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Novel Experimental Strategy for High Resolution AFM Imaging of Membrane-Associated Bacterial Toxins

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Abstract: Bacterial pore-forming toxins (PFTs) are essential virulence factors of many human pathogens. Knowledge of their structure within the membrane is critical for an understanding of their function in pathogenesis and for the development of useful therapy. Atomic force microscopy (AFM) has often been employed to structurally interrogate many membrane proteins, including PFTs, owing to its ability to produce sub-nanometer resolution images of samples under aqueous solution. However, an absolute prerequisite for AFM studies is that the samples are single-layered and closely-packed, which is frequently challenging with PFTs. Here, using the prototypical member of the cholesterol-dependent cytolysin family of PFTs, perfringolysin O (PFO), as a test sample, we have developed a simple, highly robust method that routinely produces clean, closely-packed samples across the entire specimen surface. In this approach, we first use a small Teflon well to prepare the supported lipid bilayer, remove the sample from the well, and then directly apply the proteins to the bilayer. For reasons that are not clear, bilayer preparation in the Teflon well is essential. We anticipate that this simple method will prove widely useful for the preparation of similar samples, and thereby enable AFM imaging of the greatest range of bacterial PFTs to the highest possible resolution.
Key words: atomic force microscopy (AFM), perfringolysin O (PFO), bacterial pore forming toxins (PFTs), supported lipid bilayers
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0 Introduction

Many human pathogenic bacteria secrete water-soluble pore-forming toxins (PFTs) as critical virulence factors[1-2]. These proteins bind to a cellular membrane as monomers and then self-assemble on the membrane surface, finally forming bilayer-inserted pores[1-3]. There is presently a great deal of interest in understanding the functioning of these proteins in pathogenesis and also, from a biophysical perspective, the mechanisms by which they undergo such a remarkable transition.

For both purposes, structural information is vital. Yet obtaining structural details of the membrane-bound form of these proteins, like many integral membrane proteins, is often challenging. X-ray crystallography requires water-soluble proteins. Therefore, membrane proteins must be solubilized in an appropriate detergent (while remaining in a functional conformation), which is often challenging. Cryo-electron microscopy can generate images of membrane proteins within a lipid bilayer, but the inherently low signal-to-noise ratio of this technique requires averaging of many different images, and this can be problematic with inherently heterogeneous samples, as are many membrane-bound PFTs. In this regard, atomic force microscopy (AFM) is a particularly effective structural approach for membrane protein studies since it is capable of determining the structures of proteins in lipid bilayers to sub-nanometer resolution under nearly physiological solutions directly from unprocessed images[4-7]. A number of membrane proteins, and PFTs, have indeed been well imaged with AFM[8-11].

With these successes, the present AFM technology appears sufficient for high resolution structural investigations of a wide range of PFTs. However, in the end, what prevents many AFM investigations is not a failure in the technology but a difficulty in preparing the sample in a suitable manner for optimal interrogation with AFM: ultimately, it is absolutely essential that the sample is single-layered and closely-packed. Highly

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dense samples demand incubation with sufficiently high protein concentrations but incubating with high protein concentrations invariably leads to multiple layers of bound protein, often non-specifically and loosely. These additional layers obscure the membrane-associated protein from direct AFM probing, and also are easily attached to the tip during imaging, which fundamentally prevents further higher resolution imaging.

The two most common methods of preparing supported lipid bilayers for membrane protein studies with AFM are vesicle fusion (VF) and exclusively using small Teflon wells (described below)[12]. Yet these methods are often not effective for studies of PFTs. For reasons that are not clear, many PFTs do not insert into supported lipid bilayers prepared by VF. Using Teflon wells produces samples that vary greatly in density across the specimen surface, from completely protein-free regions to multi-layered regions, which makes it difficult to find a good region for imaging other than trial-and-error.

Here, we have investigated methods to optimally produce useful samples for AFM imaging employing the PFT, perfringolysin O (PFO), as a test sample. PFO is a prototypical member of the large family of cholesterol dependent cytolysins[13]. These proteins are known to self-assemble into a large range of differently-sized membrane-inserted pores. Therefore detailed structural information of such a heterogeneous sample will likely require single-molecule methodologies like AFM.

We have identified a simple, highly robust method that routinely produces clean, closely-packed samples of PFO in a supported bilayer across the entire specimen surface. As this method is reasonably simple and is not dependent on any unique property of PFO, we expect this method will turn out to be broadly useful for the preparation of membrane-associated PFTs, and thereby enable structural studies using AFM that would otherwise not be possible.

1 Materials and Methods

PFO was expressed and purified by Sangon Biotech (Shanghai, China). Egg phosphatidylcholine (eggPC) and cholesterol were purchased form Avanti Polar Lipids (Alabaster, AL, USA). The Langmuir trough used in these experiments was from KSV Instruments (Espoo, Finland). The Teflon wells were 5 mm in diameter and 2.5 mm deep, and the diameter of side port was 1 mm. The muscovite mica was from Mei Feng Industry, Limited Liability Company, China.

