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Assemblies of pore-forming toxins visualized by atomic force microscopy☆

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ABSTRACT

A number of pore-forming toxins (PFTs) can assemble on lipid membranes through their specific interactions with lipids. The oligomeric assemblies of some PFTs have been successfully revealed either by electron microscopy (EM) and/or atomic force microscopy (AFM). Unlike EM, AFM imaging can be performed under physiological conditions, enabling the real-time visualization of PFT assembly and the transition from the prepore state, in which the toxin does not span the membrane, to the pore state. In addition to characterizing PFT oligomers, AFM has also been used to examine toxin-induced alterations in membrane organization. In this review, we summarize the contributions of AFM to the understanding of both PFT assembly and PFT-induced membrane reorganization. This article is part of a Special Issue entitled: Pore-Forming Toxins edited by Mauro Dalla Serra and Franco Gambale.

1. Introduction

Electron microscopy (EM) and atomic force microscopy (AFM) provide high-resolution images of biological samples. EM has the advantage of resolving both the 2D and 3D structures of specimens at the subnanometer level; however, experiments must be performed under a vacuum to achieve such high resolution and at cryogenic temperatures, far below the physiological temperature, to reduce the effects of radiation damage [1]. AFM, which was invented nearly 30 years ago [2], has become a powerful tool for the 2D characterization of biological structures, such as cells, DNA and membrane proteins, in an aqueous environment [3–5]. The advantage of AFM compared to EM is the nanometer to subnanometer resolution achieved under physiological conditions without pretreatment. Image processing techniques in EM, such as correlation averaging, are frequently applied to high-resolution AFM images to improve the signal-to-noise ratio. In this technique, selected unit cells are aligned and added to yield an averaged, clearer image. Unresolved surface features in repeating unit cells can be recovered using this approach. This detailed image analysis is described by Schabert and Engel [6].

1.1. Achievements in bioimaging with conventional AFM

Visualization of the 2D crystals of gap junctions [7,8], the OmpF porin [6,9,10] and the bacterial hexagonally packed intermediate (HPI) layer [11,12] using both AFM and EM has verified that AFM can provide high-resolution images of membrane proteins in an aqueous environment. Assemblies of bacteriorhodopsin, the OmpF porin and the HPI layer could be extensively studied using this feature of AFM [13–18]. Aquaporins and light-harvesting complexes were also visualized by AFM with subnanometer resolution [19–22].

Some of the studies mentioned above also show the conformational changes in individual proteins. AFM studies demonstrated that the applied force and pH can induce structural changes in bacteriorhodopsin [13,14]. Furthermore, switching of the pores in the inner surface of the HPI layer between the open and closed states [15], closure of the OmpF porin channel under an applied potential or at low pH [18], and the Ca^{+2}-induced reduction in the diameter of connexin pores [23] could be detected. The diffusion of membrane proteins could also be tracked. The time-lapse AFM images recorded in a buffer solution every 90 s for single sodium-driven rotors from a bacterial ATP synthase show the diffusion of single proteins within the lipid membrane [24]. Because AFM can provide *in situ* observations of such changes in

Abbreviations: PFT, pore-forming toxin; EM, electron microscopy; AFM, atomic force microscopy; Ld, liquid disordered; Lo, liquid ordered; Tm, melting temperature; SLB, supported lipid bilayer; LB, Langmuir–Blodgett; LS, Langmuir–Schaefer; MLV, multilamellar vesicle; SUV, small unilamellar vesicle; GUV, giant unilamellar vesicle; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; egg-PC, egg-yolk phosphatidylcholine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPOPC, 1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine; DPhPC, diphytanoyl phosphatidylcholine; DOC, deoxycholate; GM1, monosialotetrahexosyl-ganglioside; CTX-B, cholera toxin B-oligomer; VacA, vacuolating toxin A; αHL, α-hemolysin; TCM, truncated channel mutant; γHL, γ-hemolysin; CDC, cholesterol-dependent cytolysin; PFO, perfringolysin O; TMH, transmembrane amphipathic β-hairpin; DTT, dithiotreitol; LLO, listeriolysin O; Tet C, tetanus toxin C-fragment; Sts, sticholysins I and II; hcp, hexagonal close packed.

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addition to the substructure of membrane proteins, it is a powerful complementary technique for characterizing biological samples.

Initially, the high-resolution AFM imaging of membrane proteins was usually conducted in contact mode, in which an appropriate adjustment of the electrolyte concentration is required for an electrostatically balanced interaction between the AFM tip and the protein surface [17]. Images of similar resolution could also be recorded in tapping mode [25], which does not require such adjustment. A lateral resolution of 1.1–1.5 nm and a vertical resolution of 0.1–0.2 nm could be achieved for the HPI layer in both imaging modes [25]. Compared to contact mode, tapping mode is a more suitable technique, especially for studying fragile and weakly immobilized structures, because of its reduced contact time and friction forces.

High-resolution imaging was also performed on water-soluble proteins in addition to the previously mentioned membrane proteins. However, the formation of 2D crystals of soluble proteins is more complicated than the assembly of membrane proteins. The conditions for the binding of soluble proteins to the substrate must be controlled to immobilize the protein without denaturation [5]. Molecular resolution images of some water-soluble proteins, such as ferritin, immunoglobulin, catalase, lysozyme and annexin V, could be obtained using conventional AFM [26–30]. Pore-forming toxins (PFTs), which also exist as soluble monomers in an aqueous medium, can self-assemble into well-defined structures upon oligomerization on a lipid membrane. The PFT assemblies with structures that could be resolved by AFM are described in Section 3.2.

