Chapter 19

Isolation of Plasma Membranes from the Nervous System by Countercurrent Distribution in Aqueous Polymer Two-Phase Systems

Jens Schindler and Hans Gerd Nothwang

Summary

The plasma membrane separates the cell-interior from the cell's environment. To maintain homeostatic conditions and to enable transfer of information, the plasma membrane is equipped with a variety of different proteins such as transporters, channels, and receptors. The kind and number of plasma membrane proteins are a characteristic of each cell type. Owing to their location, plasma membrane proteins also represent a plethora of drug targets. Their importance has entailed many studies aiming at their proteomic identification and characterization. Therefore, protocols are required that enable their purification in high purity and quantity. Here, we report a protocol, based on aqueous polymer two-phase systems, which fulfils these demands. Furthermore, the protocol is time-saving and protects protein structure and function.

Key words: Brain, Plasma membrane, Countercurrent distribution, Two-phase system, Enrichment

1. Introduction

Plasma membrane (PM) proteins mediate signal transduction, solute transport, secretion, and cell-cell contact. They are also the central players in the propagation and transmission of action potentials, which are the lingua franca in the nervous system. Finally, ~70% of all known drug targets act on them *(1)*. PM proteins are thus of prime interest in many areas of both basic and biomedical research. However, their proteome analysis is rather difficult, as they encompass only 0.4–2.5% of the total cellular protein amount *(2)*. This renders their identification difficult in the bulk of other, more abundant proteins of the cytoskeleton,

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the energy metabolism, and alike. Furthermore, many properties of PMs such as density overlap with those of other membranous compartments, mainly the endoplasmic reticulum. Classical purification protocols of PM proteins are therefore rather cumbersome and material-consuming. This is for instance the case with the most popular method, consisting of a combination of differential and density gradient centrifugation steps *(3, 4)*. Other methods are either quite expensive and contamination-prone (e.g. immunoprecipitation) or apply only to cultured cells such as surface labeling, which cannot be applied to bulky tissue.

Interest in novel, more efficient subcellular purification protocols has recently emerged from the impressive progress in the analytical part of proteomics. Current mass spectrometry can identify proteins at concentrations of less than 1 pM. This allows for detection of proteins such as the neuronal PSD-95, which is present at 300 copies/postsynaptic density (Chen et al., 2005) in as few as 2×10^6 neurons, based on 1,000 postsynaptic densities/cells. Hence, proteomics studies on functional or anatomically well-characterized small tissue samples or scarce biopsy material come into reach. However, protocols for the isolation of defined subcellular compartments did not keep pace, despite the recognized need to analyze the subproteome of the various compartments separately. This cellular dissection is mandatory to detect low abundant proteins and to identify compartment-specific post-translational modifications or significant changes in protein localization. Changes therein often underlie physiological and pathophysiological processes *(5)*.

A highly selective and efficient method to separate membranes of different subcellular origin was developed more than 30 years ago by Albertsson and colleagues and was based on the use of aqueous polymer two-phase systems *(6, 7)*. These systems often form, when aqueous solutions of two structural different water-soluble polymers are mixed above a defined concentration. Most often, poly(ethylene glycol) (PEG) and dextran are used as polymers, as they are cheap, require only moderate concentrations, separate easily, and preserve protein structure and function well. When mixed, phases will form, and the upper phase will be enriched in PEG, whereas the bottom phase will mainly contain dextran. Interestingly, the various cellular membranes have different affinities to partition in either of the two phases. PMs prefer the upper phase, whereas mitochondria partition rather to the bottom phase. This different behavior can be attributed to differences in hydrophobic and hydrophilic surface properties of membranes, most likely arising from differences in their phospholipid composition.

