Purification of Lipid Rafts from Bacterial Membranes

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Abstract

The membranes of eukaryotic cells contain microdomains that are different in lipid composition to the surrounding membrane and aggregate a number of proteins related to signal transduction and protein trafficking. These are referred to as *lipid rafts* or *membrane rafts* and are specialized in the regulation of cellular processes related to signal transduction, protein sorting, and membrane trafficking. The integrity of lipid rafts is important for the correct functionality of these raft-harbored cellular processes, and their alteration is related to the occurrence of severe diseases. We recently discovered that the membranes of bacterial cells also organize their signal transduction pathways in functional membrane microdomains that are structurally and functionally similar to the lipid rafts of eukaryotic cells. The existence of lipid rafts in the membrane of bacteria suggests that bacteria are more complex organisms than previously appreciated, and thus, their cellular complexity should be explored in more detail. In this protocol, we provide a detailed description of the materials and techniques that are necessary to purify the lipid rafts from bacterial membranes, which is a necessary step to explore the number of proteins and lipid species that constitute these membrane platforms. This is an essential protocol for any laboratory interested in exploring any aspect related to organization of lipid rafts in bacterial membranes.

Keywords: *Bacillus subtilis*, Bacteria, Flotillin, Lipid rafts

1 Introduction

Compartmentalization of cellular processes in organelles improves their efficiency and the specificity of biological reactions. Physical compartmentalization of proteins in subcellular domains increases proximity and the probability of the assembly of specific protein complexes and signaling cascades (1). Because of this, there are a number of cellular compartments that specialize in specific cellular processes (2). For instance, mitochondria are specialized in respiration and generation of energy. The endoplasmic reticulum is functionally specialized in the translation of proteins (2). However, possibly one of the most recent examples of cellular compartmentalization is the aggregation of a large number of membrane proteins related to signal transduction and protein trafficking into membrane microdomains that are different in lipid composition to

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the surrounding membrane (3). These membrane domains are referred to as *membrane rafts* or *lipid rafts*, and they are responsible for the organization and functionality of many diverse cellular processes related to signal transduction and protein trafficking (3–5).

The organization of the cellular membrane into membrane microdomains or lipid rafts is a relatively recent concept in biology. The first membrane model proposed by Sanger and Nicolson suggests that lipid and protein components of cellular membranes diffuse freely and randomly (hereof, its name of "fluid mosaic model") (6). Therefore, it was assumed that all protein and lipid constituents were homogeneously distributed across the biological membrane. However, the pioneering fluid mosaic model has been subjected to several interpretations in the past decades. Specifically, it was discovered that biological membranes are composed of several distinct lipid species that tend to coalesce in specific areas simply due to their physicochemical affinities (4, 7). Importantly, the heterogeneous organization of distinct lipids into discrete membrane microdomains leads to a diverse distribution of the proteins. The particular subcellular distribution of proteins appears essential for their functionality (7–9). The lateral organization of lipids and proteins in specific regions of the membrane is nowadays referred to as membrane domains (10, 11).

Many types of cells organize membrane domains that are specialized in regulating diverse cellular processes. For example, polarized epithelial cells show basolateral and apical membrane macrodomains that contain a different composition of lipids and proteins and are specialized in different biological functions (12–14). Neurons also show different membrane domains exhibiting particular lipid and protein composition, according to their role in synapses (15, 16). The membranes of eukaryotic cells also organize a variety of proteins related to signal transduction and membrane trafficking into the lipid rafts. Those are discrete membrane regions at the nanoscale level that are enriched in specific lipids, like cholesterol or sphingolipids (3). Lipid rafts are well-organized membrane structures whose organization requires the activity of specific proteins that are known to exclusively localize within lipid rafts. One of these raft-associated proteins is flotillin (17–21). Flotillin is a membrane chaperone that acts as a scaffolding protein to facilitate the recruitment of the proteins that need to localize in lipid rafts to be active and facilitates their interaction (17–21).

