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TITLE:

Bottom-up in vitro methods to assay the ultrastructural organization, membrane reshaping and curvature sensitivity behavior of septins.

AUTHORS AND AFFILIATIONS:

Brieuc Chauvin*; Brieuc.chauvin@curie.fr; Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, Sorbonne Université, CNRS UMR168, 75005, Paris, France

Koyomi Nakazawa*; Koyomi.nakazawa@curie.fr; Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, Sorbonne Université, CNRS UMR168, 75005, Paris, France

Alexandre Beber; Alexandre.Beber@ibt.cas.cz; Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, Sorbonne Université, CNRS UMR168, 75005, Paris, France ; current address : Institute of Biotechnology, Czech Academy of Sciences, BIOCEV, Prague, Czech Republic

Aurélie Di Cicco; Aurilie.dicicco@curie.fr; Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, Sorbonne Université, CNRS UMR168, 75005, Paris, France

Bassam Hajj; Bassam.hajj@curie.fr; Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, Sorbonne Université, CNRS UMR168, 75005, Paris, France

François Iv; iv.francois@yahoo.fr; Institut Fresnel, CNRS UMR7249, Aix Marseille Univ, Centrale Marseille, 13013 Marseille, France

Manos Mavrikakis; manos.mavrikakis@fresnel.fr, Institut Fresnel, CNRS UMR7249, Aix Marseille Univ, Centrale Marseille, 13013 Marseille, France

Gijsje Koenderink; G.H.Koenderink@tudelft.nl, Department of Bionanoscience, Kavli Institute of Nanoscience Delft, Delft University of Technology, Van der Maasweg 9, 2629 HZ Delft, The Netherlands

João T. Cabral; j.cabral@imperial.ac.uk, Department of Chemical Engineering, Imperial College London, London SW7 2AZ, UK

Michaël Trichet ; michael.trichet@sorbonne-universite.fr, Sorbonne Université, CNRS, Institut de Biologie Paris-Seine (IBPS), Service de microscopie électronique (IBPS-SME), F-75005, Paris

Stéphanie Mangenot[#]; stephanie.mangenot@parisdescartes.fr, Laboratoire Matière et Systèmes Complexes (MSC), Université Paris Cité, CNRS UMR 7057, 75006 Paris, France.

Aurélie Bertin[#]; aurelie.bertin@curie.fr, Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, Sorbonne Université, CNRS UMR168, 75005, Paris, France

* and #: Both authors equally contributed to the work

SUMMARY:

Septins are cytoskeletal proteins. They interact with lipid membranes and are able to sense but also generate membrane curvature at the micron scale. We describe in this protocol bottom-up in vitro methodologies meant to analyze membrane deformations, curvature-sensitive septin binding, and septin filament ultrastructure.

ABSTRACT:

Membrane remodeling occurs constantly at the plasma membrane and within cellular organelles. To fully dissect the role of the environment (ionic conditions, protein and lipid compositions, membrane curvature) and of the different partners implicated in specific membrane reshaping processes, we undertake in vitro bottom-up approaches. In the recent years, we have been particularly interested in revealing the role of septin proteins, implicated in major diseases. Septins are essential and ubiquitous cytoskeletal proteins that interact with the plasma membrane. They are implicated in cell division, cell motility, neuro-morphogenesis and spermiogenesis among other functions. In that context, we want to understand how septins interact and are organized at membranes, subsequently induce membrane deformations and can be sensitive to specific membrane curvatures. We aim at deciphering the interplay between the ultra-structure of septins at a molecular level and the membrane remodeling occurring at a micron scale. To this end, budding yeast and mammalian septin complexes are recombinantly expressed and purified. We use a combination of in vitro assays to analyze the self-assembly of septins at the membrane. SLBs (Supported Lipid Bilayers), GUVs (Giant Unilamellar Vesicles), LUVs (Large Unilamellar Vesicles) and wavy substrates are used to study the interplay between septin self-assembly, membrane reshaping and membrane curvature.

INTRODUCTION:

Septins are cytoskeletal **filament-forming proteins** interacting with lipid membranes. Septins are ubiquitous in eukaryotes and essential to numerous cellular functions. They were identified as main regulators of cell division in budding yeast and in mammals^{1, 2}. They are involved in membrane reshaping events, ciliogenesis³, and spermiogenesis⁴. Within mammalian cells, septins can also interact with actin and microtubules⁵⁻⁷ in a BORG dependent manner⁸. In various tissues (neurons⁹, cilia³, spermatozoa¹⁰), septins have been identified as regulators of diffusion barriers for membrane-bound components¹¹. Septins have also been shown to regulate membrane blebbing and protrusion formation¹². Septins, being multi-tasking proteins, are thus implicated in the emergence of various prevalent diseases¹³. Their mis-regulation is associated with the emergence of cancers¹⁴ and neurodegenerative diseases¹⁵.

Depending on the organism, several septin subunits (from two (**Caenorhabditis elegans**) to thirteen (**humans**)) assemble to form complexes whose organization varies in a tissue-dependent fashion¹⁶. The basic septin building block gathers two to four subunits, present in two copies, self-assembled in a rod-like palindromic manner. In budding yeast, septins are octameric^{17, 18}. In situ, septins are often localized at sites with micrometer curvature: they are found at division constriction sites, at the base of cilia and dendrites, and at the annulus of spermatozoa^{19, 20}. At

the membrane, the role of septins seems to be dual: they are implicated in reshaping the lipid bilayer and in maintaining membrane integrity²¹. Hence, investigating the biophysical properties of septin **filament-forming proteins** and/or subunits at the membrane is crucial to understand their role. To dissect specific properties of septins, in a well-controlled environment, bottom-up in vitro approaches are appropriate. So far, only **a limited number of labs have described the biophysical properties of septins in vitro**^{20, 22, 23}. Hence, **as compared with other cytoskeletal filaments, our current knowledge on the behavior of septins in vitro remains limited.**

