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Application of Higher Density Iron Oxide Nanoparticle Pellicles to Enrich the Plasma Membrane and Its Proteome from Cells in Suspension

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Abstract

Enrichment of the plasma membrane represents one valuable method to characterize the surfaceome, along with other plasma membrane and structural proteins. Currently, the overlapping densities of many subcellular organelles hinder enrichment of the plasma membrane by centrifugation. However, external access to the plasma membrane of intact cells allows the attachment of a nanoparticle pellicle to enhance its density and facilitate enrichment. We describe the synthesis of iron oxide nanoparticles, attachment of the pellicle to suspended cells, and recovery of plasma membrane proteins for proteomic analysis.

Keywords

Plasma membrane; Nanoparticles; Pellicles; Proteomics; HPLC-MS/MS

1 Introduction

The plasma membrane (PM) provides access to transmembrane proteins and glycoproteins that extend onto the surface, as well as membrane proteins that extend into the cytoplasm, cytoskeletal proteins and, depending on the method, secreted proteins. The ability to enrich the plasma membrane from other cellular components represents an excellent approach to study the surfaceome of different cell types. This chapter presents a procedure for enriching the plasma membrane from intact cells by attaching a nanoparticle pellicle that increases its density relative to that of other subcellular organelles. This facilitates the separation by centrifugation of the plasma membrane from other lysed cellular components. Chaney and Jacobson first reported the use of a nanoparticle pellicle using an in-house preparation of cationic colloidal silica [1, 2]. Following attachment of the cationic nanoparticles to the anionic eukaryotic cell surface by ionic attraction, the pellicle is stabilized by crosslinking with poly(acrylic) acid (PAA). The pellicle method was incorporated into proteomic strategies in 2004, using commercially available colloidal silica [3] and the original Jacobson preparation [4]. Recently a comparison has been made of pellicles comprising nanoparticles of differing densities [5]. Iron oxide particles coated with cationic alumina [6]

were found to provide better enrichment than the lighter colloidal silica particles, and form the basis of the procedure presented here for enrichment of the plasma membrane from cells in suspension and, thereby, cells from tissue [7].

In brief, eukaryotic cells grown in suspension are washed with the plasma membrane coating buffer A (PMCB A), pH 5.3, to remove cell debris. Alumina-coated iron oxide nanoparticles are synthesized using polyol procedures and dispersed in PMCB A. A solution of suspended cells is added dropwise to the nanoparticle solution to ensure complete coating of the cationic nanoparticle on the anionic cell surface. After removing excess nanoparticles, the unbound charges of the nanoparticles are neutralized by anionic PAA solution in PMCB A, which also provides higher strength to the plasma membrane–nanoparticle pellicles by cross-linking of the polymer around the cells. The coated cells were then resuspended in hypotonic buffer and lysed by nitrogen cavitation. After lysis, open sheets of plasma membrane–nanoparticle pellicles can be produced as observed under the scanning electron microscope. The coated plasma membrane possesses higher density than other cellular organelles and thus can be separated by low-speed centrifugation (Table 1). The integral plasma membrane proteins are extracted from the pellicles using microwave-assisted method in the presence of sodium dodecyl sulfate. The extracted proteins can be subjected to identification or quantification using bottom-up proteomic analysis procedures.

2 Materials

2.1 Iron Oxide Nanoparticle Construction

1. For the preparation of iron oxide nanoparticles prepare 0.5 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 1.5 mM sodium acetate ($\text{C}_2\text{H}_3\text{NaO}_2$) in 25 mL of propylene glycol.
2. For coating the iron oxide particles with alumina, prepare 0.01 M $\text{Al}(\text{NO}_3)_3/0.1$ M $\text{K}(\text{NO}_3)$ as a 10 mL aqueous solution.
3. Glassware required: 100 mL round bottom flask with a 24/40 ground glass joint (catalog number UX-34706–10; ColeParmer, Vernon Hills, IL, USA) connected to the condenser via a female 24/40 ground glass joint.
4. Centrifugal Filters (Cat No. UFC500396, Millipore, Billerica, MA, USA).