Initially, the existence of lipid rafts was exclusively associated with eukaryotic cells because prokaryotic cells are unable to produce cholesterol and, therefore, unable to organize cholesterol-rich membrane microdomains (3–5). In addition to this, bacteria were considered too simple organisms to require a complex compartmentalization of their membranes and signaling networks. Consequently, it was unlikely that bacterial cells were able to assemble lipid rafts in their membranes. However, recent subcellular compartmentalization

structures have been found in bacteria (22), including the discovery that bacterial cells are able to assemble membrane platforms that are functionally and structurally similar to the lipid rafts of eukaryotic cells (23). Bacterial lipid rafts are formed by the self-aggregation of noncyclic polyisoprenoid lipids and their co-localization with raft-associated flotillin proteins (23–27). Similarly to eukaryotic flotillins, bacterial flotillins seem to act as scaffold proteins to recruit the protein cargo of the lipid rafts and facilitate the interaction and oligomerization (23–27). Bacterial lipid rafts were discovered in the model organism *Bacillus subtilis*, but it is believed that many other bacterial species are able to assemble lipid rafts in their membranes.

As many prokaryotes lack cholesterol in their membranes (28), the functional organization of the membrane into microdomains or lipid rafts depends on the existence of lipid species different from cholesterol. The exact nature of these has not yet been shown, but there is experimental evidence that other polyisoprenoid lipids that may have similar physicochemical properties to cholesterol play a role in the assembly of lipid rafts in bacteria (28, 29). For instance, *B. subtilis* produces membrane-associated polyisoprenoid lipids of carotenoid nature (30, 31). When the synthesis of carotenoids is inhibited in *B. subtilis* cells, the integrity of the microdomains is perturbed (23). Importantly, the integrity of the microdomains can be reconstituted by adding carotenoids to the cultures of the deficient *B. subtilis* strain (23). The integrity of the bacterial functional membrane microdomains also depends on the activity of the raft-associated protein flotillin. This protein is well conserved in all kingdoms of life and is indeed expressed in many bacterial species (32). It is a scaffold protein that facilitates the recruitment and interaction of the raft-associated proteins (17–21).

In summary, regardless of the species under consideration, lipid rafts could be described as rigid, compact, and superhydrophobic membrane microdomains that concentrate a specific composition of lipid and proteins and are specialized in regulating certain cellular processes (3–5). One of the key aspects regarding the research of lipid rafts has been the development of reliable techniques to allow the purification and further examination of lipid rafts. The most reliable purification technique is based on the ability of the lipid rafts to resist disaggregation when the membrane fraction is treated with a mixture of nonionic detergents. This treatment generates one membrane fraction that is sensitive to detergent disaggregation (detergent-sensitive membrane fraction, DSM) and another fraction that is composed of larger membrane fragments because they were more resistant to detergent disruption (detergent-resistant membrane fraction, DRM) (33). These two different membrane fractions can be physically separated according to their size in a sucrose gradient and analyzed independently. It is important to emphasize that the DRM fraction should not be equated to the lipid raft fraction, although it is known that this fraction is highly

enriched in lipid rafts (33). However, one should always consider that this is an artificial technique for lipid raft purification that could generate biased results, causing proteins to migrate to one fraction or another due to their efficiency to bind the detergent, their size, the temperature during the separation process, or the time that the separation took place (4, 5). In fact, the non-rigorous use of this methodology in the past led to the generation of data difficult to reconcile and opened a debate about the existence of lipid rafts and whether lipid rafts were artifacts generated during the purification process (34, 35). Therefore, the purification of the DRM fraction is an excellent point to start the analysis of lipids and proteins that constitute lipid rafts, but further experiments need to be performed to validate any potential lipid and protein candidate. Biochemical procedures based on protein-protein interaction assays using pull-down assays or a bacterial two-hybrid system are generally preferred to address further questions related to raft-associated lipids and proteins.

We present in this section a detailed methodology for isolating the DRM fraction from the cellular membrane of the bacterial model *B. subtilis*. This methodology can be applied to other bacterial models with minor modifications. This protocol for the purification of the DRM fraction should be of interest to any laboratory interested in the study of bacterial lipid rafts (Fig. 1).

2 Materials

2.1 Cell Growth, Harvesting, and Lysis

1. Growth medium: Luria-Bertani medium (LB) 0.5% NaCl, 1% tryptone, 0.5% yeast extract, pH 7. To prepare solid media, bacto-agar at the final concentration of 1.5% was added to the final growth medium.
2. Lysozyme solution 1 mg/ml in PBS buffer (Roth).
3. Phenylmethanesulfonyl fluoride (PMSF) solution 100 μM in DMSO (AppliChem).
4. DNase I (New England Biolabs®).
5. Cell lysis device: French press (10,000 psi, 4 passes) (SLM Aminco Instruments).