Membranes differ only subtly in their surface properties and the isolation of PMs by aqueous polymer two-phase systems cannot be achieved in a single step. One possibility to increase the

purity of the PM fraction is multistep extraction procedures such as countercurrent distribution (CD) (**Fig. 1**). CD is based on the Nernst distribution law. Membranes are separated by this method on the basis of their different solubilities in two immiscible aqueous solutions of structurally different polymers. These two phases, flowing into opposite directions, are brought into contact, mixed, and allowed to separate. PMs preferentially partition to the top phase and will reside there throughout the multiple extractions. Intracellular membranes such as mitochondria will preferentially partition to the bottom phase throughout the procedure. We recently adapted this principle to the isolation of

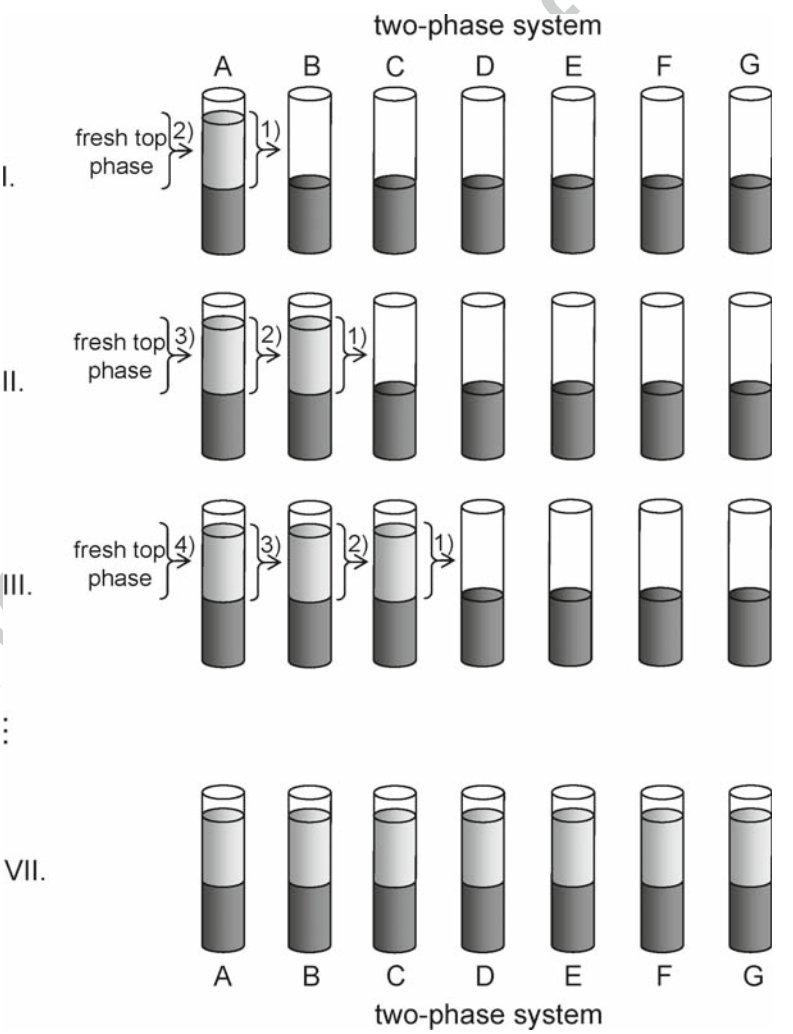

Fig. 1. Scheme of countercurrent distribution. In CD experiments, the top-phase of the first two-phase system A is transferred to a fresh bottom-phase B and the bottom-phase of two-phase system A is re-extracted with a fresh top-phase. After six iterations, biomaterial is efficiently separated.

PMs from the nervous system *(8)*. The protocol is fast, easy to perform, and yields up to 30% of the initial PMs with high purity. Contaminations by endoplasmic reticulum and mitochondria, the major contaminations in standard protocols, were low.

2. Materials

Owing to the strong influence of ions on membrane partitioning in the two-phase systems, double distilled water should be used throughout the experiments.

2.1. Two-Phase Systems

1. Glass-Teflon homogenizer.
2. Dextran stock solution: Dextran T500 (20%, w/w) (*see* **Note 1**).
3. PEG stock solution: PEG 3350 (40%, w/w).
4. Tris–H_2SO_4: Tris (200 mM), pH 7.8 adjusted with H_2SO_4.

3. Methods

3.1. Two-Phase Partitioning

All steps of the affinity two-phase partitioning protocol should be performed at 4°C. Working at room temperature prevents phase separation. The procedure is illustrated in **Fig. 1**. The numbers in **Fig. 1** correspond to the numbered two-phase systems given in **Table 1**, and the letters refer to the phases as indicated in the protocol given below.