We describe in this protocol how the organization of septin filaments, membrane reshaping and curvature sensitivity can be analyzed¹⁹. To this end, we use a combination of optical and electron microscopy methods (fluorescence microscopy, cryo-electron microscopy (cryo-EM) and Scanning Electron Microscopy (SEM)). The membrane reshaping of **micrometric**-sized Giant Unilamellar Vesicles is visualized using fluorescence optical microscopy. The analysis of the arrangement and ultrastructure of septin filaments bound to lipid vesicles is performed using cryo-EM. Septin curvature sensitivity is carried out using SEM, by studying the behavior of septin filaments bound to solid supported lipid bilayers deposited on wavy substrates of variable curvatures, **which enables the analysis of curvature sensitivity for both positive and negative curvatures. As compared with previous analysis**^{20, 24} **we propose here to use a combination of methods to thoroughly analyze how septins can self-assemble, synergistically deform membrane and be curvature sensitive. We believe our protocol will be useful and adaptable to any filamentous protein which displays an affinity for membranes.**

PROTOCOL:

1. Determination of membrane reshaping using Giant Unilamellar Vesicles (GUVs)

Giant Unilamellar Vesicles are generated to mimic the membrane deformations possibly induced by septins in a cellular context. Indeed, in cells, septins are frequently found at sites with micrometer curvatures. GUVs have sizes ranging from a few to tens of micrometers and can be deformed. They are thus appropriate to assay any micrometric-scale septin-induced deformations. Fluorescent lipids as well as fluorescently labelled septins (Green Fluorescent Protein (GFP)) are used to follow the behavior of both the lipids and the proteins by fluorescence microscopy.

1.1. Buffers and solution preparation

1.1.1. Prepare the 'Growth Buffer' (50mM NaCl, 50mM Sucrose and 10 mM Tris (pH = 7.8)) and the 'Observation Buffer' (75mM NaCl and 10 mM Tris (pH=7.8)).

1.1.2. Measure and adjust the osmolarity of the Observation and Growth Buffers (of 170 mOsmol.L⁻¹, in theory) by adding small amounts of NaCl using a commercial osmometer until their respective osmolarity are equal. Filter the buffers using a 0.2 µm filter. Aliquot the Growth buffer and store it at -20°C for further use. Store the Observation Buffer at 4°C.

Note: The difference of osmolarity between both buffers should not exceed 5%.

1.1.3. Prepare a 5 mg.mL⁻¹ β-casein solution in the Observation Buffer. Complete dissolution must be achieved (after several hours at 4°C and stirring with a magnetic stirrer). Filter the solution with a 0.2 µm filter, aliquot and store the solution at -20°C.

1.2. Lipid preparation

Prepare the lipid mixtures at a total lipid concentration of 3 mg.mL⁻¹ in chloroform. A molar ratio of 56.8 % EggPC (Egg L-α-phosphatidylcholine), 15 % cholesterol, 10 % DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), 10 % DOPS (1,2-dioleoyl-sn-glycero-3-phospho-L-serine), 8 % brain PI(4,5)P₂ (L-α-phosphatidylinositol-4,5-bisphosphate), and 0.2 % Bodipy-TR-Ceramide is used to enhance the protein-lipid interactions and favor the incorporation of PI(4,5)P₂²⁵.

NOTE: Chloroform must be handled under a fume hood using nitrile gloves and safety glasses. Plastic should be avoided given that chloroform can dissolve plastic. Glass syringes must be used to pipette chloroform solutions. Before and after use, the syringes must be rinsed by pipetting chloroform 5 to 10 times. Separate syringes should be specifically used to pipet specific fluorescent lipids to prevent any contamination from one experiment to another. Lipids can be stored in chloroform at -20°C in an amber glass vial capped with Teflon. The vials must be filled with argon before closing and sealed with parafilm to prevent any lipid oxidation.

1.3. GUVs electro-formation using a platinum wires setup.

A scheme of the experimental steps (1.A) and picture of the chamber at each step of the preparation procedure (1.B) is presented in Figure 1.

1.3.1. Chamber cleaning. Clean the platinum wires and the chamber first thoroughly to remove

any lipids. Hence, rub the wires thoroughly as detailed below. Plunge the wires and the chamber in acetone and sonicate for 10 min. Wipe everything carefully with a paper tissue using acetone. Assemble the chamber by inserting the wires, plunge again in acetone, and sonicate for 10 min. Wipe the chamber once more with acetone, making sure the wires are fully cleaned. Plunge the chamber in ethanol, sonicate for 10 min, and wipe with ethanol. Finally, plunge the chamber in deionized water, sonicate for 10 min, and dry with a stream of nitrogen or air.

Note: The Teflon chambers (Figure 1.B) were custom-made from our in-house workshop. They accommodate several chambers that can be sealed on both sides using glass coverslips. Platinum wires can be inserted all along the chamber through specific holes (1.3 mm in diameter).

1.3.2. Deposition of lipids: Deposit about 3 to 4 drops (less than 1 μL) of the lipid mixture at 3 mg. mL^{-1} on each of the platinum wire. The wires are rotated by 180° and deposit 3 to 4 to lipid droplets on the opposite side.

Note: The drops should not contact one another. About 5 μL of the lipid mixture are required per chamber.

1.3.3. Insert the growth chamber within a vacuum chamber for 30 min to remove any trace of chloroform.

NOTE: When dried, the lipids are vulnerable to oxidation. Deep vacuum (0.1 mbars) is best. The dried lipids must not be left in air for over a few min.

