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Short communication

Sample preparation method for plasma membrane proteome analysis

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ABSTRACT

The preparation of plasma membrane (PM) proteome samples is seriously difficult and time-consuming, owing to their profound hydrophobicity and low abundance. We have developed an efficient PM sample preparation method using Ultracentrifugation with Percoll and an aqueous two-phase extraction. The developed method was rapid (3 h) and provided high purities (26-fold of cell lysate) with a high yield (2.6% of whole cell lysate proteins). This method is especially useful for PM proteome studies using 2D gel electrophoresis.

1. Introduction

Plasma membrane (PM) proteins perform crucial roles in the function of cells. Within the PM, signal transduction is initiated and materials and energy are exchanged. Cell–cell interaction, as well as cell–environment interaction, is especially necessary for the survival and differentiation of stem cells. It has been reported that once mesenchymal stem cells (MSCs) are transplanted into uninjured nude-mouse hearts, 99% of MSCs perished within 4 days after transplantation [1]. This is explained as an Anoikis, and underlines the fact that cell adhesiveness is a crucial factor in the prevention of cell death. In addition, more than 50% of all membrane proteins have been implicated as possible pharmacological targets. Therapeutic monoclonal antibodies can be employed in the targeting of cell surface proteins that are uniquely expressed on diseased cells or tissues [2]. Therefore, the investigation of cell surface proteins is clearly a vital research direction.

Although many researchers have attempted to identify and characterize the PM proteome from cells or tissues, serious problems have occurred in this regard. First, the purification of PM proteins remains quite difficult, as the PM contains a large quantity of lipids and sugars, which are interrupted upon the detection of PM proteins [3]. Another problem is that substantial quantities of PM proteins are lost during preparation, when employing currently available preparation protocols [4]. The other problem is the relatively low abundance of PM proteins as compared with other cellular proteins, as the PM comprises only approximately 2–5% comparable that all membrane proteins is 30% of all cellular proteins [5]. In order to acquire sufficient quantities of PM proteins, a large quantity of cells, media, time, and labor are currently required. These studies tend to be more limited on PM proteins at the cellular level than on animal subjects, such as rats [6].

The Ultracentrifugation method unfortunately generates a large amount of intracellular organelle contaminants, and is also notoriously time-intensive. In this study, we have attempted to overcome these difficulties, using a method involving Ultracentrifugation with 30% Percoll solution, coupled with a two-phase extraction procedure. The major merits of this method include convenience, rapidity, excellent yield, and high purity.

2. Experimental

2.1. Cell culture

Human MSCs obtained from healthy donor and human melanoma A375 cells purchased from ATCC (Manassas, USA) were cultured in α-MEM and DMEM, respectively, with 10% FBS and 1% Gentamicin (Gibco, Grand Island, NY, USA) at 37 °C, in a 5% CO_2 atmosphere. The cells were harvested by scraping from nine 150 mm dishes.

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2.2. Ultracentrifugation and two-phase partition

The harvested cells were then homogenized with a Dounce homogenizer and centrifuged for 10 min at 1000 × *g*. After the pellet containing the nuclei was removed, the post-nuclear supernatant (PNS) was obtained. The collected PNS was then loaded into 30% Percoll (w/v) and centrifuged at 84,000 × *g* for 35 min (Beckman SW28 Ti rotor), and the visible band of the crude PM fraction was collected [7] (Supplementary Fig. 1).

For aqueous two-phase extraction, two sets of a Dextran 500 (6.6%, w/w)/polyethylene glycol (PEG) 3350 (6.6%, w/w) were prepared via the addition of stock solutions of 20% (w/w) Dextran 500 (GE Health Care, Uppsala, Sweden) and 40% (w/w) PEG 3350 (Sigma–Aldrich, Steinheim, Germany). The crude PM sample prepared was then added into a set of two-phase mixtures. The potassium phosphate buffer (0.2 M, pH 7.2) was added to the other set of two-phase mixtures. After 40 inversions at 4 °C, the two-phase mixtures were centrifuged for 5 min at 750 × *g* at 4 °C. The two-phase mixtures were then phase-separated via centrifugation (upper phase: PEG, lower phase: Dextran). The two upper phases of the two-phase systems were exchanged. The mixtures were inverted 40 times at 4 °C and subjected to 5 min of centrifugation at 750 × *g* at 4 °C. Two upper phases were recovered and pooled together. The pooled samples were centrifuged for 2 h at 100,000 × *g* with 1 mM sodium bicarbonate [6,8]. The purified PM proteins were then recovered as a pellet (Supplementary Fig. 2). In order to verify the reproducibility of the sample preparation method developed herein, the entire procedure was repeated 5 times.

