similarly, the apical and basolateral plasma membranes of bovine aortic endothelial cells were characterized (24). An adapted version of the method allowed Sambuy and Rodriguez-Boulan to

investigate the apical membrane of canine endothelial kidney cells closely on the microscopic level to further our understanding of epithelial cell-surface polarity (25). Stolz and coworkers used the silica-bead procedure to monitor changes in both extracellular matrix components and epithelial growth factor receptor occurring on the surface of sinusoidal endothelial liver cells in response to partial hepatectomy in rats (26).

The more recent coupling of the silica-bead method to powerful MS platforms has led to an enhanced detection of proteins in isolated fractions, thus making the approach an attractive technique in proteomics today. For instance, Rahbar and Fenselau integrated an adapted protocol successfully into their proteomics work-flow to characterize the plasma membrane of suspension and adherent cell cultures. In their study with human multiple myeloma (RPMI 8226) and human breast cancer cells (MXR MCF-7), a yield of 50% plasma membrane-annotated proteins was reported (23). They also exploited a similar strategy to quantify changes in cell-surface proteins between drug-susceptible and drug-resistant human breast cancer cell lines (MCF-7 and MXR MCF-7 cells) (27). Recently, Hör and colleagues combined the technique with quantitative proteomics to identify novel plasma membrane protein substrates for the ubiquitin ligase MARCH9 (28).

Several studies have reported the feasibility of silica-bead coating *in vivo*, thus enabling the capture of cell-surface proteins in their native microenvironment, which is grossly modified under tissue culture conditions (20). The group of Schnitzer used a modified protocol to isolate endothelial cell-surface membrane proteins *in vivo* from different rat tissues by perfusing the vasculature of animals with a silica-bead solution (20, 29). Not only did their work facilitate proteomic mapping of the targeted cells, but it also revealed putative receptor targets that are accessible to biological agents from the vasculature, which could in turn have great implications, e.g., for antibody-based cancer therapy. Li *et al.* recently adapted this strategy for the isolation of plasma membrane proteins from freshly isolated mouse hepatocytes and from rat liver sinusoidal endothelial cells (30, 31).

Arjunan and colleagues have reported a conflicting observation regarding the silica-bead coating method for the isolation of cardiac microvascular surface proteins. Although the authors could convincingly demonstrate the adherence of the silica-beads to the luminal surface of the cardiac microvasculature, only a small number of proteins/plasma membrane proteins were identified. The authors argue that the rigid structure of the cardiac muscle tissue results in the loss of silica-beads during tissue homogenization (19). Although proteins annotated to other sub-cellular structures are often identified in any organellar proteomics study, and reasons for this have been intensely debated in the field for decades (32), the protocol has proven successful in our hands (33), even for the isolation of cardiac plasma membrane proteins

(unpublished data). Interestingly, the silica-bead approach promises to be especially valuable for the characterization of endothelial cells in tissue types with more penetrable vascular systems (e.g., fenestrated endothelium), which in turn hampers alternative approaches, like biotinylation (31).

With the advent of high-throughput mass spectrometry and sophisticated bioinformatics platforms, the field of cell-surface proteomics has rapidly evolved, allowing for comprehensive coverage of cell-surface proteomes, cell signaling pathway analyses, as well as the discovery of therapeutic targets. The silica-bead coating method of cell-surface protein isolation, in particular, has held a marked position in membrane proteomics and, in many cases, proven to be superior to other cell-surface isolation techniques. It is expected that the technique will continue to facilitate the expansion of our understanding of this important class of proteins.

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## 2. Materials

### 2.1. Reagents

1. HPLC-grade solvents (water, methanol, acetonitrile, formic acid, trifluoroacetic acid (TFA), acetone, HCl).
2. MES (2-(N-morpholino)ethanesulfonic acid), NaCl, sucrose, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), urea, Triton<sup>®</sup> X-100, CaCl<sub>2</sub>, KCl, NH<sub>4</sub>HCO<sub>3</sub>, and Na<sub>2</sub>CO<sub>3</sub> are of biotechnology grade.
3. Colloidal silica-beads (LUDOX<sup>®</sup> CL colloidal silica, suspension in water), polyacrylic acid (PAA), Nycodenz<sup>®</sup> (Histodenz<sup>™</sup>), and iodoacetamide (IAA) (Sigma-Aldrich Co).
4. Protease inhibitor (Complete Mini, EDTA-free) (Roche Applied Science).
5. Dithiothreitol (DTT).
6. PPS Silent<sup>®</sup> Surfactant (Protein Discovery, Inc).
7. MacroSpin<sup>™</sup> Columns (The Nest Group, Inc).
8. Proteomics-grade trypsin.

