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Research Article

Effect of Antimicrobial Peptide-Amide: Indolicidin on Biological Membranes

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Indolicidin, a cationic antimicrobial tridecapeptide amide, is rich in proline and tryptophan residues. Its biological activity is intensively studied, but the details how indolicidin interacts with membranes are not fully understood yet. We report here an *in situ* atomic force microscopic study describing the effect of indolicidin on an artificial supported planar bilayer membrane of dipalmitoyl phosphatidylcholine (DPPC) and on purple membrane of *Halobacterium salinarum*. Concentration dependent interaction of the peptide and membranes was found in case of DPPC resulting the destruction of the membrane. Purple membrane was much more resistant against indolicidin, probably due to its high protein content. Indolicidin preferred the border of membrane disks, where the lipids are more accessible. These data suggest that the atomic force microscope is a powerful tool in the study of indolicidin-membrane interaction.

1. Introduction

Antimicrobial cationic peptides are host defense molecules produced by the innate immune system of organisms all across the evolutionary spectrum. They play a key role in the host defense system of many higher organisms [1]. Indolicidin, encoded by a member of cathelicidin gene family, a cationic antimicrobial tridecapeptide amide (H-Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH_2), was isolated from cytoplasmic granules of bovine neutrophils [2]. It is one of the shortest known natural-occurring antimicrobial peptide [3], toxic to both prokaryotes and eukaryotes [3, 4]. The high percentage of proline and tryptophan residues makes indolicidin a unique antimicrobial. Unlike several other antimicrobial peptides, the structure of indolicidin upon membrane interaction is not a well-defined helix or a β-turn and does not display their characteristic amphipathic nature [4–7]. It has been reported that indolicidin expresses its antimicrobial activity by creating pores through cell membranes [8]. Other studies showed that indolicidin treatment resulted total disintegration of membrane structures [9] or that it did not cause cell lysis even at high concentration [4]. Compared to α-helical antibiotic peptides, indolicidin is less able to dissipate the bacterial inner membrane potential and forms smaller pores, yet it kills bacteria rapidly [10]. It was reported that an interfacial membrane location was preferred by indolicidin [6, 11]. These results point to a mechanism of action that is different from well-defined channel formation.

Lipid bilayer on polyelectrolyte films can be used as a useful experimental approach to study basic problems of biological membrane structures [12–14]. Bacteriorhodopsin is a light-driven proton pump in the plasma membrane of *Halobacterium salinarum*. This integral membrane protein is tightly packed in two-dimensional crystalline from termed purple membrane with high (75% w/w) bacteriorhodopsin content, with no other protein. The remaining 25% is lipid [15]. In situ atomic force microscopic (AFM) experiments could provide detailed information about these systems [16].

Here, we present an AFM study on the interaction of indolicidin with an artificial and a natural membrane.