2.3. Western blotting and 2D electrophoresis

The quantity of proteins in each of the samples was determined by a Bradford assay.

The cell lysate, PNS, crude PM, and purified PM samples were prepared for Western blotting (Supplementary Fig. 1). Each sample was separated via SDS-PAGE and blotted onto nitrocellulose membranes for Na^+/K^+ ATPase and Cadherin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Calnexin. Anti-Na^+/K^+ ATPase, anti-Cadherin and anti-Calnexin antibodies were obtained from Abcam (Cambridge, UK), and anti-GAPDH, horseradish peroxidase-conjugated anti-mouse, anti-rabbit, and anti-goat Ig G secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Band detection was conducted using an Enhanced Chemiluminescence (ECL) system (Pierce Biotechnology, Inc., IL). Bands from Western blotting were scanned using a flat-bed scanner and digitized using Scion image analysis software (Scion Corp., MD, USA). The values were expressed as means ± standard deviation (S.D.) following to [9].

For 2D-electrophoresis, the samples were solubilized in a sample buffer consisting of 8 M urea, 2 M thiourea, 0.5 M Tris (pH 8.5), 2% ASB 14, 4% CHAPS, 65 mM DTT, 0.5% IPG buffer (pH 3–10 NL). Ready-to-use Immobiline DryStrips (24 cm, pH 3–10 NL) were rehydrated with the sample (40 and 100 μg of protein) in 450 μl of the rehydration solution containing 8 M urea, 2% CHAPS, 12.97 mM DTT, 1% IPG buffer (pH 3–10 NL), and a trace of BPB for 5 h without current and 5 h with current at 80 V. IEF was conducted for a total of 72 000 V h, using the IPGphor IEF system (AP Biotech, Sweden). The IEF strips were then applied to 12.5% constant gel. SDS-PAGE was conducted at the following settings: 55 V for 1 h, 140 V for 1 h, 300 V for 5 h using an Ettan DALT system (Amersham Biosciences). The protein spots were silver-stained via the method described previously [10,11].

Table 1
Protein concentration and yields at each purification step

	Volume (ml)	Concentration (μg/μl)	Total protein (mg)	Yield (%)*
Cell lysate	20.0	0.968 ± 0.04	19.3 ± 0.80	100.0
PNS	20.0	0.708 ± 0.06	14.2 ± 1.20	73.6
Crude PM	6.0	0.306 ± 0.04	1.8 ± 0.24	9.3
Purified PM	0.5	1.060 ± 0.11	0.5 ± 0.05	2.6

* Yield of each sample was determined by the ratio of protein concentration at each sample to whole cell lysate proteins. Values are shown as mean ± S.D. ($N = 5$).

3. Results and discussion

3.1. Evaluation of PM yield

Samples of the cell lysate, post-nuclear supernatant, and crude PM proteome following the Ultracentrifugation with Percoll and purified PM proteome samples after aqueous two-phase extraction were collected from hMSC, and their protein concentrations were determined (Table 1). The average quantity of total protein was 19 mg in the whole cell lysates, 14 mg in the PNS, 1.8 mg in the crude PM protein fraction, and 0.53 mg in the purified PM protein. The final yield of the purified PM protein was 2.6% of the whole cell lysate protein. The sample preparation method developed herein provided high yields of the PM proteome, a quantity sufficient

