Nanoscale imaging of proteins' filament assembly on membrane in rebuilt cell membrane

Fouzia Bano[1], Gerard Castro-Linares[2], Agata Szuba[3]

[1]Department of Clinical Microbiology & Wallenberg Centre for Molecular Medicine, Umeå University, 901 87, Umeå, Sweden

[2]Department of Bionanoscience, Kavli Institute of Nanoscience Delft, Delft University of Technology, 2629 HZ Delft, The Netherlands

[3]School of Electronic and Electrical Engineering, University of Leeds, LS2 9JT, Leeds, United Kingdom

Atomic force microscopy (AFM) is a multipurpose technique that can simultaneously produce a high-resolution 3D image while also obtaining mechanical information of samples on surfaces. These abilities make AFM a very useful technique for studying filamentous membrane-bound proteins in their native environment with minimal sample preparation. Here we report how we used AFM together with other imaging methods to investigate a biomimetic cell membrane. Specifically, we studied how septin proteins, which are part of the cell's cytoskeleton, bind and polymerise on flat supported lipid bilayers. With AFM, single filaments and their substructures were visualised which were not resolvable via fluorescence microscopy, while also obtaining height and mechanical stability information. Taken together, this multi-technique study showed that septin form ordered arrays of single and paired filaments on lipid membranes that might mechanically support cells.

Introduction:

Protein polymerisation on the cell membrane is crucial for multiple cellular processes including cell motility, cell signaling, and intracellular transport. Sometimes this leads to disease, for instance in the case of amyloid fibers leading to Alzheimer's disease. These processes are often very complex and require the presence of multiple accessory proteins, but the basic mechanisms always involve creating forces leading to plasma membrane remodeling. The polymerisation dynamics of proteins and the nature of their association with lipid membranes is vital to understand the mechanisms leading to complex membrane shape changes. Here, we focus on the characterisation of septins, proteins involved in diverse cellular mechanisms including cell migration and cell division. Septins are filamentous proteins that have been recently recognised as a fourth element of the cell's cytoskeleton, a filamentous network that provides cells with shape and mechanical strength. Of the four cytoskeletal filament systems, the septin cytoskeleton is the only one interacting directly with the plasma membrane.