2. Materials and Methods

2.1. Sample Preparation. Freshly cleaved mica (SPI-Chem Mica Sheets, Structure Probe, Inc., West Chester, PA, USA)

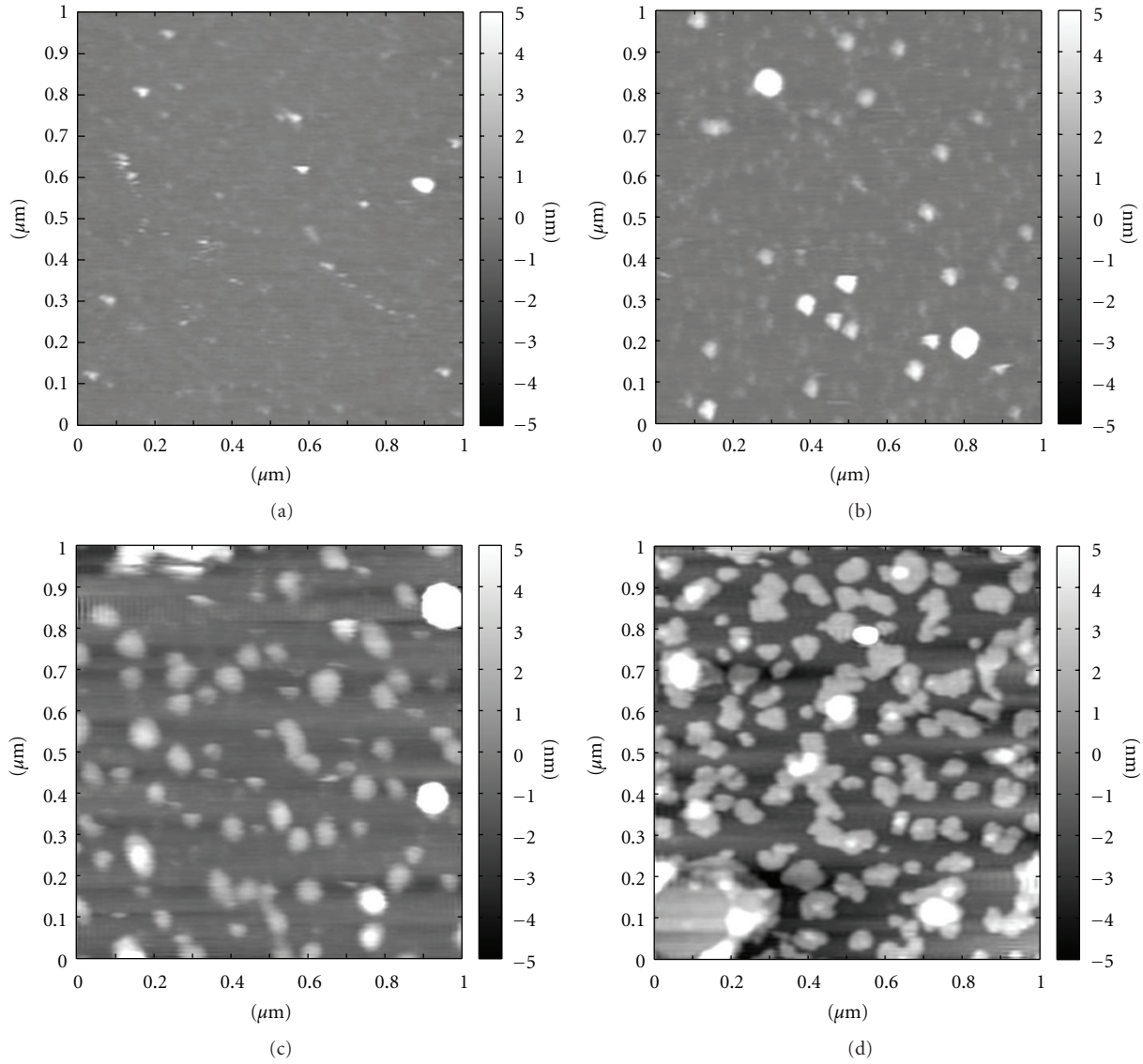

Figure 1: Effect of indolicidin on DPPC membrane on polyelectrolyte film at 0 (a), 2.6 (b), 5.2 (c), and 15.7 μM (d) concentrations after 2 hours of treatments. DPPC layer covered the PLL/PGA film on mica surface. $1 \times 1\,\mu m^2$ images were taken by AFM in AC mode.

surface was covered with poly(L-lysine)-poly(L-glutamic acid)-dipalmitoyl phosphatidylcholine (PLL-PGA-DPPC) layers [14, 17], or with purified purple membrane of *Halobacterium salinarum* [18–20] on the following ways:

Experiments were performed in tris(hydroxymethyl)aminomethane (TRIS-HCl, 10 mM), and sodium chloride (NaCl, 0.15 M) buffer at pH = 7.4. The polyelectrolytes PLL (M_r = 32600 g/moL) and PGA (M_r = 17000 g/moL) have been both dissolved in the above mentioned buffer, at a concentration of 1 mg/mL. DPPC was dissolved in chloroform: methanol (2 : 1) at final concentration of 20 mg/mL. Afterward the solvent was evaporated by N_2 flow. Buffer was added to the dried DPPC (250 μg/mL), and liposomes were formed by sonication. Sonication was applied in several steps until turbidity was observed in the test tube. No special care was taken to have unilamellar liposomes.

To form polyelectrolyte layers, PLL was adsorbed first to the freshly cleaved mica, then it was washed with buffer, and PGA was adsorbed on PLL layer, and it was washed again. These steps have been followed by covering the treated mica with DPPC layer; it was heated for 1 hour at 46°C, and let to cool slowly.

Purple membrane of *Halobacterium salinarum* was prepared according to the method of Oesterhelt and Stoeckenius [21], and it was adsorbed from a buffer containing 10 mM TRIS and 150 mM NaCl at pH = 8.0 to the freshly cleaved mica surface treated with the same buffer containing 10 mM $CaCl_2$.

Indolicidin was added just before the measurements to the same buffer in which the membrane surfaces were prepared at 0.52, 2.6, 5.2, 7.9, and 15.7 μM concentrations (1, 5, 10, 15, and 30 μg/mL, resp.) [10, 22].