1.1 Sample Preparation with the Vesicle Fusion Method

First, eggPC and cholesterol dissolved in chloroform were combined to amount-of-substance ratio of 1 : 1 and then the chloroform was evaporated under nitrogen gas. The dried film was then re-suspended in aqueous buffer (PB buffer, 10 mmol/L sodium phosphate, pH 7.4) to a concentration of 1 g/L, yielding a multi-lamellar vesicle suspension. The suspension was next sonicated to form small unilamellar vesicles (SUVs) (until the suspension was clear). A droplet (20—40 μL) of this solution was then applied to freshly cleaved muscovite mica. After incubation for about 40 min at 60 °C, the sample was cooled to room temperature and then rinsed with PB buffer to remove excess SUVs. Finally, PFO was added to the sample surface to a final concentration of about 30 mg/L, followed by incubation for 2 h at room temperature, and then rinsed with PB buffer to remove excess proteins. The sample was taken to the AFM, always under solution.

1.2 Sample Preparation with the Teflon Well Method

The mica fragment was first submerged within the aqueous solution of the Langmuir trough. EggPC dissolved in hexane/ethanol (1/1, V/V) at a concentration of 2 g/L was then added to the air/water interface of the trough. Following evaporation of the organic solvent, the monolayer was compressed to a final surface tension of 32 mN/m. The mica was then slowly raised up through the air/water interface, which deposited the monolayer on the mica. Separately, a droplet (about 1 μL) of 1 g/L eggPC/cholesterol (amount-of-substance ratio of 1 : 1) dissolved in chloroform/methanol (2/1, V/V) was applied to the air/water interface of the Teflon well. The solution in the well was the PB buffer. Following evaporation of the organic solvent, the monolayer-coated mica was horizontally lowered through the monolayer in the Teflon well, forming the supported bilayer. Finally, PFO to a final concentration of about 30 mg/L was injected into the well through a side port, followed by 30 min incubation. The sample was then removed from the well and taken to the AFM, always keeping the sample under solution.

1.3 New Sample Preparation Method

The supported bilayer was prepared following the same method as described above in the Teflon well. The first monolayer (facing mica) was eggPC, while the second monolayer was eggPC/cholesterol (amount-of-substance ratio of 1 : 1). After the supported bilayer was formed in the Teflon well, the sample was removed from the well and then the PFO was added to the sample surface at a final concentration of about 30 mg/L. Following 1 h incubation at room temperature, the sample was rinsed with PB buffer and taken to the AFM, always under solution.

1.4 AFM Imaging

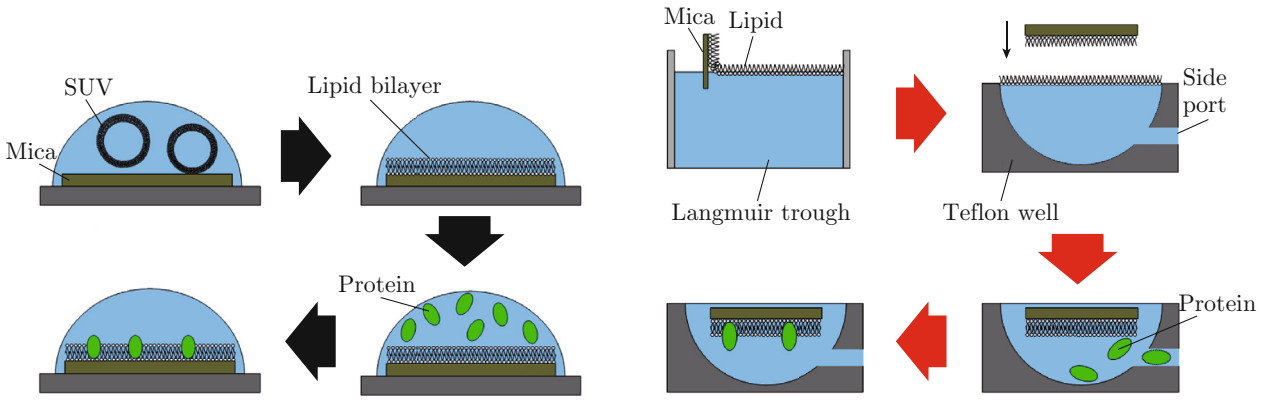
Imaging was performed in the contact mode with a Nanoscope Multimode^{TM} AFM (Veeco, Santa Barbara, CA) or Nanoscope Multimode 8 (Bruker, Santa Barbara, CA) using oxide-sharpened "twin-tip" Si_3N_4 cantilevers with a spring constant of 0.06 N/m. The scan rate was about 8 Hz and the applied force was minimized to 0.1 nN. All images were reproducible with

different tips and different fast-scan directions.