1.2. Achievements in bioimaging with recently developed AFM techniques

In the previous section, we mentioned the successful applications of AFM in scanning 2D protein crystals in a physiological environment with subnanometer resolution. However, the dynamics of biomolecules or fast biological processes cannot be probed by conventional AFM due to the slow image acquisition rate, ranging from one to several minutes per frame. Recently, this limitation has been overcome by the introduction of high-speed AFM, which can provide *in situ* images of dynamic biological systems at subsecond time resolution [31–33]. Dynamic protein and live cell imaging are discussed in a recent review by Ando *et al.* [34]. Using this new technique, the motion of membrane proteins could be examined at a time interval shorter than 1 s [35–39]. Additionally, for the first time this technique was used to visualize the assembly of a PFT on a lipid membrane [40], elucidating the subsecond dynamics of toxin oligomers.

Another recent improvement allows high-resolution imaging in an aqueous medium using frequency modulation AFM (FM-AFM) [41], which is known as noncontact AFM and is commonly operated in an ultra-high vacuum for atomic resolution imaging. This method was applied to a molecular-scale study of artificial lipid membranes in pure water or buffer solution [42–44]. FM-AFM revealed the different structuring of water on the surfaces of gel- and fluid-phase bilayers and the variation in the ordering of gel-phase lipid molecules in different salt solutions. These studies show that this technique can monitor single lipid molecules in an aqueous environment. Although lipid bilayers have been well studied using the contact or tapping modes [45–51], the lateral resolution has never been as high as that which can be achieved by this recently developed AFM.

2. Lipid bilayers as model cell membranes

Cell membranes have a complicated structure that is composed of lipids, proteins and carbohydrates [52]. Despite this complexity, lipids constitute the basic structure of natural membranes. They are arranged in two layers with their polar heads facing the exoplasmic and cytoplasmic spaces. These structures are known as lipid bilayers and can be prepared artificially. Below, the physicochemical properties of lipid bilayers are briefly explained.

2.1. Phase behavior of lipid bilayers

Lipid bilayers exhibit gel, fluid/liquid-disordered (Ld) or liquid-ordered (Lo) phases. The gel and fluid phases depend on the lipid structure [53]. A stronger interaction between lipids with longer acyl chains increases the phase transition/melting temperature, T_m. In addition to the length of the acyl chains, the degree of saturation also determines lipid phase behavior. Saturated phospholipids with long acyl chains (*e.g.*, sphingomyelin (SM), a type of sphingolipid) exist in the gel-phase state at room temperature. The high-T_m lipids segregate into a thicker gel phase within a thinner Ld phase of the low-T_m lipids. In the presence of cholesterol, the high-T_m lipids form an Lo phase that has the characteristics of the gel and fluid phases, *i.e.*, a high degree of acyl-chain ordering with increased lateral lipid diffusion. In natural membranes, sphingolipids and cholesterol form lipid rafts within a fluid phase of unsaturated phospholipids [54]. Raft-like domains within artificial membranes are created using a combination of both high- and low-T_m phospholipids in the presence of cholesterol. In addition to using specific types of lipids, mixing lipids in appropriate ratios is also crucial for the construction of a phase-separated membrane at a certain temperature [55,56].

2.2. Supported lipid bilayers (SLBs)

Supported lipid bilayers (SLBs) are employed as simple models of the cell membrane for AFM imaging [57]. SLBs are usually prepared by the Langmuir–Blodgett (LB) technique or the fusion of lipid vesicles [58]. In the LB technique, a hydrophilic solid substrate is immersed into an aqueous subphase in a Langmuir trough. A lipid monolayer is formed by spreading the lipid-containing solvent at the air/water interface. The solvent must be volatile and immiscible with water. After the complete evaporation of the solvent, the lipid monolayer is compressed by the movable barriers. At a constant surface pressure, the substrate is withdrawn vertically from the aqueous subphase, resulting in the deposition of the monolayer onto the substrate with the lipid tails exposed to the air. The substrate is subsequently dipped into the Langmuir trough or lowered horizontally onto the Langmuir film to form the bilayer. The latter approach is called the Langmuir–Schaefer (LS) technique. Asymmetric bilayers, which are found in biological membranes, can also be prepared using the LB or LB/LS method. Vesicle fusion, a simpler and more popular method, involves the adsorption of liposomes on a solid substrate and their subsequent rupture. The liposomes are first prepared by dissolving the lipid in an organic solvent and evaporating the solvent under nitrogen and/or a vacuum to yield a lipid film. The dried lipid film is then hydrated with an aqueous buffer solution to form multilamellar vesicles (MLVs). The MLVs are sonicated to obtain small unilamellar vesicles (SUVs). The SUVs are deposited onto the hydrophilic substrate at temperatures above the melting point of the lipids. Some AFM studies showed the fusion mechanism of lipid vesicles on solid supports, such as mica and silica [59–62].

Lipid molecules can diffuse laterally in SLBs because of the thin water layer (~1 nm) separating the bilayer from the solid support [57,63]. However, the lateral diffusion of lipids in SLBs is slower than that in free-standing membranes. Under identical conditions, the diffusion coefficients of dye-labeled lipids within a lipid bilayer created on mica and a cover slip were found to be approximately half of that in giant unilamellar vesicles (GUVs) [64,65]. The results obtained from these studies also indicated the existence of a strong interleaflet coupling, *i.e.*, lipids in the inner and outer leaflets of SLBs diffuse with the same velocity. However, this conclusion cannot be generalized because the bilayer–support interaction and accordingly the interleaflet coupling are strongly affected by the ionic strength of the solution [66]. If the interaction between the inner leaflet and the solid support is sufficiently strong, the lipids in the inner leaflet will be packed more densely than those in the outer leaflet. This vertical asymmetry weakens the interleaflet coupling; thus, the lateral mobility is higher for the lipids