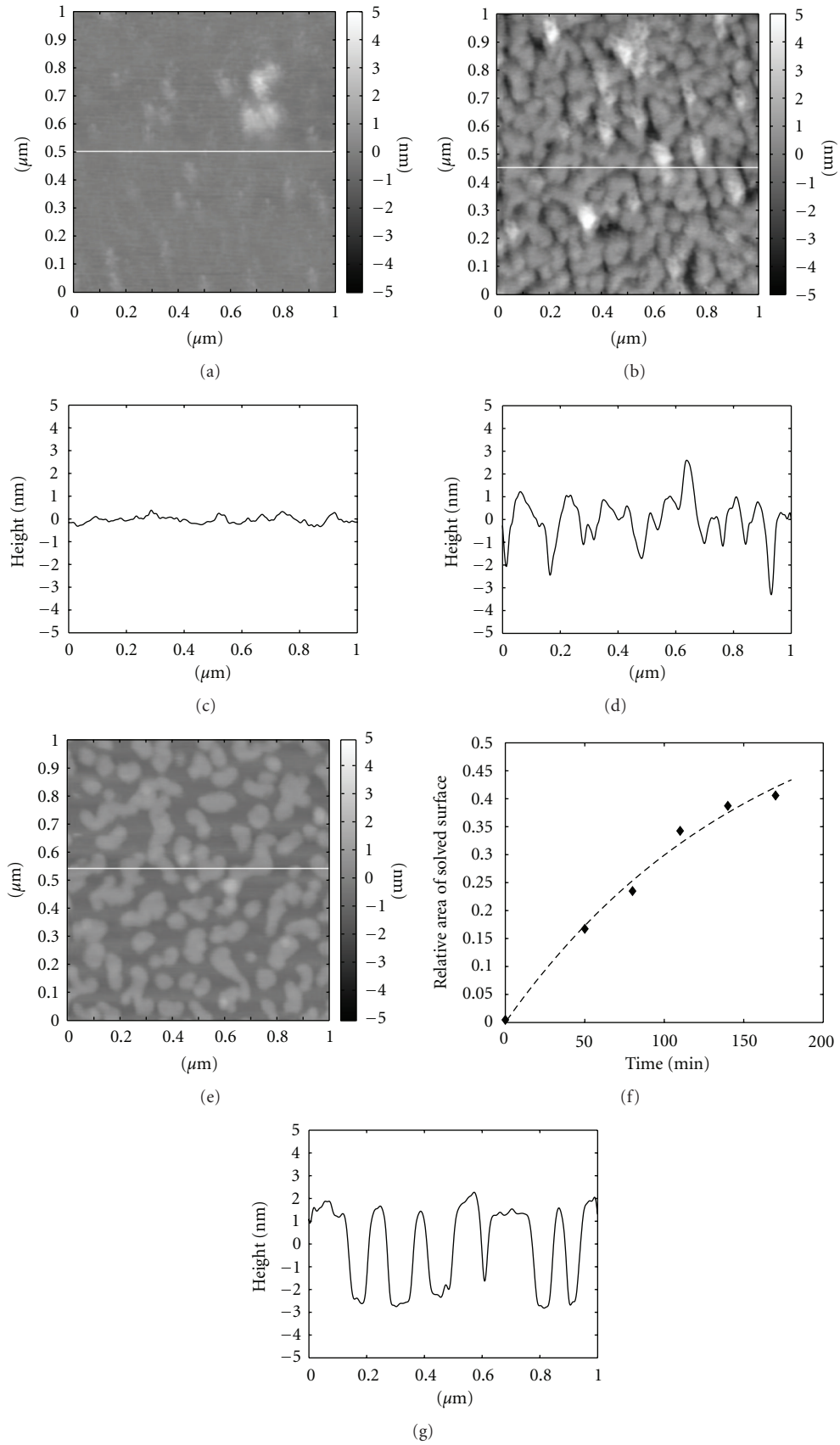

FIGURE 2: 15.7 μM indolicidin mediated DPPC membrane disruption after 0 min (a, c), 50 min (b, d), and 140 min (e, g). $1 \times 1\ \mu m^2$ images are presented (a, b, e) with sections (c, d, g) marked with lines on the original images. Relative area of degraded membrane surface in function of time (f).