2.2 Pellicle Construction and PM Analysis

1. Plasma Membrane Coating Buffer A (PMCB A): 800 mM sorbitol, 20 mM 2-(N-morpholino)ethanesulfonic acid buffer (MES), 150 mM NaCl, pH 5.3. Solutions of 10 M NaOH or concentrated HCl are recommended to adjust pH of PMCB A.
2. 10 mg/mL poly(acrylic) acid (PAA) in PMCB A, pH 6–6.5. After adding PAA, the pH of PMCB A will be decreased, and a portion of 10 M NaOH solution is needed to adjust the pH to be 6.0–6.5.
3. Lysis buffer: 2.5 mM imidazole with protease inhibitor cocktail (catalog number P8340–1ML) (Sigma-Aldrich, St. Louis, MO, USA).
4. 1 M Na_2CO_3 , pH 11.4.

5. 1 M KCl.
6. Protein extraction buffer: 2% SDS, 62.5 mM Tris-HCl, and 5% β -mercaptoethanol.
7. Resolubilization buffer: 8 M urea, 50 mM NH_4HCO_3 .
8. 20 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 .
9. 40 mM iodoacetamide (IAA) in 50 mM NH_4HCO_3 .
10. 0.1% formic acid.
11. Endoproteinase Lys-C, sequencing grade (catalog number V1071) (Promega, Madison, WI, USA).
12. Trypsin, mass spectrometry grade (catalog number V5280; Promega).
13. C18 spin column (Pierce™, Thermo Fisher Scientific, San Jose, CA, USA).

2.3 Tissue Culture Supplies

1. Human multiple myeloma cells (ATCC RPMI 8226).
2. Tissue culture incubator with CO_2 regulator.
3. Refrigerated tissue culture compatible centrifuge for 15 and 50 mL tubes.
4. 75 cm^2 cell culture flask.
5. RPMI 1640 medium.
6. Fetal Bovine Solution (FBS).
7. Hemocytometer.
8. Light microscope (200 $\times$ magnification).
9. 50 mL conical tube.
10. 3 mL syringe with 21G needle.

2.4 Other

1. Ultrasonic bath sonicator.
2. Cavitation chamber (4635 Cell Disruption Vessel, Parr Instrument Company, Moline, IL, USA).
3. Microwave compatible glass tubes and stir bar.
4. Reducing Agent and Detergent Compatible assay, RC DC™ (catalog number 500-0122; Bio-Rad, Hercules, CA, USA).
5. Water bath at 56°C.
6. Lyophilizer (*see* Note 1).
7. Microwave (CEM Corporation, Matthews, NC, USA).

3 Methods

3.1 Iron Oxide Nanoparticle Construction [6] (See Fig. 1)

1. To begin the nanoparticle preparation dilute $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and sodium acetate in 25 mL of propylene glycol to a final concentration of 0.5 mM and 1.5 mM, respectively.
2. Add 500 μL of deionized water to this mixture and reflux for 12 h in a 100 mL round bottom flask at room temperature; be sure to fit a condenser to the round bottom flask via a female 24/40 ground glass joint. Particles will form (see **Note 2**).
3. Collect and wash the particles four times with water via centrifugation (8500 g for 30 s) in Millipore centrifugal filter.
4. Resuspend the particles in 10 mL of 0.01 M $\text{Al}(\text{NO}_3)_3/0.1$ M $\text{K}(\text{NO}_3)$ for 12 h at room temperature on a rocking platform.
5. Collect the particles via centrifugation (8500 $\times g$ for 10 min), discard the supernatant, and resuspend the pellet in PMCBA for use in cell coating and pellicle construction.

3.2 Coating of Multiple Myeloma Cells with Nanoparticle Pellicles (Figs. 2 and 3, See Note 3)

1. Human multiple myeloma cells were cultured in suspension in T-75 flasks at 37°C in RPMI 1640 medium containing 10% fetal bovine serum under a 5% CO_2 atmosphere. The cell density should be maintained between 5×10^5 and 2×10^6 cells/mL. Do not allow the cell density to exceed $2\text{--}3 \times 10^6$ cells/mL. Approximately, 5–10 flasks containing 20 mL media are required for one experiment.
2. To estimate cell culture concentration, remove a sample and count the cells with a hemocytometer. Use the counts to determine the volume of culture needed to collect 6×10^7 cells.
3. To collect cells, each cell culture flask should be poured into a sterile 50 mL conical tube utilizing aseptic technique (see Note 4).
4. Pellet the 6×10^7 cells via centrifugation at $900 \times g$ for 5 min. The temperature is controlled to be 4° C (see Notes 5 and 6).
5. Wash cells with cold PMCBA buffer three times by resuspending the cell pellet gently by trituration with a 10 mL automatic pipet. After each wash, repellet the cells via centrifugation at $900 \times g$ for 5 min (see Note 7).
6. After the final wash, resuspend the cell pellet in 2 mL of PMCBA buffer and use a syringe to add the suspension dropwise to the 10% (w/v) suspension of cationic nanoparticles prepared under Subheading 3.1 (see Notes 8 and 9).
7. Incubate the suspension for 15 min at 4°C with gentle rocking.