2 Results and Discussion

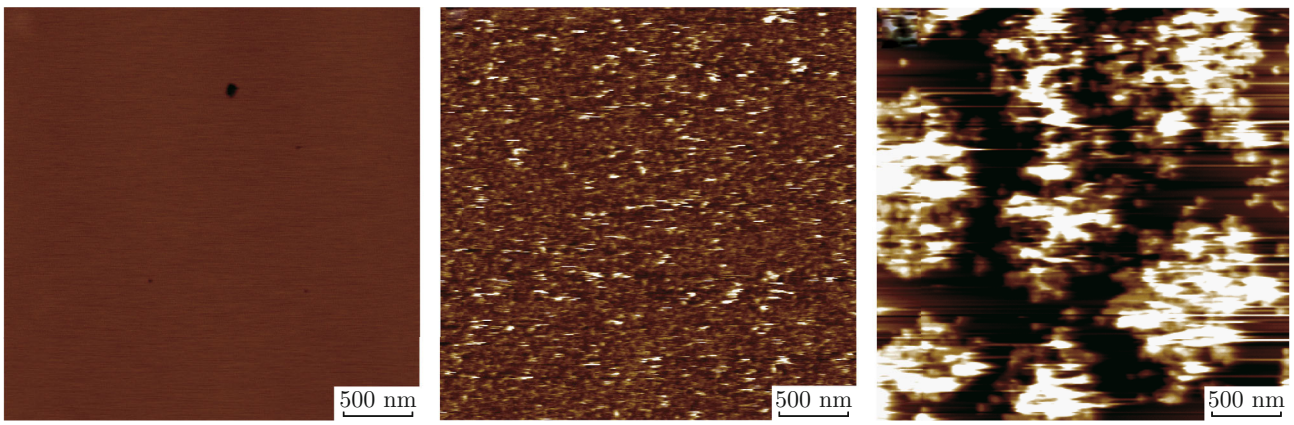
We first investigated the quality of samples of membrane-associated PFO prepared using the two most commonly used procedures for membrane proteins in AFM: VF and exclusively using small Teflon wells. For VF, small lipid vesicles are added directly to the substrate (muscovite mica), where they spontaneously break open and fuse to form a single supported bilayer (Fig. 1(a)). The proteins are then directly added to this bilayer, where they would presumably associate with and then insert within the supported bilayer. For those samples prepared exclusively using small Teflon wells, a lipid-monolayer coated mica fragment (prepared using a Langmuir trough) is lowered through a lipid monolayer at the air/water interface in the small Teflon well, thereby forming the supported bilayer (Fig. 1(b)). Proteins are next injected through a side-port of the Teflon well, and then would associate with and insert into the bilayer.

Figure 2(a) shows an AFM image of a supported bilayer, before the addition of protein, prepared with the VF method. The dark regions are stable holes in the bilayer, which thereby indicates the presence of bilayer. This method is thus indeed effective for the preparation of high quality supported pure lipid bilayers.



(a) Preparation with the VF method (b) Preparation exclusively using the Teflon well method

Fig. 1 Procedures for conventional sample preparation of membrane proteins for AFM



(a) Supported bilayer prepared with the VF method (b) Addition of PFO to the supported bilayer (c) AFM image of PFO samples prepared exclusively with Teflon wells

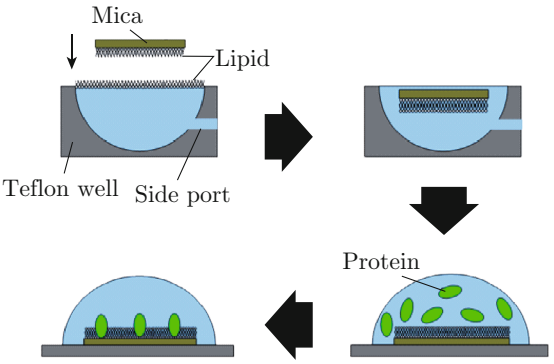
Fig. 2 AFM images of PFO samples prepared by the conventional methods

However, addition of PFO to such supported bilayers does not result in membrane-associated protein (Fig. 2(b)). Instead the images resemble those obtained if the protein is simply added directly to mica (data not shown). We speculate that, rather than inserting into these bilayers, PFO binds directly to mica (perhaps initially at the membrane defects shown in Fig. 2(a)) and, by virtue of this interaction, destabilizes the lipid, eventually resulting in its complete detachment from the mica surface and complete coverage of PFO on mica. Nonetheless, regardless of the mechanism, it is clear that this strategy is not effective with these proteins.