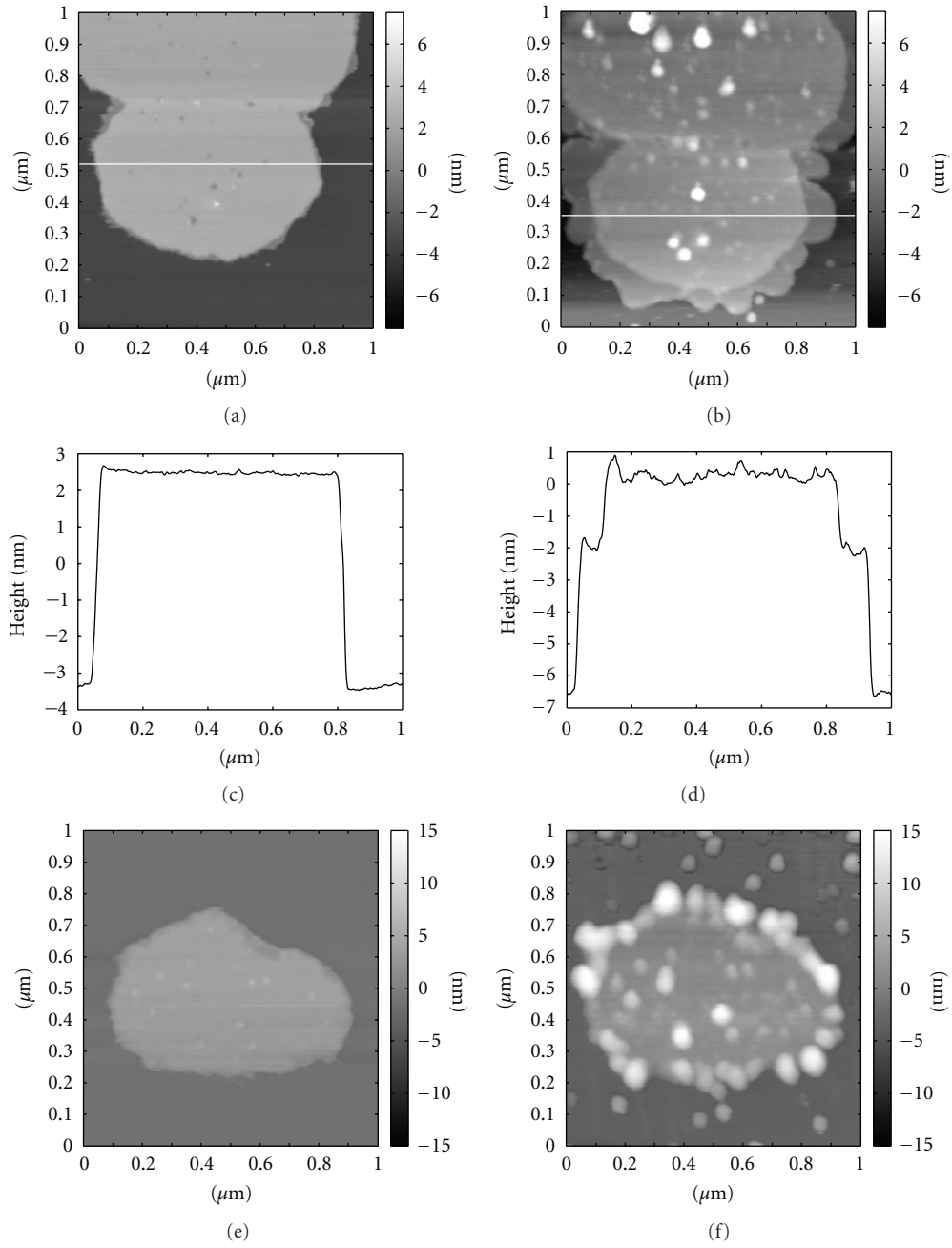

Figure 3: Effect of indolicidin on purple membrane of *Halobacterium salinarum* at 0 (a), 7.9 (b) with sections (c, d) and 0 (e), 15.7 μM (f) concentrations. Same membrane disks are presented before (a, e) and after (b, f) treatment. The images are $1 \times 1\,\mu m^2$.

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Atomic Force Microscopy. AFM measurements were carried out with an Asylum MFP-3D head and controller (Asylum Research, Santa Barbara, CA, USA) in tapping/noncontact (AC) mode. The driver program MFP-3D Xop was written in IGOR Pro software (version 5.05a, Wavemetrics, Lake Oswego, OR, USA). Silicon nitride (BioLever Mini BL-AC40TS) cantilevers (Olympus Optical Co., Ltd., Tokyo, Japan) were used for the experiments (resonance frequency was 25 kHz in water with 0.09 N/m spring constant). Typically 512×512 points were taken at 1 line/s scan rate in AC mode under buffer solution. The measurements presented here are $1 \times 1\,\mu m^2$ flattened heights. All experiments were repeated from 3 to 9 times.