8. After the incubation, repeat the washing outlined in **step 5** using fresh PMCBA buffer. This process removes any excess nanoparticle suspension.
9. Resuspend the coated cells in 2 mL PMCBA buffer and add dropwise to a solution of 10 mg/mL PAA for cross-linking. This step should be carried out in the same manner as **step 6**.
10. Incubate the cross-linking reaction for 15 min at 4°C with rocking.
11. To collect coated cells spin at 900×*g* for 5 min.
12. Perform three washes with PMCBA as outlined in **step 5**.
13. After the final wash, resuspend the cell pellet in 10 mL of lysis buffer and incubate at 4°C for 30 min with gentle rocking. This causes cell swelling for efficient cavitation.
14. Perform N₂ cavitation within cavitation chamber for 30 min at 1500 psi (*see Notes 10 and 11*).
15. Visually confirm cavitation by observing a small aliquot of the sample under a light microscope (*see Note 12*).
16. Collect pellicle fragments using low speed centrifugation at 100×*g* for 7 min at 4°C (*see Note 13*).
17. Wash the pellicle three times with 1 M Na₂CO₃ (pH 11.4) via low-speed centrifugation at 100×*g* for 7 min at 4°C to remove soluble proteins and randomly attached proteins from the pellicles. The plasma membrane nanoparticle pellicles will be pelleted by centrifugation, while supernatant containing other organelles should be discarded.
18. Repeat washing steps three times with 1 M KCl to further-remove randomly attached proteins from the pellicles.

3.3 Protein Extraction

1. Resuspend the pellicle pellet in 200 µL of protein extraction buffer and place samples into microwave compatible glass tubes, with a small stir bar.
2. Microwave samples for 5 min at 100°C with gentle stirring while irradiating with 300 W, using a CEM Discover Microwave.
3. Remove samples from microwave and transfer to a 1.5 mL Eppendorf tube (*see Note 14*).
4. Spin samples at 100 × *g* for 7 min at room temperature to pellet the pellicle/membrane fragments. Following pellet formation, collect the supernatant as the first protein extract.
5. Resuspend the pellicle/membrane fragments in Protein extraction buffer and transfer back to the microwave compatible tube and repeat the extraction two additional times to get the second and third protein extracts (*see Notes 15 and 16*).

6. Perform a Reducing Agent and Detergent Compatible assay on each protein extract to determine protein concentration, and combine fractions of the extracts as needed to obtain 100 µg of protein.

3.4 Preparation of Samples for High Performance Liquid Chromatography (HPLC)-Mass Spectrometry (See Note 17)

1. Precipitate 100 µg of protein from the extraction sample using a standard chloroform–methanol procedure (*see* Note 18).
2. Resuspend the protein pellet in 100 µL 8 M urea and 50 mM NH₄HCO₃.
3. Reduce protein sulfhydryl groups by adding 2 µL of 1 M DTT stock solution for a final concentration of 20 mM DTT. Incubate the sample at 56 °C for 20 min in a water bath.
4. Alkylate protein thiol groups by adding 4 µL of the 1 M IAA stock solution to the sample for a final concentration of 40 mM IAA. Incubate at room temperature for 20 min.
5. Add protease Lys-C to a final concentration of 1:50 enzyme–substrate and incubate at 37°C for 3 h.
6. Following Lys-C digestion dilute the approximately 8 M urea solution to 1.6 M urea with 50 mM NH₄HCO₃.
7. Add trypsin to a final concentration of 1:25 enzyme–substrate and incubate at 37°C for 16 h.
8. Desalt and concentrate samples using C18 spin columns. Recover peptides in 85% acetonitrile (*see* Notes 19 and 20).
9. Freeze samples at 80°C to prepare for lyophilization.
10. Lyophilize samples until the final volume is negligible and resuspend in 100 µL of 0.1% formic acid for analysis via HPLC interfaced to a tandem mass spectrometer.