2.2 Membrane Purification

1. Buffer H (see general buffers)
2. Purification device: Ultracentrifuge Optima™ L-80 XP (Beckman Coulter) (100,000 × *g*)
   Rotor 70.1 Ti (fixed angle) with appropriate polycarbonate tubes
3. Glycerol 10% solution in buffer H

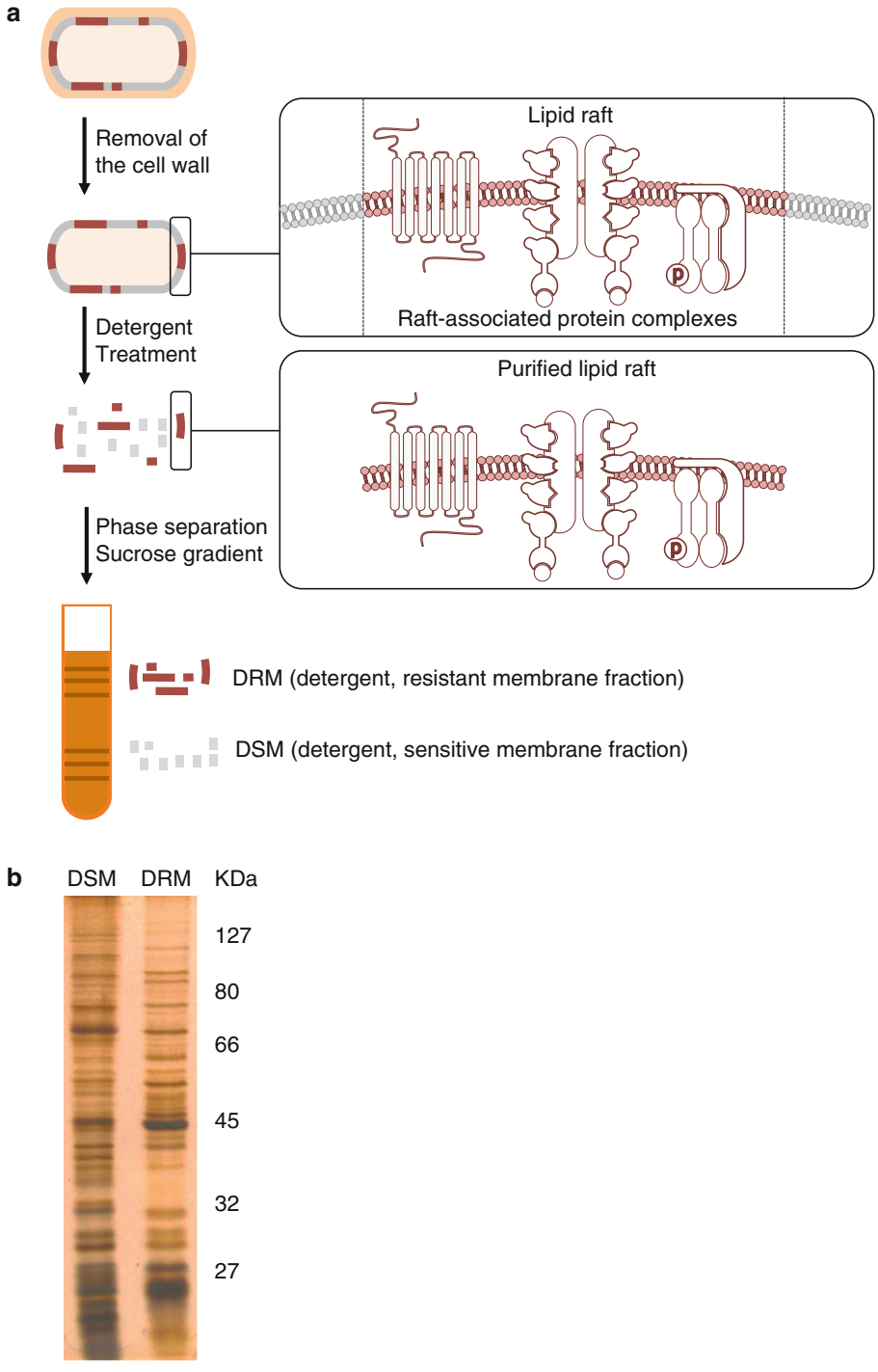

Fig. 1 Purification of the DRM fraction from *B. subtilis* membranes. (**a**) Schematic representation of the protocol for the purification of the DRM fraction of *B. subtilis* membranes. This approach could be applied to other bacterial species. After cell wall removal using a lysozyme treatment, the membrane fraction is disaggregated using a mixture of nonionic detergents. Lipid rafts are resistant to detergent disruption and

2.3 Membrane Disaggregation

1. Detergent cocktail: Triton X-100, Lubrol, Brij 96, Nonidet, CHAPS, and octylglucoside (concentrations from 1% to 4%) (all from Sigma-Aldrich).
2. Buffer H.