### 2.2. Silica-Bead Coating of Plasma Membranes and Cell Rupture

1. *MES-buffered saline (MBS)*: 25 mM MES, pH 6.5, and 150 mM NaCl.
2. *Colloidal silica-bead solution*: 1 or 10% for coating, dissolved in MBS.
3. *PAA solution*: 0.1%, dissolved in MBS.
4. *Sucrose/HEPES solution*: 250 mM sucrose, 25 mM HEPES, pH 7.4, 20 mM KCl, and 20 µl/ml protease inhibitor (see Note 1).
5. Glass dounce homogenizer.
6. Syringe and needle (for suspension cells, see Note 2).

**2.3. Density Gradient  
Centrifugation of  
Plasma Membrane  
Proteins**

1. *Density gradient medium*: Nycodenz<sup>®</sup> gradient of 27.5–40.0% in sucrose/HEPES solution.
2. Ultracentrifuge capable of reaching 100,000×*g* (our lab uses rotor # SW40 Ti, Beckman Coulter).
3. Ultracentrifuge tubes (Ultra-Clear Centrifuge Tubes, Beckman Instruments, Inc).

**2.4. Protein Elution/  
Solubilization**

1. *TX-100 Buffer*: 1% Triton<sup>®</sup> X-100, 400 mM NaCl, and 25 mM HEPES.
2. *Urea Buffer*: 8 M urea, add 100 mM DTT for a final concentration of 2 mM (see Note 3).
3. *PPS Silent<sup>®</sup> Surfactant solution*: 0.2% PPS Silent<sup>®</sup> Surfactant in 50 mM NH<sub>4</sub>HCO<sub>3</sub> and 40% methanol.
4. *Wash Buffer*: 0.025 M Na<sub>2</sub>CO<sub>3</sub>.

**2.5. Protein  
Precipitation for  
TX-100 Buffer-Treated  
Samples**

1. Acetone (prechilled to –20°C).

**2.6. Preparation  
of Sample for MS**

1. *Resuspension Buffer*: 8 M Urea, 100 mM Tris, and pH 8.5. Before use, add 100 mM DTT at a ratio of 1:50.
2. 100 mM IAA, freshly prepared.
3. 100 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.5.
4. 100 mM CaCl<sub>2</sub>.
5. MS-grade trypsin.
6. 2.5% TFA.
7. Desalting column; (e.g., MacroSpin<sup>™</sup> column kit).
8. Acetonitrile.
9. HPLC-grade water.
10. 0.1% TFA.
11. *Elution Buffer*: 70% acetonitrile, 0.1% TFA.
12. 5% acetonitrile, 0.1% formic acid buffer.
13. 200 mM HCl.

**2.7. General Materials**

1. Shaker or rotator.
2. Cell culture supplies (scraper, plates, microscope).
3. Vortex.
4. Bench-top centrifuge.
5. Microfuge tubes.
6. Incubator set at 37°C.

### 3. Methods

#### 3.1. Silica-Bead Coating of Plasma Membranes

For adherent cells (recommended volumes are for the confluent monolayer adherent cells growing on 100-mm diameter plates, see Note 4):

1. Discard cell culture media.
2. Place tissue culture plates containing the monolayer of cells on a flat bed of ice.
3. Gently wash the cells three times with 10 ml of ice-cold MBS.
4. Overlay with 1% ice-cold colloidal silica-bead solution. Incubate for 10 min on ice and adjust the plates accordingly to ensure complete coverage (see Note 5). Discard solution.
5. Wash the cells three times with 10 ml of ice-cold MBS.
6. Overlay with 10 ml of ice-cold 0.1% PAA solution. Incubate for 10 min on ice and adjust the plates accordingly to ensure complete coverage (see Note 6). Discard solution.
7. Add 2 ml sucrose/HEPES solution to the first plate, scrape the cells into the solution, then transfer cell suspension to the next plate, and harvest the cells into the same solution. After the harvesting of all plates, combine cell suspensions (see Note 7).