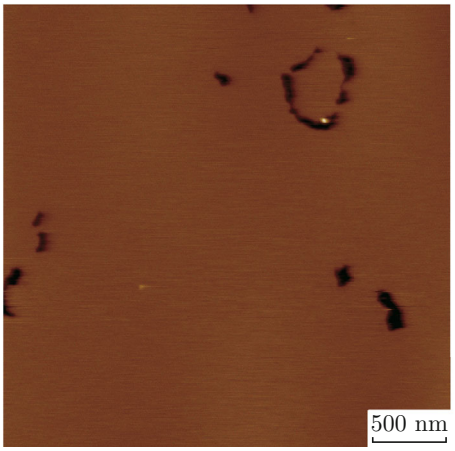
Similar results have also been obtained with other PFTs (data not shown).

Samples of PFO prepared exclusively with Teflon wells are markedly different. In some regions of the specimen surface, there is a suitable protein density for AFM imaging (see for example, Ref. [14]). However, by far, most of the specimen surface either contains no protein (and images resemble Fig. 2(a)) or contains many, highly variable layers of protein (Fig. 2(c)). A priori, it is not clear which region of the specimen contains the suitable protein density, which can thus fundamentally prevent AFM study since searching for these regions can be time-consuming and the sample deteriorates with time. We thus explored alternate methods to prepare these samples.

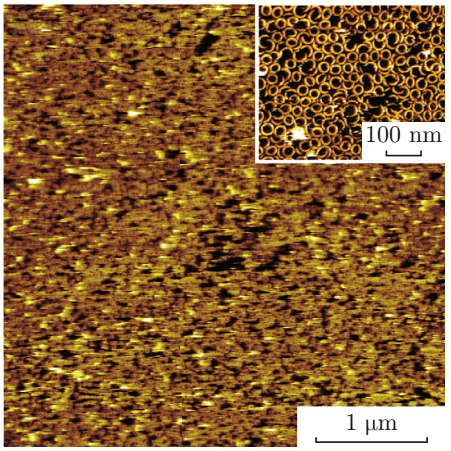
As the addition of the protein in the VF method enables the greatest possible control of protein density across the sample, and the supported lipid bilayer prepared in the Teflon well effectively supports PFO association and insertion, we thus tested a procedure whereby the supported lipid bilayer prepared in the Teflon well was removed from the well, and then subsequently the protein was added directly to the bilayer (as in the VF method) (Fig. 3(a)). Unlike in the previous method using Teflon wells, in the new method, the sample is removed from the wells before the protein is added.



(a) Schematic of the new method



(b) AFM image of the supported eggPC/ cholesterol bilayer prepared with new method, before the addition of protein



(c) Large-scan size AFM image showing a high density of PFO pores associated with the bilayer

Fig. 3 Optimal PFO samples prepared with the new method

The supported bilayer prepared in this way, prior to protein addition, resembles that obtained with VF (Fig. 3(b)), exhibiting a thickness of (4.2 ± 0.3) nm, and is consistent with that of a single bilayer[15]. However, unlike the VF supported bilayer, addition of PFO to these supported bilayers results in a highly uniform, high density of membrane-associated PFO complexes (Fig. 3(c)). In Fig. 3(c), the inset shows a slightly smaller scan size image that better shows individual PFO complexes. Similar images could be obtained across large regions of the sample, thereby greatly facilitating higher resolution AFM. Indeed, as shown in Fig. 4, smaller scan sized images of such samples resolve individual subunits within the complexes, revealed as a periodicity of (2.5 ± 0.3) nm along the arc of the complex, consistent with the atomic model of the

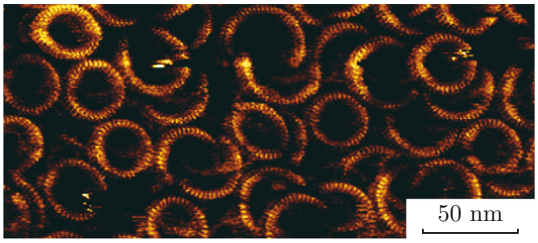


Fig. 4 High resolution AFM image of PFO pores

water-soluble monomer[16].

3 Conclusion

Since its invention in 1986, there has been tremendous interest in using AFM to image biological samples to high resolution. The number of recent successes exhibiting sub-nanometer resolution demonstrates that the present-day technology is likely adequate for many biological samples. What are needed are generally applicable sample preparation strategies that produce a single-layer of closely-packed proteins, regardless of what type of sample it is. The method described in this work does not depend on any special property of PFO, nor of the lipid composition. Hence we expect that this will indeed prove to be a highly robust effective method for a wide range of PFTs. Atomic models of the water-soluble versions of many PFTs, like PFO, are presently available, but not of their membrane-associated forms. We thus expect that, with this method, AFM will provide a useful link in determining how the water-soluble structures assemble into the functionally relevant membrane-associated pores.

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