2.4 Sucrose Gradients

1. Eighty percent sucrose solution in 0.2 M sodium carbonate.
2. Twenty percent sucrose solution in buffer H.
3. Sucrose gradient device 1: Ultracentrifuge (40,000 × *g*)
   Rotor SW40 Ti (swinging bucket) with appropriate polycarbonate tubes
4. Optional: Sucrose gradient maker

2.5 Fraction Examination

1. Ice-cold acetone.
2. Buffer PBS.
3. 1× Protein loading buffer: 62 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue.

2.6 Bacteria Strain

1. *Bacillus subtilis* strain 168 was used a reference strain.

2.7 General Buffers and Reagents

1. Buffer PBS = 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7
2. Buffer *H* = 20 mM HEPES, pH 8, 20 mM NaCl, 1 mM DTT, 1 mM PMSF

3 Methods

3.1 Cell Growth, Harvesting, and Cell Lysis

We provide in this protocol the general procedure to grow bacterial cells in liquid medium. Alternatively it is also possible to collect cells from solid medium. Growth conditions could change depending on the species under consideration.

1. Grow *B. subtilis* strain 168 in 100 ml of LB liquid medium at 37°C for 24 h. It is important to allow cells to reach stationary phase before harvesting (*see* **Note 1**).

Fig. 1 (continued) remain larger fragments that can be further separated from the rest of the membrane by zonal centrifugation using a sucrose gradient. Zonal centrifugation renders two different membrane fractions: a DSM fraction (detergent-sensitive membrane fraction) that is composed of smaller fragments and is the consequence of a successful disruption by detergent treatment and another detergent-resistant membrane fraction (DRM) that is composed of larger fragments because they were not disrupted by the detergent treatment and is supposed to be enriched in lipid rafts. (**b**) Analysis of the protein composition of the DRM and the DSM fractions using SDS-PAGE. The electrophoresis analysis shows that there is a different protein composition in the DSM and the DRM fraction, suggesting that the subcellular localization of membrane proteins is strongly influenced by the different lipid composition of the cellular membrane

2. Pellet the cells by centrifugation (10 min, 4,000 rpm, 4°C) and remove the supernatant. Wash the cells twice with PBS buffer. At this point, the washed dry pellet can be stored at –20°C until further usage.
3. Resuspend the cell pellet in 10 ml of buffer H. It is important to ensure that cells are well resuspended and dispersed in buffer H. The presence of cell clumps will prevent a correct cell lysis.
4. To lyse the cells, add 200 µl of lysozyme (1 mg/ml), 100 µl of PMSF (100 µM), and 5 µl of DNase I to the suspension and incubate the sample at room temperature for 30 min. This step is necessary to dissolve the peptidoglycan cell wall of bacterial cells, which is especially prominent in gram-positive bacteria. Next, pellet the cells by centrifugation (10 min, 4,000 rpm, 4°C) and carefully resuspend in 10 ml buffer H (*see* **Note 2**).
5. Perform cell disruption using a French press (10,000 psi and 4 passes). After disruption, eliminate cell debris by centrifugation (10 min, 12,000 rpm, 4°C) and use the supernatant for membrane purification.

3.2 Membrane Purification

1. The membrane fraction can be purified using an ultracentrifuge with a fixed angle rotor (100,000 × *g* for 1 h at 4°C).
2. Discard the supernatant and dissolve the membrane fraction in 200 µl buffer H supplemented with glycerol 10% by persistent pipetting (*see* **Note 3**).
3. At this point, the membrane fraction dissolved in 200 µl buffer H + glycerol 10% can be stored at –20°C until further usage. Alternatively, you can immediately proceed with the membrane disaggregation step.