For suspension cells:

1. Transfer the suspension cells from flask to centrifuge tubes and sediment the cells by centrifugation at  $1,000 \times g$  and  $4^\circ\text{C}$  for 5 min.
2. Wash the cell pellet three times with ice-cold MBS.
3. Prepare a 10% silica-bead solution and add the cells drop-wise, using a syringe and needle or a pipette (see Note 2). Place on ice and rock gently for 10 min (see Note 8).
4. Transfer the cells to a centrifuge tube and sediment by centrifugation at  $1,000 \times g$  and  $4^\circ\text{C}$  for 5 min.
5. Wash the pellet three times with ice-cold MBS.
6. Prepare a 0.1% PAA solution and add cells drop-wise. Place on ice and rock gently for 10 min.
7. Transfer the cells to a centrifuge tube and sediment by centrifugation at  $1,000 \times g$  and  $4^\circ\text{C}$  for 5 min.
8. Wash the pellet three times with ice-cold MBS.

#### 3.2. Cellular Rupture

1. Place the harvested cells in a centrifuge tube and centrifuge at  $1,000 \times g$  and  $4^\circ\text{C}$  for 5 min.
2. Remove the supernatant. If desired, save an aliquot of the supernatant for Western Blotting (see Note 8).

3. Resuspend the cell pellet in 1 ml of sucrose/HEPES. Place in a glass dounce homogenizer and homogenize to rupture the cells (at least 5 strokes or until the solution becomes cloudy, see Note 9).
4. Take a small aliquot (50  $\mu$ l) and check under a microscope to ensure the complete rupture of cells. If desired, save an aliquot of this fraction for Western Blotting (see Note 8).

### **3.3. Density Gradient Centrifugation of Plasma Membrane Proteins**

1. Prepare a discontinuous Nycodenz<sup>®</sup> sucrose/HEPES gradient (as shown in Fig. 2 and Table 1, see Note 10).
2. Dilute sample with Nycodenz<sup>®</sup> and place it on top of the Nycodenz<sup>®</sup> sucrose/HEPES gradient (recommended: 25% Nycodenz<sup>®</sup>, see Note 11).
3. Gently add 1 ml of sucrose/HEPES solution to cover the sample.
4. Prepare an exact counterbalance to each tube.
5. Ultracentrifuge at 100,000  $\times g$  and 4°C for 1 h (see Note 12).
6. Once the membrane pellet has reached the bottom of the tube, discard the supernatant (see Note 13).
7. Resuspend the membrane pellet in 500  $\mu$ l of 0.025 M Na<sub>2</sub>CO<sub>3</sub>. Vortex and shake or rotate for 30 min at 4°C.
8. Centrifuge on a bench-top centrifuge at 5,000  $\times g$  and 4°C for 20 min. Remove the supernatant and proceed to the Subheading 3.4 – Protein Elution/Solubilization.

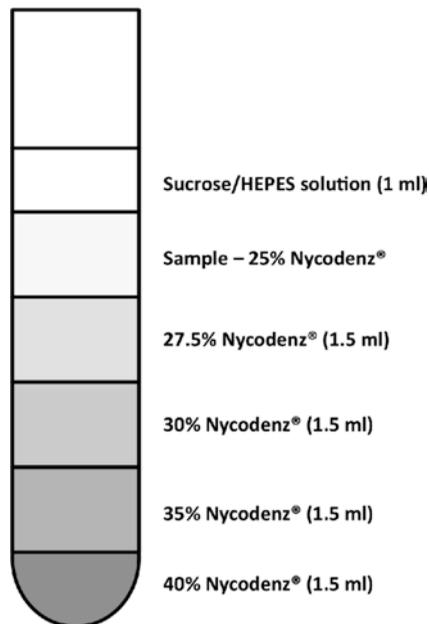


Fig. 2. Careful layering of the Nycodenz<sup>®</sup> solutions (as prepared according to Table 1) in increasing density yields a discontinuous gradient for comprehensive purification of silica-bound membrane proteins.