1.3.4. Chamber bottom sealing: deposit high vacuum grease at the bottom of the chamber (side closest to the wires) at the periphery of the three wells using a syringe. Press a clean (22*40 mm) coverslip against the grease to ensure a perfect sealing. Both extremities of the chamber are sealed (at the entry/exit sites of the wires) using sealing paste (wax plates). Place vacuum grease in a similar fashion on the other side of the chamber.

1.3.5. Electro-formation. Fill the **chambers** with growth buffer (about 1 mL is required per chamber). Do not stir too rapidly and strongly the solution to prevent any detachment of the lipid film from the wires. Seal the top of the chamber hermetically using a 22*40 mm coverslip by pressing it against the grease. Discard air bubbles from the chamber. Place the chamber at 4°C , in a fridge, and connect the wires to a wave function generator with the sine function at 500 Hz. For short growths (6 h), set an effective voltage of 350 mV while for longer growths (12 to 16 h) apply a voltage of 250 mV **as already presented and optimized in Beber et al²⁵**.

1.3.6. Chamber cleaning after use. Remove the windows. Wipe off all sealant and grease. Remove the wires, and wash and scrub the chamber with a tissue using water and ethanol ($\geq 70\%$) alternately.

NOTE: The optimal voltage and time scale for GUVs growth depend on many parameters, from the buffer salt concentration to the chamber geometry (distance between the wires and size). The same chamber should be used every time the experiment is repeated to ensure reproducibility.

NOTE: The wires are in proximity with the bottom of the chamber, so that the lipids can be imaged using fluorescent microscopy. Imaging the lipids at each step (See Figure 1.B) is advised to make sure the electro-formation process is successful.

[Place Figure 1 here]

1.4. Septin incubation with the vesicles

- 1.4.1. GUVs gentle collection. Collect GUVs from the wires by using pre-cut pipette tips that are brought in close vicinity of the wires. Then, pipet the solution all along the wire. This procedure prevents the generation of strong lamellar flows that could disrupt GUVs. Following this step, cutting pipette tips is no longer required. Indeed, lamellar flows do not damage GUVs in solution.

NOTE: Because of the presence of PI(4,5)P₂ in the lipid mix, GUVs that have been collected must be stored for no more than 2-3 hours prior to the experiment. Indeed, PI(4,5)P₂ gets solubilized rapidly and septins no longer bind to the membranes a few hours after their formation. Once septins are bound, however, they remain bound to the membrane for a few days.

- 1.4.2. Dilute the septins stock solution in Tris 10 mM (pH 8) **exclusively** to reach an osmolarity equal to the one of the Growth Buffer. The septin solution can, if needed, be further diluted in the Observation Buffer. Add the intended volume of collected GUVs. The incubation can be performed directly in the observation chamber after passivation with β -casein (see below). 20 to 30 min waiting time is required to reach equilibrium.

NOTE: The expression and purification of septin octameric complexes (human or budding yeasts) are extensively described in other articles^{26, 27}. But briefly, **septins are expressed in E. coli and purified following the dedicated protocol, in the lab. Affinity, size exclusion and ion exchange purification steps are performed. After production, septins are kept at -80° in a Tris-HCl 50mM, pH 8, KCl 300 mM, MgCl₂ 5 mM solution at about 1mg.mL⁻¹ (3 μ M). Septin complexes should not be concentrated through a filter centrifugation device, which induces aggregation and thus yield reduction.**

1.5. Imaging with confocal and/or spinning disk microscope.

- 1.5.1. Passivation: To prevent the GUVs from sticking to the surface and/or explode, passivate the observation chamber with a 5mg.mL⁻¹ β -casein solution which is left for a 30 min incubation.
- 1.5.2. Remove the β -casein solution and insert the collected GUVs in the observation chamber, **in the observation buffer, using a pipette**. The composition discrepancy between the interior of the GUV and the external buffer creates both a density and refractive index mismatch. Let the GUVs sediment at the bottom of the chamber for 10 to 15 min.
- 1.5.3. Observation: Thanks to the refractive index mismatch, GUVs are visible with transmission light optical microscopy. Using confocal microscopy, visualize the fluorescent signal of the lipids to check for the GUV quality and membrane lamellarity state. Assess the density of

septins bound to GUVs by recording the septins fluorescent signal, after performing a proper calibration²⁵. Perform Z-stack acquisitions at 0.4 μm spatial interval to analyze and visualize the 3D deformations of the vesicles induced by the interaction between the septins and the membrane.

NOTE: Oil immersion objectives with 60- or 100-fold magnifications were used. Nikon confocal or spinning disk microscopes with pixel sizes of 250 nm and 110 nm, respectively were used. To collect images, a EZ-C1 Nikon software was employed. Those microscopes are standard microscopes that can be found in any imaging facility. Any given lab would have to adapt their imaging to their own piece of equipment. No specific anti-photo bleaching agent were added in solution.

2. Analysis of septin filaments ultrastructural organization by cryo-electron microscopy

Vesicles are not suitable for imaging with standard electron microscopy methods. Indeed, using standard negative stain methods, samples are dried. Upon dehydration, vesicles are likely to undergo unspecific deformations, frequently resulting in lipid protrusions. Cryo-electron microscopy is thus a much better strategy to observe specific deformations of vesicles. Indeed, using cryo-EM, the samples are embedded within a thin (about 100-200 nm) layer of vitrified ice, which preserves the samples in a close to native state. GUVs are too large (several tens of micrometers) to be embedded within thin ice and thus imaged by transmission electron microscopy. Hence, Large Unilamellar Vesicles (LUVs), whose diameters range from about 50 to 500 nm, are generated to determine both how septins can deform vesicles and how they arrange on vesicles.