3. Results and Discussion

In order to clarify which model may be the more likely explanation of indolicidin's antimicrobial activity (i.e., pore formation that disturbs the metabolism/homeostasis/ion

balance of a cell, or the cell membrane destruction), we developed a modified supported membrane model: a polyelectrolyte film composed of PLL and PGA attached to flat mica surface and covered with a lipid bilayer of DPPC. The reason why we chose this membrane model is that it contains a layer between the hard surface (mica) and the lipid bilayer through which small peptides are able to penetrate. In other words, if indolicidin creates a pore through a membrane, this structure may ensure space for indolicidin on the other side of the membrane, and it can access the membrane from the side that is close to mica, too. In models used previously for studying the action mechanism of indolicidin [8, 23, 24], lipid bilayers were directly attached to mica, leaving no space between the membrane and the hard surface, which may be important for full pore formation.

To test the effect of indolicidin on our membrane model, various concentrations of peptide solutions were used (in a range of 0.52 to 15.7 μM) [10, 21]. On one hand, when we used 0.52 μM of indolicidin, no membrane alterations could be detected with AFM (Figure 1(a)). If the concentration was increased above 2.6 μM, instead of creating pores, indolicidin induced the appearance of aggregates, but the membranes were still intact (Figures 1(b) (2.6 μM), 1(c) (5.2 μM)). We hypothesize that these aggregates are formed from excess amount of indolicidin that is present in the system. However, the aggregation process was too fast, and it did not allow us to follow the formation of these aggregates during AFM imaging (taking one image required approximately 10 minutes of scanning).

When we further increased the concentration of the antimicrobial peptide (7.9 μM (data not shown) or 15.7 μM), after about 2 hours, the membrane bilayer structure was destroyed; this phenomenon was not observed at lower concentrations (Figure 1(d)).

In summary, in our model membrane, we could not find a concentration where pore formation occurred. This suggests that the mechanism through which indolicidin expresses its antimicrobial activity is more likely via disintegrating membranes.

To make sure that smaller concentrations have a different effect relative to higher concentrations, and for better understanding of the membrane destruction process, the time dependence of indolicidin treatment was also measured (Figure 2). 15.7 μM of indolicidin started to induce detectable changes in the membrane structure after the first 40–50 min (Figures 2(b) and 2(d)). The collapse of the membrane required 140 min at room temperature (Figures 2(e) and 2(f)). Based upon the above observations, that is, (1) indolicidin needs a minimal concentration to affect the bilayer structure (below which there is no detectable impact, and above which there is a major/significant impact). These results have a good agreement with the proposed mechanism reported by Melo et al. [25]. (2) It takes time even for high concentrations to induce visible changes. Indolicidin may behave as a surfactant-like material in a sense that it breaks down the continuity of membranes.

The purple membrane disks of *Halobacterium salinarum* have been checked also to test the importance of PLL/PGA layer. These membrane disks were deposited directly on the Ca^{2+} covered mica surface, without any polyelectrolyte film. In this case, the interaction was slower and more specific. As it can be seen, indolicidin binds to the membrane, but the edges were preferred (Figures 3(b) (7.9 μM), 3(d) (15.7 μM)), since the lipids were more accessible in those regions. Also the roughness of the membrane increased, probably caused by the aggregation of the indolicidin on the surface. Even at higher concentration (15.7 μM), indolicidin attached to the membrane surface and especially on the border of membrane disk only but did not break the membrane integrity. The surface area of the transient layer increased. This transient layer has about half height (3-4 nm) of the purple membrane, in agreement with the results of Shaw et al. [23] and Mecke et al. [26]. Based on the height of the layer, it is probably a monolayer of lipids stabilized with indolicidin.

4. Conclusion

The subject of our present publication is an *in situ* atomic force microscopy study of interaction of indolicidin with supported planar bilayer membranes of DPPC, and purple membrane of *Halobacterium salinarum*. The effect of the peptide on membranes was concentration dependent for DPPC and it resulted in the destruction of intact membranes. Among the samples examined, the purple membrane was less sensitive to indolicidin treatment, most likely due to the high membrane protein content. For these membranes, indolicidin tended to bind to the border of membrane disks, where the lipids are easier to interact with. In this paper, we demonstrated that the association of indolicidin with supported planar bilayers causes membrane thinning similar to the work of Shaw et al. [23, 24] and Mecke et al. [26] or solubilization of supported membrane on polyelectrolyte film rather than initiating pore formation [8]. These results are in good correlation with molecular dynamics simulations [27, 28].

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