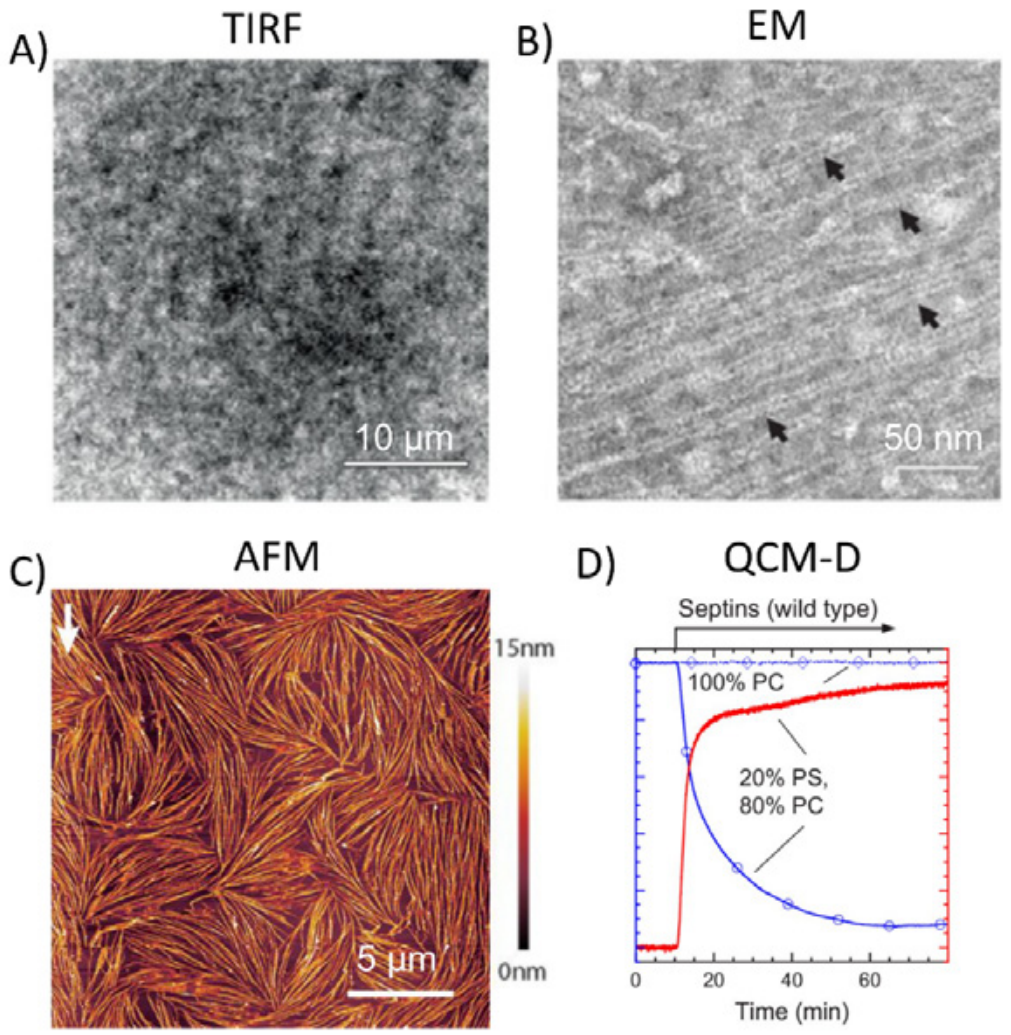

Figure 1. A multi-technique approach is needed for comprehensive understanding of fibrous protein assembly on membranes. An example is shown for septin filament assembly on a reconstituted membrane containing 20% negatively charged lipids. PS = Phosphatidylserine and PC = Phosphatidylcholine, a zwitterionic phospholipid. This figure is adapted from (Szuba et al. 2021). Each imaging technique (TIRF (A), EM (B) and AFM (C)) provided unique information about the organisation of the septin filaments on the membrane in steady state, whereas QCM-D (D) revealed the kinetics of the binding and assembly process. Black arrows in (B) indicate examples of individual paired septin filaments and white arrow in (C) show the direction of slow scan axis.

In the course of our recent journey to understand the process of septin self-assembly reported in *elife*, we used one of the most powerful nanoscale imaging tools – AFM. It is an exceptional imaging tool since it allows imaging of biomolecular systems in their native form, in liquid, and without the requirement of labelling. AFM has been successfully used for imaging of other filamentous proteins assembled in the bulk (e.g. amyloid fibers, collagen, fibrin). However, we quickly realised that AFM, in parallel with other imaging techniques, also is a unique tool to examine septin organisation on lipid membranes. This multi-technique approach

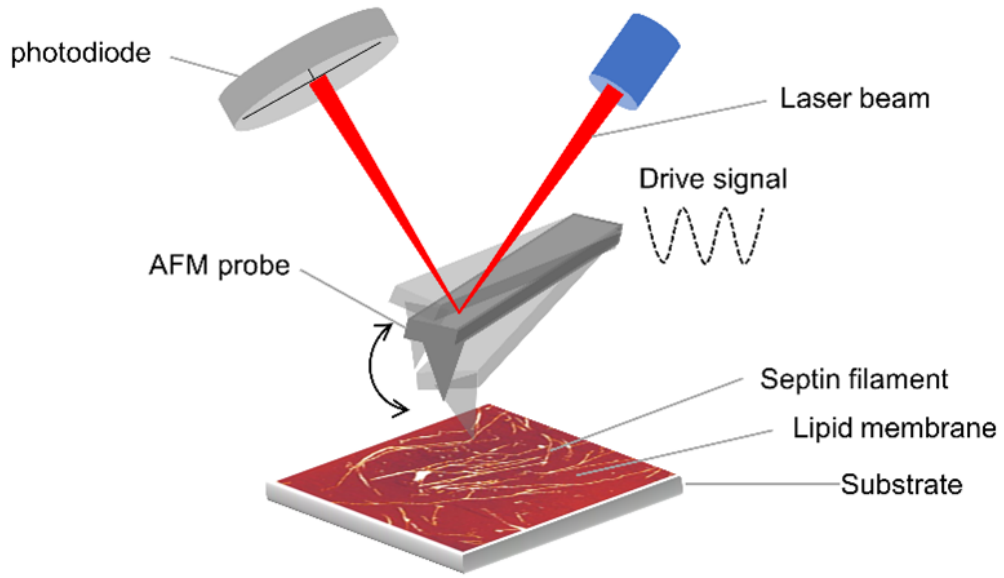

Figure 2. Schematic representation (not to scale) of imaging septins bound to a lipid membrane supported on a SiO_2 substrate with the AFM operating in PeakForce Tapping mode. In this mode, the AFM probe is oscillated with a drive frequency that is much lower than its resonance frequency, which permits imaging of the surface with intermittent contact as well as with low imaging force.