3.5 Analysis of Samples (See Note 21)

1. The LC gradient utilized should be appropriate for the expected complexity of the sample. For analysis of the multiple myeloma PM 15 µg of sample was utilized per injection.
2. To ensure thorough desalting, load samples for 10 min at a flow rate of 10 µL/min onto the trapping column desired. For analysis of the sample discussed, an Acclaim PepMap 300 C18 column was used.
3. Samples should be analyzed using a column of the same chemistry as the trapping column, such as a Grace Vydac Everest C18 column.
4. The HPLC gradient should contain a linear phase. In this case, a 90 min gradient from 10–60% solvent B (90–40% solvent A) was used, followed by 20 min from

60 to 85% solvent B (40–15% solvent A). Solvent B contains 97.5% acetonitrile, 2.5% water and 0.1% formic acid; while solvent A contains 97.5% water, 2.5% acetonitrile and 0.1% formic acid.

5. Mass spectrometric parameters should be adjusted to desired purpose of sample analysis.

4 Notes

1. A centrifugal evaporator can be used instead of a lyophilizer.
2. During the reflux, the solution should be boiled, and the color of the solution should change from yellow to dark orange. When the particle forms, changes in the color of the solution should be observed.
3. This protocol for iron oxide nanoparticle pellicle experiment is suitable for suspended cells either from cell culture or from blood sample. It is also applicable for other types of mammalian cells or tissues; however, the procedure should be optimized. Since each type of cells possesses different degrees of negative charge on the surface, you should bring an aliquot of samples after **steps 5, 8, 12 and 14** to observe the cell morphology, coating ability, cross-linking ability and cell lysis efficiency by using the scanning electron microscope. If incomplete coating of the nanoparticle or PAA is observed, the concentration of nanoparticles or PAA and coating time should be optimized. If intact cells are presented after cell lysis, the pressure for nitrogen cavitation should be increased.
4. Care should be taken to maintain a sterile environment when transferring cultured cells to conical tubes to ensure minimal environmental contamination.
5. Every step should be performed at 4°C to avoid the risk of internalizing nanoparticles.
6. If cells do not readily pellet under the experimental conditions, increase spin times rather than speeds. This minimizes the risk of pelleting debris present in the medium.
7. Resuspension of cell pellets at any point in the procedure should be done via gentle pipetting rather than vortexing, in order to avoid premature lysis.
8. Before coating the cells, the nanoparticle suspension should be sonicated and vortexed to ensure good dispersion of the nanoparticle in solution.
9. When adding cell solutions dropwise to either cationic particle suspensions or PAA solutions, care should be taken to add samples slowly. This allows thorough mixing and thus more efficient and complete coating and cross-linking. Use of 3 mL syringes with 21G needles is recommended for these additions. The inner diameter of the syringe needle should be larger than the cell diameter to avoid cell lysis.