**Table 1**  
**Recipe for a 27.5–40% discontinuous Nycodenz® gradient preparation**

100% Nycodenz® (ml)	Sucrose/HEPES solution (ml)	Final volume (ml)	Gradient (%)
1.375	3.625	5	27.5
1.5	3.5	5	30
1.75	3.25	5	35
2.0	3	5	40

### 3.4. Protein Elution/ Solubilization

For the elution/solubilization of membrane proteins, three alternative reagents may be used as follows (see Note 14):

1. *TX-100 buffer*: Resuspend the pellet in 200  $\mu$ l of TX-100 Buffer. Transfer the suspension to a microfuge tube. Shake or rotate for 1 h at 4°C. Centrifuge on a bench-top centrifuge at 5,000  $\times g$  and 4°C for 20 min to bring the pellet back down. Collect the supernatant (if desired, take an aliquot of this membrane-enriched fraction for Western Blotting) and proceed to the Subheading 3.5 – Protein Precipitation for TX-100 Buffer-Treated Samples.
2. *8 M Urea with 2 mM DTT*: Resuspend the pellet in 200  $\mu$ l Urea Buffer. Incubate with shaking or rotation for 30 min at 37°C. Centrifuge on a bench-top centrifuge at 5,000  $\times g$  and 4°C for 20 min. Collect the supernatant (if desired, take an aliquot of this membrane-enriched fraction for Western Blotting) and proceed to the Subheading 3.6 – Preparation of Sample for MS step 2 (see Note 15).
3. *PPS Silent® Surfactant solution*: Resuspend the pellet in 100–250  $\mu$ l of PPS Silent® Surfactant solution. Gently pipette repeatedly (if desired, take an aliquot of this membrane-enriched fraction for Western Blotting). Proceed to the Subheading 3.6 – Preparation of Sample for MS step 1.

### 3.5. Protein Precipitation for TX-100 Buffer-Treated Samples (See Note 16)

1. To the volume of recovered supernatant, add 5 times the volume of acetone (at –20°C).
2. Allow proteins to precipitate overnight at –20°C.
3. Bring down protein pellet by centrifugation at 5,000  $\times g$  and 4°C for 20 min.
4. Resuspend the pellet in ice-cold acetone and centrifuge again. Repeat.
5. Discard acetone and dry pellet for 30 min at 37°C.
6. Proceed to the Subheading 3.6 – Preparation of Sample for MS step 1.

### 3.6. Preparation of Sample for Mass Spectrometry

For approximately 30–200 µg of protein

1. For TX-100 Buffer or PPS Silent® Surfactant-treated samples, prepare Resuspension Buffer and add 50 µl to the recovered membrane-enriched fractions. Shake or rotate at 37°C for 30 min.
2. Add freshly prepared 100 mM IAA for a final concentration of 8 mM. Shake or rotate at 37°C for 30 min.
3. Dilute sample with 100 mM NH<sub>4</sub>HCO<sub>3</sub>. Add enough to bring the urea concentration from 8 to ~1.5 M.
4. Add 100 mM CaCl<sub>2</sub> for a final concentration of 2 mM.
5. Add trypsin for enzyme:protein of 1:30–1:40.
6. Digest overnight at 37°C with shaking or rotation (see Note 17).
7. Stop the digestion:
 

For samples solubilized in TX-100 Buffer or 8 M Urea with 2 mM DTT, stop the digestion by adding ~50 µl of 2.5% TFA and proceed to desalting.

For samples solubilized in PPS Silent® Surfactant solution, stop the digestion by adding HCl to a final concentration of 200 mM. Incubate at 37°C for 45 min. Centrifuge sample at 5,000×g at 4°C for 10 min (see Note 18). Collect the supernatant and proceed to desalting.