provided a comprehensive understanding of how septin proteins bind and self-assemble on lipid membranes. In this feature article, we will mainly focus on the role of AFM and discuss how AFM advanced our understanding of septin assembly on lipid membranes while at the same time providing information about septin filament mechanics.

A multi-technique approach for comprehensive understanding of fibrous protein assembly on membrane:

Self-assembly of proteins can lead to highly ordered structures such as filaments, virus capsids, 2D lattices, or less ordered and irreversible aggregates. Considering the highly complex and dynamic nature of such processes, knowledge about both the structural properties of individual or monomeric proteins and how the individual components are arranged relative to each other is needed. Septins provide an interesting example of a filament-forming protein whose assembly is tuned by membrane binding. Septins are a family of eukaryotic cytoskeletal proteins involved in important cell processes in animal cells such as cell division, vesicle trafficking, pathogen-host interactions, and regulation of cell surface rigidity. Septin monomers form hetero-oligomers that act as building blocks for their polymerisation into filaments and higher order structures. These structures are able to interact in turn with vital cell components such as the cell membrane, actin filaments, and microtubules (Mostowy and Cossart 2012).

We have recently investigated the interactions of recombinant fly septins with model lipid biomembranes using a set of techniques in a cell-free setting. Using optical microscopy, in our case total internal reflection fluorescence (TIRF) microscopy, we could observe that septins bind to membranes containing anionic lipids and form dense layers (Figure 1A, septin bulk concentration = 500 nM). However, due to the limitations of diffraction-limited optical microscopy, we were unable to resolve individual filaments of septins within these layers even at low bulk concentration of septin (50 nM, Figure 3A). Alongside TIRF, we used transmission electron microscope (TEM), which taught us that the septins arrange themselves in paired filaments (Figure 1B, septin bulk concentration = 65 nM). However, TEM operates in vacuum on dried samples and does not provide any direct information about the thickness of the septin layer or its mechanical properties. To gain further insights into septin's filament organisation and physical properties, we used AFM, which enabled us to record structural data at various length scales or scan sizes, (Figure 1C and Figure 3, septin bulk concentration = 60 nM) under physiological conditions, and at various experimental conditions (Figure 4) without the requirement of fluorescent labelling. Besides structural data, AFM also provided information about the mechanical properties of the septin filaments on the membranes – a unique feature of this imaging tool. We finally complemented all this information with quartz crystal microbalance with dissipation monitoring (QCM-D) experiments, which gave information about the kinetics of septin adsorption to the membrane (Figure 1D, septin bulk concentration = 60 nM). In the following sections we will describe, within the context of this septin-membrane study, how AFM is an ideal tool to obtain a whole range of information ranging from structural to mechanical.

AFM as an ideal imaging tool to study septins on lipid membrane

AFM is a powerful and multipurpose imaging technique that belongs to the family of scanning probe microscopies. It is powerful because it can create an image of scan sizes spanning from a few 100 nm to tens of micrometres in the XY direction (depending on the application or purpose of imaging) with nanometre scale information in height (or Z direction) without the requirement of sample labelling. It is multipurpose because it not only provides information about the sample topography (size and height) but also about different material properties such as mechanical (Szuba et al. 2021), electrical (Nonnenmacher et al. 1991), conductive (Murrell et al. 1993), and magnetic (Martin and Wickramasinghe 1987), to name a few. Other unique features of AFM are that (i) it is easy to work with AFM samples due to their large size (silica wafer with total surface area of ~1x1 cm^2 *vs.* ~3 mm EM grid), (ii) the sample time preparation is reduced considering no requirement for labelling step, and (iii) it can be operated in both vacuum and ambient conditions like air, water, and physiological buffer.