10. During lysis it is often beneficial to add empty conical tubes to the cavitation chamber to minimize the risk of sample tube movement (and thus sample loss) with changing pressures.
11. Nitrogen cavitation is the chosen method for this example because it uses physical forces, rather than chemical reactions, to lyse the cells. This minimizes the risk of pellicle disruption. If other lysis methods are attempted, the fragile nature of the pellicle interaction should be considered.
12. When cell lysis is achieved by nitrogen cavitation, small fragments and debris should be observed under the light microscope. In case intact cells are presented, higher pressure of the cavitation (1800 psi) is recommended.
13. It is not uncommon for the pellicle to precipitate while the sample is resting on the bench.
14. When extracting proteins from the pellicle take care when removing samples from the microwave as glass vials may retain heat for an extended period following microwaving.
15. Using protein quantitation (RC DC protein assay), we observe that recovery falls off significantly after three extractions.
16. For a new type of sample it may be wise to determine the optimal number of extractions required to recover all the protein from the pellicle fragments. After each time of protein extraction in the microwave, 25 μ L of the extracts should be subjected to RC DC protein assay to determine the concentration. If the concentration drastically decreases from the previous extraction, subsequent extraction is not necessary.
17. It is recommended for a novice to consult with a trained mass spectroscopist to perform the sample preparation, since the membrane peptides are generally hydrophobic and may require additional procedures for different type of samples.
18. To perform a protein precipitation using chloroform/methanol procedure, one volume of sample is mixed with four volumes of methanol. Then, one volume of chloroform is added to the mixture, followed by three volumes of deionized H₂O. After vigorously vortexing, the solution should become cloudy. The mixture is subjected to centrifugation at $14,000 \times g$ for 2 min at room temperature. The white protein precipitate should be observed as an interface between the top aqueous layer and the bottom organic solvent layer. The top layer should be removed by gentle pipetting to avoid disturbing the protein precipitate. Then, four volumes of methanol are added to wash the pellet, followed by centrifugation at $14,000 \times g$ for 2 min at room temperature. The protein precipitate should be observed as a white pellet, and the supernatant can be discarded. Methanol can be removed from the pellet by air-dry or using a vacuum centrifugation.
19. To desalt and concentrate the peptide samples, it is recommended to follow the protocol from manufacturer (Pierce™, Thermo Fisher Scientific, San Jose, CA,

USA). Briefly, the spin column is activated using 400 μ L of 50% acetonitrile and then equilibrated with 400 μ L of 0.5% trifluoroacetic acid in 5% acetonitrile. Sample buffer should be adjusted to contain the final concentration of 0.5% trifluoroacetic acid in 5% acetonitrile. After column equilibration, sample is applied on the resin bed and centrifuged at $1500 \times g$ for 1 min. At this point, peptides are bound to the C18 resin, and salt can be removed by washing several times with 0.5% trifluoroacetic acid in 5% acetonitrile solution. After washing, the peptides are eluted using 40 μ L of 0.1% formic acid in 85% acetonitrile. The eluted solution is then frozen to prepare for lyophilization.

20. When performing a C18 column cleanup it is not uncommon to observe a colored layer within the top of the column. These are a small number of residual nanoparticles that manage to remain in solution during extraction and digestion. They do not interfere with column desalting and sample concentration.
21. For the analysis of the sample using HPLC-MS/MS, it is recommended to consult with professional mass spectroscopists or staff at a proteomic core facility. The HPLC instrument and mass spectrometer could be different from what is mentioned in this chapter. However, it is recommended to use a nano-HPLC system to increase the sensitivity of the separation, since many membrane proteins are low abundant. Analytical column used to separate peptides should be reverse-phase; C18 is recommended. In case you work with highly hydrophobic proteins, column heater may be required to elute highly hydrophobic peptide from the column. Multiple injections and analysis of the peptide mixture by LC-MS/MS will provide additional peptide and protein identifications.

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Fig. 1. Transmission electron microscope (TEM) image of alumina-coated iron oxide nanoparticles (modified from ref. 5). A white scale bar represents 50 nm in length. An average diameter of the nanoparticles is approximately 17.6 nm. Individual nanoparticles are shown in gray. Since the TEM sample preparation requires drying process, some nanoparticle aggregates could be observed. Darker particles could result from different layers of the nanoparticles as a part of the aggregates

Fig. 2.

Pellicle construction with alumina-coated iron oxide nanoparticles. Negatively charged intact cells are added to a solution of positively charged iron oxide nanoparticles to coat the cells via electrostatic interaction. Then coated cells are cross-linked with anionic PAA to neutralize excess charges on the surfaces. After that, coated cells are lysed by nitrogen cavitation to produce plasma membrane pellicle fragments. The pellicles are isolated by low-speed centrifugation and subjected to protein extraction, followed by bottom-up proteomic analysis

Fig. 3. Scanning electron microscope images. (a) Human multiple myeloma cell, (b) cell coated with aluminacoated iron oxide nanoparticles, (c) PAA cross-linked coated cell, (d) fragment of pellicle-coated plasma membrane (modified from ref. 5)

Table 1.

Densities of subcellular organelles (adapted from ref. 8)

Subcellular component	Density (g/cm ³)
Nucleus	1.4
Endoplasmic reticulum	1.2
Golgi apparatus	1.14
Mitochondria	1.1
Ribosomes	1.6
Lysosomes	1.1
Plasma membrane	1.12