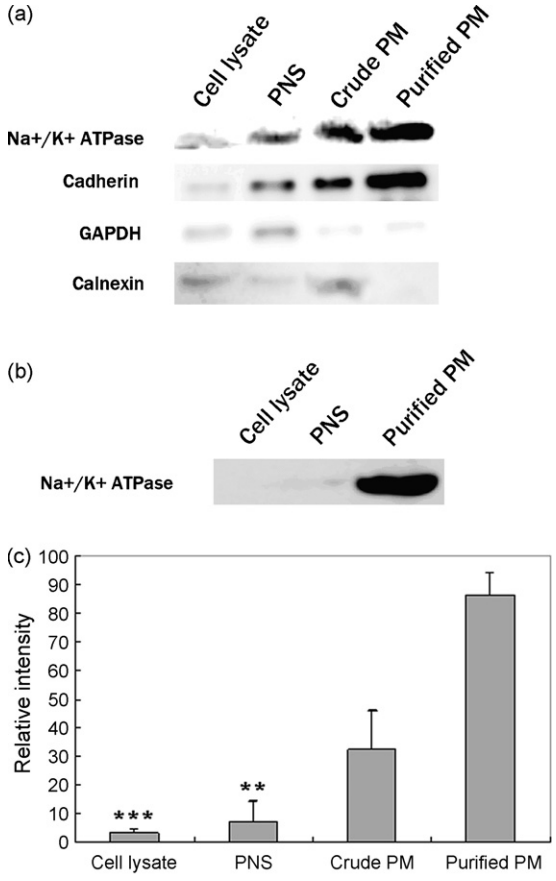

Fig. 1. Western blot of enriched PM. Relative protein levels of PM markers, Na^+/K^+ ATPase, Cadherin and inner cellular markers, GAPDH, Calnexin on each PM purification step in hMSC (a). Na^+/K^+ ATPase in melanoma A375 (b) were compared by Western blotting. Quantitative analysis of Na^+/K^+ ATPase gel bands of hMSC (c). Each bar represents the mean ± S.D. for each spot. Statistical significance was evaluated via one-way analysis of variance (ANOVA) (Cell lysate vs purified PM: $^{***}P < 0.001$) (PNS vs purified PM: $^{**}P < 0.01$) (c).

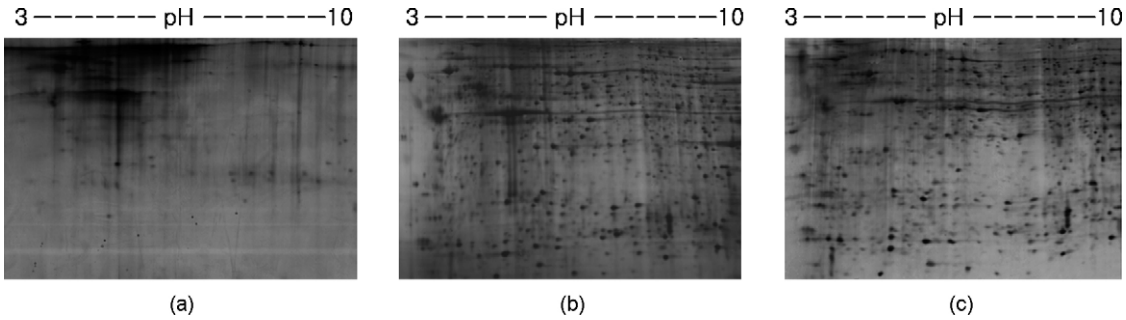

Fig. 2. 2D electrophoresis maps of the purified PM proteome. The protein spots of the crude PM sample prepared via Ultracentrifugation interfered profoundly with residual lipids and aggregated proteins, as shown in the 2DE map (a). However, only an additional single step after Ultracentrifugation, namely two-phase extraction, provided high resolution on the 2DE map with loading quantities of 40 μg (b) and even 100 μg (c) of purified PM.

Table 2
Efficiency comparisons of PM purification methods

	Ultracentrifugation with sucrose [12,13]	Commercial kit (biotinylation) [17]	Two-phase system [18]	This study
Required time	20 h	2.5 h	2–4 h	3 h
Starting material	6 g tissue	1×10^7 cells	80 mg protein	20 mg protein
Purified PM*	1 mg	30–100 μg	No data	500 μg
Enrichment of PM	No data	Very high	10–20-fold	26-fold

* Means the PM protein amount purified from starting material.

for 2DE analysis. Commercially available PM protein preparation kits generally produce 1.2–4% of total protein. The quantities of PM protein acquired in one experiment are from approximately 30 μg to a maximum of 100 μg, quantities which are insufficient for proteomic analysis [18]. In addition, commercial kits are quite expensive. Therefore, the special merit of this study is that PM quantity can be controlled as much as we need.