The working principle of AFM is rather simple. It is based on "feeling" the surface by a cantilever with a sharp tip on its free end to scan over the surface, equivalent to the tactile reading of braille. AFM images are three-dimensional (XYZ) maps of the surface topographic features. AFM can be operated in three classical imaging modes depending on how closely the tip is interacting with the surface. These modes are contact, intermittent (also known as tapping) contact and non-contact.

Contact mode offers high spatial resolution due to direct contact, hence higher lateral interaction forces. However, it is not useful when the sample is not laterally stable and can result in disruption of the sample due to interactions with the tip, which we found was the case for septin filaments on a lipid membrane. Tapping mode, in which the tip is intermittently tapping the sample during the imaging, is more suitable for soft or fragile samples. However, damping effects of the fluid together with cantilever oscillation immersed in a fluid cell can cause a change to the free amplitude of oscillation of the cantilever, which can result in a change in the applied forces during imaging. This change in applied force can fluctuate considerably and can result in a loss of resolution and damage to the tip or to the sample. In the past decade, the AFM community has witnessed the development of alternative dynamic modes that allow the users to collect "quantitative nanomechanical mapping" data of materials like polymers (Sheiko and Magonov 2012) and live cells (JPK 2011, Dokukin and Sokolov 2017). One such mode is known as "PeakForce Tapping™" (Pittenger et al. 2010) (Figure 2). By controlling the imaging force with high precision, this dynamic

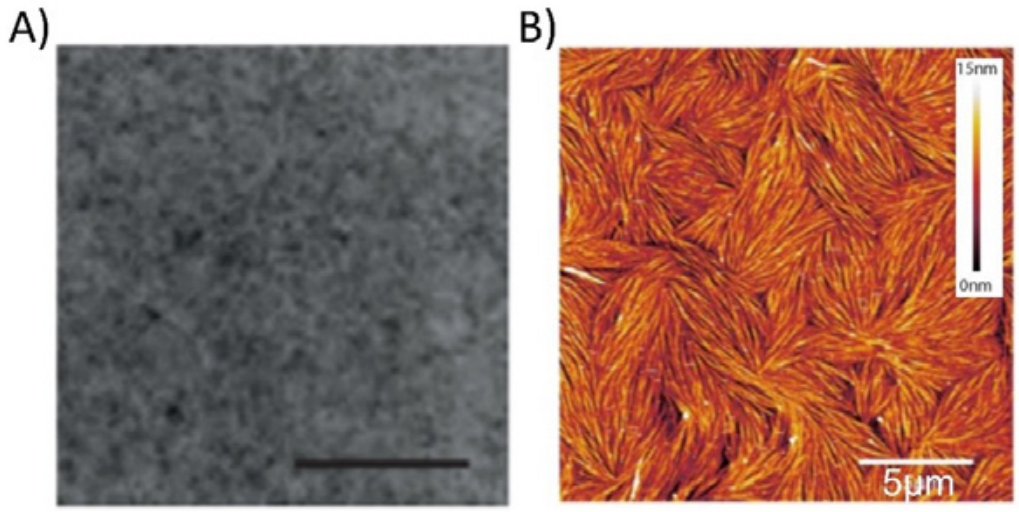

Figure 3. AFM imaging reveals what TIRF cannot about septin assembly on membrane: Purified fly septin hexamers which were deposited on a glass-supported lipid bilayer composed of 80% of net-neutral and 20% of anionic lipids and imaged using TIRF (A) and AFM (B). It is immediately clear that AFM provides a more detailed information about septin' filaments when imaged at comparable scan sizes of TIRF and at nearly similar septins bulk concentration (~50 nM). This figure is modified from (Szuba et al. 2021).

mode offers high stability of applied forces during imaging. This high stability has been achieved by the way the feedback loop is set up to control the tip-sample interactions. The applied force, also known as "peak force", is calculated from a pixel-wise force-distance curve, which is used as an input to regulate the z-piezo position for maintaining the tip-sample interaction at a certain set point or a force. This allows the users to image the sample at well-defined imaging forces, which prevents causing any major damage to the sample or to the tip. Some examples of challenging samples that were successfully imaged by this dynamic mode are DNA on mica (Pyne et al. 2014), Snf7 proteins on a lipid bilayer (Chiaruttini et al. 2015) and our own work on membrane-bound septin filaments (Szuba et al. 2021). Next, we highlight how this mode has enabled us to understand the structural arrangement of septin filaments on lipid membranes.