Desalting (each centrifugation step is performed at ~200×g, see Note 19).
8. Condition the MacroSpin™ column with 500 µl of acetonitrile. Centrifuge and discard flow-through in the collection tube.
9. Condition the column two times with 200 µl of HPLC-grade water.
10. Add sample to the column. Centrifuge and discard flow-through from the collection tube.
11. Wash the column by centrifuging with 200 µl of 0.1% TFA. Repeat.
12. Into a new collection tube, elute the peptides by adding 200 µl of Elution Buffer to the column. Centrifuge. Add an additional 200 µl of Elution Buffer. Centrifuge and collect the recovered eluate (peptides).
13. Concentrate the peptides by drying at 45°C using a vacuum concentrator.
14. Resuspend the resulting peptide pellet in ~40 µl of 5% acetonitrile 0.1% formic acid buffer (see Note 20).

## 4. Notes

This protocol has delivered acceptable results in our hands using the procedure and materials described above. We strongly recommend that users complete several quality controls prior to using the isolated membrane fractions. For example, complete rupture of cells should be ensured under the microscope. Fractions obtained from Subheading 3, steps 2 and 4 should be probed by Western Blotting against known intracellular organelle and membrane protein markers (see Fig. 3). If necessary, adjust the protocol. Critical readjustment of conditions might also be necessary when applying the protocol to a new cell type. The following notes are provided as guides for the development and optimization of silica-bead coating protocols that are suited to individual experimental needs.

1. Protease inhibitor should be added to the sucrose/HEPES solution immediately before use. The purpose of the protease inhibitor is to block the enzymatic activity of endogenous proteases that will be liberated during cell homogenization and might otherwise contribute to protein degradation.

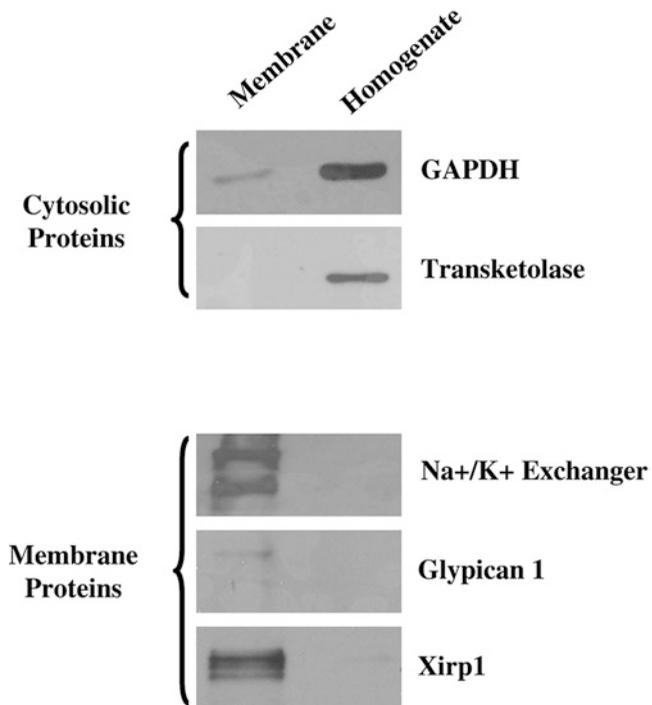


Fig. 3. Purity of sample fractions obtained from the Subheading 3, Steps 2 and 4 can be probed by Western Blotting (Figure from Elschenbroich et al. *J Proteome Res.* 2009; 10:4860–9).