3.3 Membrane Disaggregation

1. Disruption of the cellular membranes should be performed using a mixture of nonionic detergents such as Triton X-100, Lubrol, Brij 96, Nonidet, CHAPS, or octylglucoside (*see* **Note 4**).
2. Use a range of concentration of detergents between 1% and 4% for the disruption of cell membranes. Unfortunately, the optimization of the detergent treatment needs to be determined empirically. The disruptive activity of each detergent component from the mixture depends on external factors like the duration of the procedure or the temperature of the process (*see* **Note 5**).
3. In our particular case, we normally use a mixture of Triton X-100, Brij 96, and Nonidet, 1% for a membrane disruption process of 30 min at 4°C (*see* **Note 6**). We determined these conditions empirically as referred to in Sect. 3.3(2) and **Note 5**.

3.4 Sucrose Gradients

1. Mix the sample thoroughly with 800 µl 80% sucrose in 0.2 M sodium carbonate (keep sample at 4°C).

2. Transfer the sample into an ultracentrifugation tube and carefully overlay it with 4 ml 20% sucrose in buffer H and 3 ml 10% sucrose in buffer H, respectively (keep sample at 4°C) (*see* **Note 7**).
3. Separate the sucrose gradient fractions by ultracentrifugation with a swinging bucket rotor (15 h, 40,000 × g, 4°C).
4. Carefully take fractions of 1 ml volume by aspiration from the very top of the sucrose gradient and keep the samples on ice for further protein precipitation and analysis (*see* **Note 8**).

3.5 Fraction Examination

1. To analyze the protein content of each membrane fraction, protein precipitation can be performed to purify the protein content of the samples. To do this, add 4 volumes of ice-cold acetone to each sample fraction.
2. After 2 h of incubation at −20°C, precipitate denatured proteins by centrifugation (20 min, 15,000 rpm, 4°C).
3. Remove the supernatant and dry the pellets at room temperature for up to 2 h.
4. The protein pellet should be carefully and repeatedly washed with PBS buffer. After washing, resuspend the protein samples in 1× protein loading buffer and store at –20°C prior to protein analysis.
5. For separation of protein samples, use SDS polyacrylamide gels consisting of a stacking gel and a resolving gel with 12% acrylamide/bisacrylamide (37.1:1).
6. Stain the SDS gel with Coomassie to visualize the differential protein contents of the DSM and DRM.

4 Notes

1. The diversity of lipid composition in bacterial membranes is greater in cells undergoing stationary phase because many unusual membrane lipids are produced at stationary phase. Alternatively, cells grown on solid agar medium generally show more variety in the cell growth and allow detecting a more diverse lipid composition in bacterial membranes.
2. The cell pellet needs to be handled with care at this point. The absence of a cell wall makes bacterial cells extremely sensitive to any type of stress.
3. It is important to note that the purified membrane fraction is strongly enriched in membrane lipids and therefore, it is hard to dissolve in aqueous buffers like buffer H. The addition of glycerol 10% to the buffer H is highly convenient to dissolve the membrane fraction in buffer H. However, dissolving the membrane fraction still requires vigorous pipetting.

4. These nonionic detergents are optimal to particularly disaggregate cellular membranes according to their lipid composition. The unusual lipid composition of lipid rafts, which are enriched in noncyclic polyisoprenoid lipids, provides rigid, compact, and hydrophobic properties to these membrane microdomains (like a floating raft, hereof its name of lipid raft), which confer to lipid rafts the capacity to resist detergent disaggregation by nonionic detergents.
5. An alternative approach to purify the DRM can be achieved with the purification kit CelLytic™ MEM for protein extraction (Sigma-Aldrich® Ref. CE0050). The kit contains a mixture of nonionic detergents, which has been optimized to achieve a high-performance purification of the DRM fraction. The kit also contains a gel phase that separates into two fractions in response to lower temperatures (4°C). DSM and DRM membrane fractions separate with the gel phases after treatment and can be further isolated and purified.

   We performed a comparative analysis between the CelLytic MEM kit and the traditional purification method. The protein composition of the DRM fraction was similar using both approaches.
6. When using any of these two technical approaches for membrane phase separation, it is crucial that manipulation of cellular membranes and detergent disruption procedures are performed at 4°C. This is because low temperatures enhance the separation of the different membrane lipids and the stabilization of lipid ordering in bacterial membranes.
7. Distinct layers of different sucrose concentrations must be visible prior to ultracentrifugation. In order to avoid mixing of the different solutions let sucrose slowly run down the wall of the ultracentrifugation tube. Alternatively, a gradient maker can be used to make the sucrose gradients.
8. Fractions of 1 ml each are a good starting point for the analysis of raft-association of a protein. Taking more fractions improves the resolution of the assay, but also increases the necessary time and effort.

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