1. Prepare seven two-phase systems with the compositions indicated in **Table 1**, 1 day prior to use. Mix them by 20 invertations, vortexing for 10 s, another 20 invertations, and store

Table 1
Composition of two-phase systems

	Two-phase system "A" (g)	Two-phase systems "B–G" (g)
Dextran stock solution	1.035	1.035
PEG stock solution	0.518	0.518
Tris–H_2SO_4	0.750	0.750
Water	0.598	0.698

the mixtures at 4°C overnight. Two-phase systems will form overnight with the top phase enriched in PEG and the bottom phase enriched in dextran.

2. On the next day, remove all top phases from two-phase systems "B–G" and store them separately.
3. Homogenize 0.1 g brain tissue in two-phase system "A" using a glass-Teflon homogenizer followed by 45 s of sonication. Centrifuge at 700 × *g* for 5 min to accelerate phase separation.
4. Transfer the top phase (Top) of two-phase system "A" onto the bottom-phase (Bot) of two-phase system "B" (TopA → BotB) (*see* **Note 2**). Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A" (I. in **Fig. 1**). Mix both two-phase systems by 20 invertations, vortex for 10 s, then mix again by another 20 invertations. Centrifuge at 700 × *g* for 5 min to accelerate phase separation.
5. Transfer top phases in the following order (II. in **Fig. 1**): (1) TopB → BotC; (2) TopA → BotB. Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A." Mix all two-phase systems by 20 invertations, vortexing for 10 s, and another 20 invertations. Centrifuge at 700 × *g* for 5 min to accelerate phase separation.
6. Transfer top phases in the following order (III. in Fig. 1): (1) TopC → BotD; (2) TopB → BotC; (3) Top A → BotB. Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A."

   Mix all two-phase systems by 20 invertations, vortexing for 10 s, and another 20 invertations. Centrifuge at 700 × *g* for 5 min to accelerate phase separation.
7. Transfer top phases in the following order: (1) Top D → BotE; (2) TopC → BotD; (3) TopB → BotC; (4) Top A → BotB. Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A." Mix all two-phase systems by 20 invertations, vortexing for 10 s, and another 20 invertations. Centrifuge at 700 × *g* for 5 min to accelerate phase separation.
8. Transfer top phases in the following order: (1) TopE → BotF; (2) Top D → BotE; (3) TopC → BotD; (4) TopB → BotC; (5) Top A → BotB. Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A." Mix all two-phase systems by 20 invertations, vortexing for 10 s, and another 20 invertations. Centrifuge at 700 × *g* for 5 min to accelerate phase separation.
9. Transfer top phases in the following order: (1) TopF → BotG: (2) TopE → BotF; (3) Top D → BotE; (4) TopC → BotD; (5) TopB → BotC; (6) Top A → BotB. Add an equal amount of fresh top-phase (stored in **step 2**) onto bottom-phase "A."

   Mix all two-phase systems by 20 invertations, vortexing for 10 s, and another 20 invertations. Centrifuge at 700 × *g* for 5

min to accelerate phase separation. After phase separation, you end up with seven two-phase systems (VII. in **Fig. 1**). PMs are enriched in TopF and TopG.

10. PMs can be recovered from TopG or combined TopF + G (*see* **Note 3**) by diluting the phases 1:10 with water followed by ultracentrifugation at 1,50,000 × *g* and 4°C for 1 h.

4. Notes

1. Dextran can contain up to 10% water and for that reason has to be freeze-dried. For freeze-drying, dissolve dextran in distilled water in a plastic dish with a large surface (e.g. Petri dish), freeze it at –80°C, and dry it by sublimating the water under vacuum. Store the freeze-dried dextran in closed plastic tubes sealed tightly with parafilm at –20°C. Let it come to room temperature before opening to protect it from humidity.
2. In two-phase systems, interphases are always considered as part of the bottom phase.
3. The purity of PMs from TopG alone is slightly higher but combining TopF and TopG nearly doubles the yield.

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