AFM is blind but it can 'see' at high spatial resolution

Historically, when it comes to image the surface at micrometer scale at similar experimental conditions, AFM can outperform conventional optical microscopy methods such as TIRF and confocal microscopy due to its ability to 'feel' the sample without the requirement of fluorescent labelling by a blind sharp tip. For instance, Chiaruttini et al in 2015 used AFM (PeakForce Tapping mode in liquid) to resolve the molecular structure of Snf7 patches on membranes for understanding how the polymerisation of ESCRT-III filaments could drive membrane curvature. (Major component of ESCRT-III) spirals on the surface of a lipid bilayer" by "proteins on a lipid bilayer. In their study, the authors have first reported about micrometer size patches observed by TIRF imaging, which were later resolved to be packed arrays of Snf7 circular assemblies on membranes when imaged by AFM. Furthermore, AFM also revealed that each assembly was formed by concentric circle-like structures from a single Snf7 spiraling filament. On a similar note, in our TIRF data, we found that septins were absorbed to anionic lipid bilayer but failed to provide any details due to limited resolution (Figure 3A and 1A). In contrast, with AFM nanoscale imaging, we discovered that septins formed either individual or bundled filaments, depending on the septin concentration in solution (Figure 3B and 4).

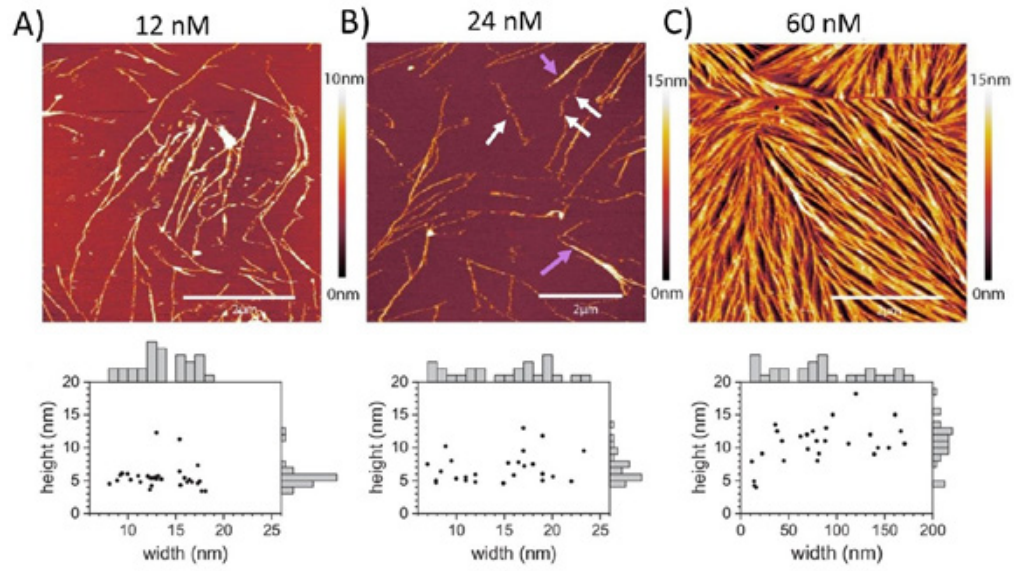

Figure 4. Height measurement of septin filaments and arrays thereof at various septin concentrations measured by AFM. Despite imaging with low force, single filaments were easily disturbed by the AFM tip (white arrows), while bundled filaments were more stable and did not change their positions (purple arrows). The figure is modified from (Szuba et al. 2021).

Beyond resolving filament/bundle forms (Figure 4A and 4B) with high resolution AFM, we also learned that septin filaments form dense arrays at high concentration of septins in solution (Figure 3B and 4C). These examples highlight how AFM can 'feel' (or image) better in comparison to commonly used fluorescence imaging techniques by avoiding the use of labelling tags. Moreover, these examples also demonstrate how, by taking advantage of imaging by a blind sharp tip, AFM can resolve the structural details of biomolecular assemblies under physiological conditions.