2.1. Generation of Large Unilamellar Vesicles

- 2.1.1. Prepare 50 μg of a mix of lipid solubilized in chloroform with a compositions (56.8% EggPC, 15% cholesterol, 10% DOPE, 10% DOPS, 8% brain PI(4,5)P₂; molar ratios) optimized to enhance septin interaction with the membrane in a glass vial.
- 2.1.2. Dry the solution under a flow of argon to generate a dried lipid film in the vial. Put the vial under vacuum for 30 min to achieve full lipid drying.
- 2.1.3. Resolubilize the lipid film in an aqueous solution (50 mM Tris-HCl (pH 8); KCl 50 mM; MgCl₂ 2 mM) at 1 $\text{mg}\cdot\text{mL}^{-1}$ (50 μL) by vortexing for 10 s and transfer the solution into an Eppendorf tube.

Note: LUVs must be used at once for incubation with septins. Otherwise, the protein-lipid interaction will be weak. This crude re-solubilization process generates a heterogeneous population of vesicles with diameters ranging from 50 nm to 500 nm. Hence, a whole range of diameters, and thus curvatures, are simultaneously assayed.

2.2. Incubation with proteins

Incubate the septins with the solubilized lipids at a final septin and lipid concentrations of 0.1 $\text{mg}\cdot\text{mL}^{-1}$ (about 300 nM) and 20 nM respectively in a high salt buffer (50 mM Tris-HCl (pH 8), 300 mM KCl, 2 mM MgCl₂). Incubate the sample for one hour at room temperature.

2.3. Plunge freezing to vitrify the sample
2.3.1. Glow-discharge holey carbon grids (300 mesh) on the carbon side for 30 s at 5 mA using a plasma generator equipment and insert the grid inside a plunge freezing machine in a humid environment.

2.3.2. Adsorb 4 μL of the sample on the grid, on the carbon glow-discharged side. Immediately before sample adsorption, add 5 to 10 nm gold beads in solution, in case the samples are used to generate tilted series by cryo-tomography.

Note: The density of gold beads must be screened and adjusted empirically and depends on the provider. Optimized density should be 10 to 15 gold bead in the field of view.

2.3.3. Blot dry the samples from the bare side to aspirate the sample drop on the opposite side.
Note: The blotting time is typically 4 s, and the position of the filter paper and blotting force are adjusted empirically by testing and screening alternative positions of the filter paper to optimize the ice quality (thickness) and the material density.

2.3.4. Transfer the grids into a microscope or store them in a liquid nitrogen container.
NOTE: Using holey carbon grids is essential to accommodate the polydispersity in sizes of the vesicles. Blotting the sample from the opposite side favors an enhanced adsorption of the biological material on the grid.

2.4. Cryo-electron microscopy imaging

2.4.1. Insert the grids into an electron microscope equipped for cryo-EM observation and cooled down with liquid nitrogen. Screen the whole grid by generating a map of the whole sample at low magnification (120 magnification, typically), to select areas displaying better ice (thin and well vitrified).

2.4.2. Cryo-electron microscopy 2D data collection. Collect images at a pixel size of about 2 \AA per pixels to check for the quality of the sample.

Note: Both the septin filaments and the lipid bilayer should be visible.

2.4.3. Cryo-electron tomography data collection.

Select areas of interest displaying deformed vesicles.

Note: Enough gold beads (at least 10) should be present in the field of view.

According to the software used for tilted series data collection, select focus and tracking positions to be far enough from the area of interest. Collect tilted series by varying the tilt angle from -60 to +60 degrees, collecting an image every two or three degrees.

Note: The total dose should be about, or less than, 100 electrons/ \AA^2 . The pixel size varies from 1.3 to 2.1 \AA , depending on the microscope used for data collection. Ideally, an angular symmetric scheme for data collection is preferred as follows: 0, -3, +3, -6, +6, -9, +9[...] -60, +60 degrees. However, goniometers of side-entry microscopes do not provide enough mechanical stability to achieve those symmetric schemes. Alternatively, the acquisition can be started at 0 degrees to 34 degrees, followed by a second angular sequence from -2 degrees to -60 degrees, and ended

by a final sequence from 36 to 60 degrees. The purpose is to collect the first images (with the lowest radiation damages) at the lowest angles.

NOTE: To visualize the ultrastructure of vesicles and bound septin filaments, a standard cryo-EM microscope can be used (200kV, LaB6 (**Lanthanum hexaboride**) filament and sample side entry). However, if one aims at pursuing further image processing (sub-tomogram averaging, for instance), it is best to use last generation FEG (**Field Emission Gun**) microscopes equipped with direct detectors. In this protocol, we will restrict our description to the acquisition of 3D reconstruction and leave out sub-tomogram averaging.

2.5. Three-dimensional reconstruction generation from cryo-tomography and segmentation

2.5.1. Image alignment and 3D reconstruction. Use the IMOD software suite for tilted series image alignment and 3D reconstruction^{28, 29} (<https://bio3d.colorado.edu/imod/>). Within IMOD, perform tilt series alignment based on fiducials (gold beads) positioning. In addition, if needed, carry out contrast transfer function (CTF) determination and correction within IMOD²⁸. Finally, achieve 3D reconstruction with IMOD following each step rigorously.

2.5.2. Segmentation. Segment manually the lipid bilayers and septin filaments using 3Dmod²⁹ from the IMOD software suite, for display.

3. Analysis of septin curvature sensitivity using scanning electron microscopy

To understand how septins can be sensitive to micrometer curvatures, we have used an in vitro approach and incubated septin filaments complexes with solid supported lipid bilayers deposited on micrometer-scale wavy undulated patterns.