2. For suspension cells, ensure that the gauge of the needle is not so small that it would force premature rupture of the cells. If cells are large or easily perturbed, use a pipette and pipette tip instead.
3. Prepare a stock solution of 8 M urea, 100 mM Tris, pH 8.5 (can be kept at room temperature), and a separate stock solution of 0.1 M DTT (which must be frozen at  $-20^{\circ}\text{C}$ ). Before use, add 100 mM DTT at a ratio of 1:50 to arrive at a final concentration of 2 mM DTT.
4. During the whole procedure, ensure cooling of sample preparation to prevent degradation of proteins.
5. Make sure the cell layer is completely coated with silica-bead solution during the entire incubation step and readjust the plate position if necessary. Gentle movement of the plates after half of the incubation time ensures thorough silica-bead coverage of cells. During this incubation step, silica-beads tightly attach to negative charges on the cell-surface. After incubation, discard silica solution completely by soaking up the remaining liquid with a laboratory wipe (to this purpose, plates can be placed onto the wipe with part of the rim facing upside-down). It is important to remove all excess silica-beads to prevent them from reacting with the PAA cross-linker.
6. During this step, PAA links silica-beads to each other, thus not only completing the formation of pellicles, but also neutralizing all exposed positive charges on the nonattached surface of the beads, which might otherwise capture intracellular proteins after cell rupture.
7. The total volume of the solution should be kept to a minimum, thus enhancing the complete sedimentation of particles during centrifugation. This is especially important when applying the protocol for the first time or to new cell types. As stated several times, complete cell harvest should be ensured using a microscope.
8. Depending on the cell type used, intact cells or cellular debris form the pellet in this centrifugation step. Cells that are easily disrupted break open during scraping, thus releasing intracellular proteins into the buffer (in this case, the supernatant might be kept if these proteins are of interest). For more sturdy cell types (e.g., skeletal muscle cells), intact cells are collected through centrifugation. If cells rupture during this step and consequently release intracellular proteins into the supernatant, they might be saved (storage at  $-80^{\circ}\text{C}$ ). An aliquot of this fraction can be subjected to Western Blotting to probe for the presence of intracellular marker proteins.
9. Homogenization of cells should be complete while avoiding the extended exposure to the shear forces. It is, therefore,

necessary to optimize the number of strokes for each cell type individually using a microscope to monitor the cell rupture. Furthermore, with sturdier cells, an additional lysis procedure may be required. This can be achieved by the addition of lysis buffer followed by alternative cellular disruption methods (e.g., nitrogen cavitation, sonication, etc.).

10. Prepare solutions as outlined in Table 1. To prepare a discontinuous Nycodenz<sup>®</sup> gradient, very carefully place solutions on top of each other, starting with the densest. Then, very slowly let the following solution slide down the wall of the centrifugation tube. This avoids mixing of solutions of different density. If the gradient is prepared correctly, clear boundaries will be visible between layers.
11. Dilute 100% Nycodenz<sup>®</sup> 1:2 with sucrose/HEPES-buffer and add to an equal amount of prepared sample (thus arriving at a final Nycodenz<sup>®</sup> concentration of 25% in the sample). Place the sample carefully on top of the gradient preparation. The sample must be the least dense of all gradient layers, so it can be assumed that only the silica-bead pellicles will migrate through the gradient during ultracentrifugation but not the sample solution as a whole.
12. The ultracentrifugation time and speed must be optimized according to the type of rotor to allow for sedimentation of the silica-coated membrane pellet.
13. The silica-bead pellicles should form a firm pellet after this step. If the pellet does not reach the bottom of the tube, transfer it to a microfuge tube with some of the gradient medium, and then spin at  $14,000 \times g$  and  $4^{\circ}\text{C}$  for 20 min. Then, add homogenization buffer to decrease the density of the gradient and spin again using the same conditions. This should allow for sedimentation of the pellet.
14. Three different reagents for the elution/solubilization of proteins are described here. Note that these reagents have produced acceptable results in our hands, but optimizations according to the unique conditions of each experiment are warranted, and thus other reagents may be substituted.
15. Samples treated with 8 M urea and 2 mM DTT in the elution/solubilization step already have sufficient urea to denature proteins. Thus, Resuspension Buffer need not be added, and IAA can be added directly to this sample.
16. Triton<sup>®</sup> X-100 detergent may interfere with peptide detection and must, thus, be completely removed prior to MS analysis. This can be achieved by precipitation of the proteins and subsequent resolubilization into another buffer.
17. Do not exceed 18 h of digestion to avoid nonspecific cleavage of proteins.

18. At low pH, the PPS molecule is cleaved and subsequently removed by centrifugation, thereby eliminating the interfering effects of the detergent on MS sensitivity.
19. Desalting is necessary to remove buffer salts, which interfere with MS analysis.
20. Store at 4°C if MS analysis is to be carried out within 1 week. Otherwise, store at -80°C until use.

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