3.2. Evaluation of PM purity

In order to determine the degree to which the PM proteins had been enriched, the quantities of Na^+/K^+ ATPase and Cadherin, indicators of the PM fraction, were detected via Western blotting at each purification step. As shown in Fig. 1a and c, the Na^+/K^+ ATPase and Cadherin bands on the purified PM sample of hMSC were significantly denser than those on the cell lysate, PNS, or crude PM samples, despite the fact that identical amounts of protein were loaded for each (10 μg). GAPDH, a marker of cytosol protein, and Calnexin, an endoplasmic reticulum marker, could not be confirmed on the purified PM fraction, as the soluble proteins and inner cellular membrane were removed via aqueous two-phase extraction (Fig. 1a). Similar results were obtained via the detection of Na^+/K^+ ATPase on purified PM of human melanoma A375 (Fig. 1b). The purification efficiency of the method developed herein was confirmed with both of the tested cell types. In order to determine the degree of enrichment, quantitative analysis was conducted on the Na^+/K^+ ATPase band, via image analysis (Fig. 1c). The relative intensity of the purified PM band was much stronger than that of other fractions, which was 26-fold that of the cell lysates. Although PM fractions were collected after Ultracentrifucation, lipid in PM fraction was remained. Following, the two-phase extraction drastically improved the purification power of the sample preparation method developed.

3.3. Confirmation of lipid removal

2D electrophoresis was conducted in order to confirm protein resolution and lipid removal. As expected, it was impossible to analyze a 2DE map of crude PM proteins samples after Ultracentrifugation, as shown in (Fig. 2a). However, a 2DE map of the purified PM sample following two-phase extraction has a much better clearance (Fig. 2b). Even at a loading quantity of 100 μg, which generally provides a high background and complexity on 2DE analysis, the protein spots on a 2DE map were clear and their image analysis was proven possible (Fig. 2c).

In previous studies of the 2DE analysis of PM proteome, aggregated proteins were usually removed [4] or treated with a variety of surfactants in order to obtain a clear PM proteome 2D map [15]. Otherwise, several extraction steps were conducted in an effort to remove the aggregated proteins [16]. As a result, a substantial quantity of PM proteins was lost, requiring far more PM fractions. In this study, the PM proteins purified via a two-step purification process were clearly separable via 2DE analysis, which is indicative of efficient lipid removal and good protein resolution.

4. Conclusion

PM analysis of stem cells is an important way to study cell adhesion and signaling, which is associated with cell survival and differentiation after transplantation. However, in order to obtain PM proteins of high purity, a substantial quantity of cells, time, and cost is required. This has prevented many researchers from working with cells for PM preparation. In this study, a method was devised involving Ultracentrifugation with Percoll coupled with two-phase extraction in order to overcome these problems. Generally, sucrose is used as a gradient media during Ultracentrifugation for the preparation of PM [12,13]. However, Ultracentrifugation methods using sucrose are quite inconvenient and time-consuming. Instead, the use of Percoll as a gradient media during Ultracentrifugation saved a significant amount of time and made the procedure far more convenient [14]. Although PM protein preparation methods employing biotinylation have some merit, in that the PM proteins can be isolated with a relatively high degree of purity, it remains difficult to acquire a substantial quantity in a single experiment and involves high cost [17]. In addition, two-phase system-method shows lower enrichment degree when the starting materials are of small quantity (40 mg) [18]. The efficiency of PM purification method was compared in Table 2.

In conclusion, the two-step procedure for PM protein purification, which involves Ultracentrifugation with Percoll followed by aqueous two-phase extraction, has many advantages: It can be performed rapidly and conveniently as compared with other manual methods. The degree of PM enrichment is also higher than results obtained by other methods. Specially, it is possible to obtain enough quantity to use proteomics tool.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.07.028.

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