3.1. Design of wavy NOA (**Norland Optical Adhesive**) replica from wavy PDMS (**polydimethylsiloxane**) patterns.

3.1.1. Typically, use patterns of 250 nm amplitude and 2 μm lateral periodicity or other dimensions to assay the curvatures suitable to your protein of interest.

Note: PDMS (polydimethylsiloxane) undulated patterns are designed and generated as described in Nania et al.^{30, 31}. Briefly, centimeter large PDMS coupons were subjected to a plasma irradiation while mechanically stretched by a linear strain stage. A thin (~ 10 nm) layer of oxidized, glassy material is formed on the surface of the PDMS. After the mechanical tension is released, the PDMS surface spontaneously buckles, adopting a wavy periodic pattern. The periodicity and amplitude of the undulations can be controlled by tuning the mechanical strain and the power and duration of the plasma treatment. Hence, wavy undulated patterns of prescribed curvature could be produced.

3.1.2. NOA resin replica. From the PDMS substrates generate NOA replicas. In a clean room environment, deposit 5 μL of liquid NOA on a circular glass coverslip of 1 cm in diameter. Appose the PDMS wavy template on the drop. Treat with a UV light treatment at 320 nm for 5 min to photo-polymerize the liquid NOA into a thin polymer film.

The slide with the freshly polymerized NOA is then gently peeled off from the PDMS piece.

NOTE: NOA is a common optically transparent glue and is thus suitable for optical microscopy

imaging. In addition, NOA is resistant to the chemical fixation and staining processes carried out prior to SEM imaging. Both NOA 71 and NOA 81 resins are used with similar outcomes. The PDMS initial pattern could be used several times to produce NOA replicas. After their production NOA replicas can be kept at room temperature, in a box for months.

3.2. Fusion of a lipid supported bilayer on the NOA pattern and protein incubation

3.2.1. Treat the NOA films using a plasma cleaner for 5 min to make the surface of the pattern hydrophilic.

3.2.2. Prepare a solution of SUVs (Small Unilamellar Vesicles) with a molar composition of 56.8% EggPC, 15% cholesterol, 10% DOPE, 10% DOPS, 8% brain PI(4,5)P₂ and total lipid concentration of 1 mg.mL⁻¹. Prepare the SUVs by resuspending a dried lipid film in the Observation Buffer, like the one prepared in section 2.1.3. Sonicate gently the solution using a bath sonicator until the solution is transparent. Sonicate the solution for 5 to 10 min.

Note: The solution of SUVs can be stored frozen for several weeks at -20°C.

3.2.3. Insert the slides supporting the NOA patterns within cell culture boxes enclosing wells whose dimensions accommodate the 1 cm circular coverslips. Deposit 100 µL of the SUV solution at 1 mg.mL⁻¹ on the freshly glow-discharged NOA patterns and leave for incubation for 30 min, at room temperature. Following this incubation, induce the fusion of SUVs with the surface of the pattern to generate a supported lipid bilayer.

3.2.4. Rinse the slides thoroughly six times with the septin buffer (50 mM Tris-HCl (pH 8), KCl 50 mM, MgCl₂ 2 mM) to remove unfused SUV. After each rinse, never let the sample completely dried.

3.2.5. Dilute the octameric septin stock solution in septin high salt buffer (50 mM Tris-HCl (pH 8), KCl 300 mM, MgCl₂ 2 mM) in septin buffer (50 mM Tris-HCl (pH 8), KCl 50 mM, MgCl₂ 2 mM) to final concentrations ranging from 10 nM to 100 nM, and volumes of 1 mL. Incubate the protein solution on the slides for about one hour at room temperature.

Note: The volume is large enough so that evaporation is not an issue.

3.3. Sample preparation for SEM analysis

Various protocols have been developed in electron microscopy to analyze protein structures and their assembly. Our protocol preserves the organization of septin filaments, using a fixation protocol derived from Svitkina et al.³² that is easy to implement. Besides, this protocol optimizes high-resolution SEM observations.

3.3.1. Reagents and preparation of stock solutions

This protocol requires several reagents and solutions that can be prepared in advance, or just prior to incubation. Follow our instructions to avoid any artifacts or lack of chemical reactivity.

Sodium cacodylate 0.2M stock solution: prepare this double-strength stock solution of cacodylate buffer by dissolving powder into distilled water with a magnetic stirrer. After complete dissolution, adjust the pH to 7.4 by gently adding 0.1 M HCl (almost 1 mL is required in case of a 50 mL solution). Add distilled water to adjust to the final volume. This solution can be stored for 24 - 48 h at 4°C.

2% glutaraldehyde (GA) in 0.1 M sodium cacodylate: Prepare this solution right before the fixation. It is important for glutaraldehyde to have a high level of purity (EM Grade). Prepare the fixative solution to obtain a final concentration of 2% GA in 0.1 M sodium cacodylate. Use a 25 to 50 % commercial stock solution of GA because of its optimized storage capacity at -20°C. Dilute this GA solution with the double strength stock solution of sodium cacodylate (see above).

1% Osmium tetroxide (OsO₄) in 0.1 M sodium cacodylate: Prepare this solution right before use. Use commercial 4% OsO₄ stock solution in cleavable glass bulbs because of their storage and handling properties. It is important to note that OsO₄ is highly reactive. Make sure that its color is slightly yellow and not dark. Prepare this second fixative solution to obtain a final concentration of 1% OsO₄ in 0.1 M sodium cacodylate. This OsO₄ solution is diluted with the double strength stock solution of sodium cacodylate described above.

1% Tannic acid (TA) in water: Prepare this solution right before use. Prepare the TA solution to obtain a final concentration of 1% TA in distilled water at room temperature. Dissolve for a few min by vortexing. TA solution cannot be stored and must be filtered with a 0.2 µm filter before being used.