AFM provides more than just topographic features

Beyond topographic mapping, AFM imaging provides information about height and width of surface features. Moreover, it can yield information about the binding stability of the biomolecules to the underlying surface through consecutive imaging of the same surface provided that (i) there are no strong tip-sample interactions, and (ii) imaging is acquired with minimum or low imaging force. These conditions are essential because if the tip-surface interactions are too strong, molecules can either stick to the tip or can be easily moved around by the tip, even when imaging is acquired in the gentlest available imaging mode. If imaging forces are too high, the tip can completely displace molecules from the imaging frame and can distort the sample. We took advantage of these AFM abilities in our recent study and have gained additional information about septins. We observed that individual filaments have an average height of 5 nm (Figure 4, left), which increased to an average height of 13 nm when septins were attached as bundles (Figure 4, right). Even more interestingly, we found those individual filaments to be more sensitive to the touch of the AFM tip as compared to bundled filaments: specifically, individual filaments were brittle and appeared as "jittering lines" (white arrows, Figure 4, centre), whereas bundle filaments remained stably bound to the membrane (purple arrows, Figure 4, center).

On top of gaining information about molecular height and binding stability of attached molecules, AFM imaging can also provide quantitative information

about adhesion energy, elastic modulus, dissipation, and deformation. This is collectively known as nanomechanical mapping and can be performed by operating the AFM in modes commonly known as amplitude modulation, QI (Quantitative Imaging introduced by JPK) and an extended version of the PeakForce tapping mode called PeakForce QNM. The basic idea behind these advanced modes is (i) collection of force-distance curves at each pixel during imaging (or within an individual oscillatory cycle of the probe) and (ii) rapid fitting of these curves (approach curves) using models like the Hertz, Derjaguin-Muller-Toporov (DMT), or Johnson-Kendall-Roberts (JKR) models to calculate the adhesion energy and elastic modules. This allows the users to gain detailed information about the nanoscale mechanical properties of the sample - an investigation yet to be performed for septins.

Closing remarks

AFM is one of the most versatile imaging tools to study biological molecules because it can provide information about their morphology, assembly, and height, as well as mechanics, all at high spatial resolution. Additionally, its comparatively easy sample preparation, the ability to image under hydrated conditions, and the possibility to operate in various imaging modes make AFM an attractive technique in biology that can be applied both to living cells and to reconstituted systems.

In our recent study, by complementing AFM with fluorescence microscopy and TEM, we obtained quantitative insights into membrane-templated septin assembly (filaments and bundle formation) and showed that bundling protects septin filaments from mechanical damage. In follow-up work investigating nanomechanical mapping of septin filaments by AFM, we aim to understand the role of septins in biological processes like cell division and regulation of cell surface rigidity.

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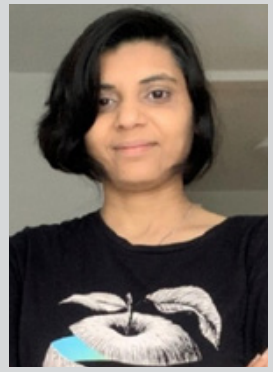
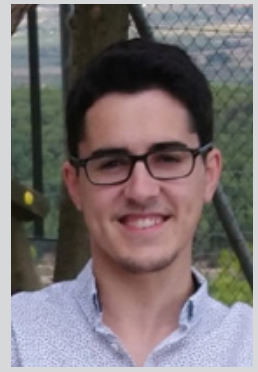
Dr Fouzia Bano

Senior Research Engineer, Umeå University



Fouzia has a MS in Physics, a PhD in Statistical and Biological Physics, and two postdocs in single molecule biophysics. Fouzia has worked in diverse research fields ranging from nanoscale surface chemistry, glycobiology and virology during her more than 12 years' research experience. Fouzia now works in the department of clinical microbiology at Umeå university as a senior research engineer to characterise the viruses-glycosaminoglycan interactions to elucidate their role in virus entry of the cell.

Gerard Castro-Linares



Gerard Castro-Linares is a PhD candidate at the Delft university of Technology, interested in cell division and synthetic biology. He is studying the interactions of septins with the cell membrane and the actin and microtubule cytoskeletons to understand the roles of septins in cell division and to explore their potential for a synthetic cell division mechanism.