1% Uranyl acetate (UA) solution in water: prepare UA solution to obtain a final concentration of 1% UA in distilled water. Dissolve for at least 30 min - 1h by vortexing or shaking at room temperature. This solution can be stored for a month at 4°C but can easily precipitate and must be filtered with 0.2 µm filters before being used.

Graded concentration of ethanol solutions: Prepare 50% - 70% - 95% - 100% ethanol solutions in water. As ethanol is highly hydroscopic, use a final bath in an anhydrous solution to remove all traces of water that might interfere with drying and coating. This last solution must be prepared from newly opened bottles of 100% ethanol, or from 100% ethanol that has been dehydrated for at least 24h with molecular sieves (nominal pore diameter: 4 nm). Be careful not to shake this solution as it might cause a resuspension of silicate particles.

Note: Be careful when handling the chemical reagents used in this protocol. Glutaraldehyde, osmium tetroxide, sodium cacodylate and uranyl acetate are all highly toxic, the last of this list being radioactive as well. All manipulation of the reagents and their waste must be done by using individual (gloves, lab coats, safety glasses) and collective protection (fume hoods and plexiglass shields), according to the procedures of your laboratory.

3.3.2. Sample fixation

3.3.2.1. Wash samples (NOA slides with fused supported lipid bilayers and incubated protein) with PBS then replace it with the fixative solution prewarmed at 37°C. Let the reaction proceed for 15 min. Samples can then be stored at 4°C.

3.3.2.2. Remove the fixative solution and wash fixed samples thrice with 0.1 sodium cacodylate, 5 min per washing with gentle shaking.

3.3.2.3. Incubate samples in the OsO₄ second fixative solution for 10 min with maximum protection from light. This second step allows for fixation of membranous structures and increases the electrical conductivity of the samples. Remove the second fixative solution and wash samples thrice in distilled water, 5 min per washing with gentle shaking.

3.3.2.4. Incubate washed samples in the filtered TA fixative solution for 10 min maximum. Remove TA solution and wash samples thrice in distilled water, 5 min per washing with gentle shaking.

3.3.2.5. Incubate washed samples in a freshly filtered UA fixative solution for 10 min with maximum protection from light. Remove UA solution and wash samples thrice in distilled water, 5 min per washing with gentle shaking.

3.3.3. Sample dehydration and critical point drying

Air-drying is not permitted, to avoid damaging samples through unwanted surface tensions when changing from the liquid to gaseous state. Two methods have been established in EM: the physical method of reaching the supercritical state of CO₂ and bypassing its critical point (31°C, 74 bar), or the chemical method of evaporating hexamethyldisilazane (HMDS), a drying agent with a reduced surface tension. CO₂ and HMDS are poorly miscible with water. Consequently, all traces of water must be substituted to a transitional solvent (Ethanol) to avoid any damage during later drying processes.

3.3.3.1. Incubate samples 2-3 min in each ethanol solution starting at 50% to reach the anhydrous ethanol bath.

Note: Be careful, as the evaporation of ethanol between baths is fast. The sample handling must also be fast to avoid any air-drying.

3.3.3.2. Transfer the glass slides inside the pre-ethanol-filled critical point dryer and follow manufacturer's instructions.

Note: In our protocol, we use an automatic apparatus, but any system can be used. Protocols may differ for each apparatus but must be optimized to completely remove ethanol (25 baths in our protocol) and reduce speeds for both the different solvent exchanges (CO₂ inlets and ethanol/CO₂ outlets) and for the final depressurization (almost 1h in our protocol).

3.3.3.3. At the end of the critical point drying, store samples immediately in a desiccator until mounting and coating. As dried samples are highly hygroscopic, we recommend coating them as soon as possible.

NOTE: Alternately, HMDS can offer a cheaper and faster method. Note that we have never used or tested it on our samples, but this approach produced great results for the observation of proteins at the inner face of cellular membranes^{33, 34}.

3.3.4. Sample mounting and coating

Since biological samples present poor electrical conductivity properties, they need to be

coated with a conductive metal film before SEM observation. Plasma-magnetron sputtering is thus used.

3.3.4.1. Attach the glass slides to stubs that will be used for future SEM observations. Use silver paint because of its improved electrical conductivity, as compared with carbon disks. Add a strip of silver paint to the upper face of the coverslip and make sure that the connection with the stub is satisfactory. Avoid any paint agglomerates.

Note: The paint strip must be thin, to avoid any contact between the sample and the objective lens of the SEM when working at high resolutions (*i.e.*, low working distance).

3.3.4.2. Wait for the solvent to evaporate completely.

Note: The duration of this step may vary depending on the amount and the thickness of the silver paint deposit. This step can be shortened by using a bell jar or the coater and a primary vacuum for 10-30 min.

3.3.4.3. Use an apparatus equipped with a plasma-magnetron sputtering head and a rotary-planetary stage. Use the standard protocols that are provided by the manufacturer. The apparatus is vacuumed to 2.5×10^{-5} mbar, purged once with high quality Argon before adjusting the pressure to 8.0×10^{-3} mbar for a pre-sputtering phase (120 mA for 60 s, to remove the oxide layer at the surface). Deposit 1.5 nm of tungsten (90 mA, working distance 50 mm) controlled by the film thickness monitor.

Note: The coater must be perfectly cleaned to ensure the reproducibility of the film evaporation. The coating must be stopped once the targeted thickness (indicated by the user) has been reached. The final film thickness is calculated and corrected afterward. Films of about 1.5 nm have, on average, a post-correction of 0.7 nm using our apparatus. Because those correction values are close to the target, a series of coatings are performed to assess and then subtract this correction from the target value.

3.3.4.4. Store the samples under vacuum to protect them from the ambient air until, and throughout all the SEM analyses.

Note: the nature of the metal used for sputtering matters. Platinum, despite being a common material, results in a coating of poor quality with a Pt film thickness adapted to septin filaments (1.5 nm). At high resolutions, a 1.5 nm platinum film exhibits a lack of cohesivity. The size of the platinum clusters and the septin filaments thereby become similar, leading to misinterpretations during the filament segmentation process³⁵ (see Figure 2.A, insert). Tungsten is a good alternative to platinum because it displays a smaller grain size, barely visible at high resolution SEM (see Figure 2.B, insert). Nonetheless, pure tungsten is easily oxidized, leading to strong charging effect artifacts during SEM observation if the procedure detailed in section 3.3.4. is not strictly followed.

[place Figure 2 here]

3.4 Sample observation by SEM

Acquire the images using a Field-Emission gun SEM (FESEM) microscope.

Note: the technology was recently upgraded with a novel electron optic design to improve resolution. This gain in resolution (accessible with other manufacturers), especially at low

accelerating voltage (0.9 nm at 1 kV), is necessary to resolve nanometric structures similar to septin networks.

Achieve high resolution imaging using the detection of primary secondary electrons (SE1) with the "in-lens" detector and with the settings below:

- accelerating voltage: 3 kV,
- the beam current is fixed with the 20 mm (34 pA) aperture or decreased with the 15 mm (18,5 pA) aperture if the suppression of charging effects is needed,
- observations are done with resolutions ranging from 21.25 to 1.224 nm/pixel, and at about 5.58 nm/pixel for data analysis (20 k magnification according to the Polaroid 545 reference),
- working Distance: between 1 and 2 mm for high resolution observation, around 3 mm if increased depth of field is required,
- scan speed and line integration are continuously adjusted to ensure a constant signal to noise ratio with an acquisition time around 30 to 45 s per image.

REPRESENTATIVE RESULTS:

1. GUVs deformations.

Typical confocal fluorescence images of GUVs reshaped after being incubated with septins are displayed in Figure 3, in conditions where septins polymerize. Bare GUVs (Figure 3.A) are perfectly spherical. Upon their incubation with more than 50 nM budding yeast septins filaments, the vesicles appear deformed. Up to a concentration of 100 nM budding yeast septin octamers, the vesicles appear faceted, and the deformations remain static and thus do not fluctuate (Figure 3.B). Above 200 nM budding yeast septins, with the membrane being saturated with septins, periodic deformations are observed (Figure 3.C). Using budding yeast septins, spikes are visualized with a periodicity varying from 2 to 6 micrometers and with an amplitude of about 1 micrometer. Hence septins strongly reshape membranes in a specific fashion. These amplitudes and associated curvatures of the observed deformations strikingly reflect the affinity of septins for micrometer curvatures observed in cells. GUVs, being deformable, are thus appropriate to assay how proteins reshape membranes.

[Place Figure 3 here]

2. Self-assembly of septin filaments bound to LUVs imaged by cryo-EM.

As compared with fluorescence microscopy, cryo-EM provides better spatial resolution. Individual septin filaments and lipid bilayers can thus be discerned unambiguously in images. Figure 4.A displays an image of LUVs decorated with budding yeast septin filaments (some of them being highlighted in blue) at 50 nM. Parallel sets of filaments, about 10 nm apart, are observed on the LUVs. This typical image is a two-dimensional projection. Hence, to decipher any 3D deformation and to know whether septin filaments directly interact with the membrane, cryo-electron tomography is carried out (Figure 4.B. and 4.C). Figure 4.B displays a slice in a three-dimensional reconstruction, while Figure 4.C. presents a segmentation of the same area highlighting the membrane in yellow and septin filaments in blue. As seen on the side view, septin filaments remain essentially straight bound to the liposome and the vesicle has been considerably flattened as compared with naked spherical vesicles.

[Place Figure 4 here]

3. Curvature dependent arrangement of septin filaments visualized by Scanning Electron Microscopy.

Septins localize, in cells, at locations displaying micrometer curvatures (e.g., at the cell division furrow, the base of cilia, etc.). In addition, in vitro studies show that septins, as seen above, can reshape membranes so they eventually display micrometer curvatures. To assay the curvature sensitivity of septins for both positive (convex) and negative (concave curvatures), we have incubated septins with a supported lipid bilayer fused with the wavy patterns described above. The outcome is displayed in Figure 5. **Figure 5.A recapitulates the different steps required to**

obtain the samples. Figure 5.B presents a wavy pattern at low magnification, where the periodicity of negative and positive curvatures is clearly visible. Two successive waves are pointed by orange solid lines. Besides defects, on average orthogonal to the waves are pointed by white arrows. On Figures 5.C and 5.D, at higher magnification, the ultrastructural organization of septin filaments is displayed. Septins assemble into sets of parallel filaments, whose orientation depends on the curvature (see filaments highlighted in blue). Indeed, septin filaments are not oriented randomly. Instead, septins can bend when interacting with negative (concave curvatures) to follow the imposed curvature. Conversely, on positive curvatures, septin filaments remain straight, unbent, and align along the convex waves. Hence, septins can interact with both positive and negative curvatures but adopt specific organizations on given curvatures. This methodology thus appears particularly relevant to highlight the curvature sensitivity of binding and self-assembly of filamentous proteins.

[Place Figure 5 here]

FIGURE AND TABLE LEGENDS:

Figure 1.

A. Schematic representation of the electro-formation process using platinum wires. B. Picture of the teflon home-made device used to generate GUVs by platinum wire electron formation. Wires are 0.5 mm in diameter and 3 mm apart. C. GUVs (**spherical objects**) observed by transmission optical microscopy during the growth process. The opaque zone at the bottom of the image is the platinum wire. D. GUVs (**round-shaped fluorescent objects**) observed with fluorescent microscopy during the growth on the platinum wire. Scale bars are 100 μm .

Figure 2.

Effect of the material deposited on septin filaments on wavy PDMS patterns. SEM of filaments which are coated by sputtering with either A) 1.5 nm of platinum, exhibiting the “arid cracked soil” pattern typical of a lack of cohesivity between the clusters of platinum nuclei, or B) 1.5 nm of tungsten covered with a smooth and cohesive layer. Scale bar 200 nm, white-boxed squares represent magnified views on lower right. The spherical globules are small lipid vesicles interacting with the septins.

Figure 3.

A. Equatorial plane of a Control GUVs labelled with 0.5% rhodamine PE, images by confocal microscopy. Scale bar 3 μm . B. GFP-septins (100 nM) bound to a deformed GUVs and imaged by fluorescence confocal microscopy. Scale bar 1 μm . C. GFP-septins (600 nM) bound to a deformed GUVs and imaged by fluorescence spinning disk microscopy. Scale bar 10 μm . The GUVs are made of a molar ratio of 56.8 % EggPC, 15 % cholesterol, 10 % DOPE, 10 % DOPS, 8 % brain PI(4,5)P₂, and 0.2 % Bodipy-TR-Ceramide. The observation buffer includes 75mM NaCl and 10 mM Tris (pH=7.8).

Figure 4.

A. Cryo-EM image of septin filaments bound to a vesicle. Some of the filaments are highlighted in blue for visibility. The dark dots are gold fiducials usually used to align data for tomography. Scale bar 100 nm. B. and C. 3D reconstruction obtained from a tilted series. The lipids are highlighted in yellow while the septins are segmented in blue. Scale bars: 200 nm.

Figure 5.

A. Schematic representation of the steps required to obtain the “wavy” samples meant to analyze septin curvature sensitivity imaged by SEM. B. Low magnification SEM image of a wavy pattern with septin filaments bound but not resolved at this resolution. Two consecutive waves are highlighted in orange. Orthogonal defects are pointed by white arrows. Scale bar 10 μm . C. SEM Image at 10 k magnification where septin filaments are visible. Scale bar 1 μm . D. SEM Image at 20k magnification. Some of the filaments are highlighted in blue for visibility. Scale bar 200 nm. The spherical objects present in the images are small vesicles interacting with the septins and substrate. The septin concentration was set at 200 nM for B, C and D.

DISCUSSION: (Instructions)

As stated above, we have used a lipid mixture which enhances PI(4,5)P₂ incorporation within the lipid bilayer and thus facilitates septin-membrane interactions. Indeed, we have shown elsewhere²⁵ that budding yeast septins interact with vesicles in a PI(4,5)P₂ specific fashion. This lipid composition was adjusted empirically from screening multiple compositions and is now widely used in our lab. PI(4,5)P₂ lipids have to be handled carefully. Stock solutions must be aliquoted in small volumes so that a specific vial is not opened more than twice for pipetting. Besides, lipid aliquots should be stored under argon to prevent lipid oxidation. Finally, once the lipid mixes are solubilized in an aqueous solution, the mixture must be used at once and cannot be stored for further experiments. We have noticed that after GUVs reconstitution or supported lipid bilayer fusion, the interaction between septins and lipids is greatly reduced after two hours. We have chosen to present some of the results obtained with octameric budding yeast septin complexes (enclosing Cdc10, Cdc11, Cdc12 and Cdc3). However, the experimental procedures followed to analyze the behavior of septin complexes from other species should be identical.

To generate GUVs, we prefer the electro-formation method using platinum wires over electro-formation on ITO plates or PVA swelling. As demonstrated earlier²⁵, this method reproducibly produces unilamellar GUVs of good quality without any defects.

The wavy substrates can be designed with a wide range of curvatures. After screening various designs, we have consistently used patterns with a periodicity of 2 μm and an amplitude of 250 nm which generate curvatures ranging from $-3.5 \mu\text{m}^{-1}$ to $3.5 \mu\text{m}^{-1}$ ($\pm 0.5 \mu\text{m}^{-1}$). Using patterns with a short periodicity and higher amplitude, the lipid bilayers did not nicely conform to the surface. Often, in the case of higher curvature, the bilayer would be suspended in between two convex waves instead of being fully supported on the NOA substrate. With lower amplitudes and larger periodicity, septins organized with random nematic-like orientations, indicating that the patterns were too flat. Besides, using wavy patterns instead of cylindrical tubes²⁰ or spheres³⁶ has the benefit that both positive and negative curvatures can be tested simultaneously. In the future, examining the behavior of filaments on gaussian “saddle-like” curvatures would be

relevant to mimic the curvature of more realistic cellular contexts.

In solution, mixing LUVs with septins induced the generation of septin-lipid aggregates even at low concentrations of proteins and lipids. The samples thus were heterogeneous, and it is wise to look for thinner ice domains where smaller and more defined septin-vesicle objects are found. This is particularly crucial when choosing areas of interest where cryo-tomography will be performed. Often, even at low magnifications, vesicles displaying protrusions of strong deformations as compared with spherical vesicles are more likely to have a high density of septin filaments bound. **This protocol focuses on obtaining a description of the global ultrastructure of filaments on biomimetic membranes. Currently higher resolutions are reachable. The resolution limitation will result from the limited number of views available by imaging. However, this remains beyond the scope of this protocol.**

We have shown through this protocol that a combination of complementary methods is essential to understand and dissect how the specific arrangement of cytoskeletal septin filaments can sense and reshape membranes in a curvature dependent manner.

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DISCLOSURES: ([Instructions](#))

The authors declare no conflicts of interest to be disclosed.

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