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Comparison of coarse-grained (MARTINI) and atomistic molecular dynamics simulations of α and β toxin nanopores in lipid membranes[†]

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Abstract. Pore forming toxins (PFTs) are virulent proteins whose primary goal is to lyse target cells by unregulated pore formation. Molecular dynamics simulations can potentially provide molecular insights on the properties of the pore complex as well as the underlying pathways for pore formation. In this manuscript we compare both coarse-grained (MARTINI force-field) and all-atom simulations, and comment on the accuracy of the MARTINI coarse-grained method for simulating these large membrane protein pore complexes. We report 20 μ s long coarse-grained MARTINI simulations of prototypical pores from two different classes of pore forming toxins (PFTs) in lipid membranes - Cytolysin A (ClyA), which is an example of an α toxin, and α -hemolysin (AHL) which is an example of a β toxin. We compare and contrast structural attributes such as the root mean square deviation (RMSD) histograms and the inner pore radius profiles from the MARTINI simulations with all-atom simulations. RMSD histograms sampled by the MARTINI simulations are about a factor of 2 larger, and the radius profiles show that the transmembrane domains of both ClyA and AHL pores undergo significant distortions, when compared with the all-atom simulations. In addition to the fully inserted transmembrane pores, membrane-inserted proteo-lipid ClyA arcs show large shape distortions with a tendency to close in the MARTINI simulations. While this phenomenon could be biologically plausible given the fact that α -toxins can form pores of varying sizes, the additional flexibility is probably due to weaker inter-protomer interactions which are modulated by the elastic dynamic network in the MARTINI force-field. We conclude that there is further scope for refining inter-protomer contacts and perhaps membrane-protein interactions in the MARTINI coarse-grained framework. A robust coarse-grained force-field will enable one to reliably carry out mesoscopic simulations which are required to understand protomer oligomerization, pore formation and leakage.

Keywords. Pore forming toxins; cytolysin A; α -hemolysin; molecular dynamics; MARTINI force-field; membrane protein.

1. Introduction

Pore forming toxins (PFTs) belong to a class of pathogens specifically evolved to form pores in plasma membranes.^{1,2} Once secreted from the host organism which range from bacteria to sea anemones, these proteins which are secreted as water soluble monomers bind to the membrane and rapidly oligomerize to form

functional pores in target cells. Unregulated pore formation disrupts ion transport leading to osmotic stress, compromised cell signaling, and eventually cell lysis,^{1–4} and thus bacterial PFTs in particular are potent molecular weapons used to propagate infection in humans. The initial process of monomer binding to the target cell membrane typically involves a significant conformational change, wherein the water soluble monomer converts into a membrane bound “protomer”. Pore formation can occur in one of two ways. In the growing pore mechanism, membrane-inserted protomers oligomerize to form the pore complex, while in the pre-pore pathway,

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[†]Dedicated to the memory of the late Professor Charusita Chakravarty.

pore formation occurs on the membrane interface followed by an insertion step of the pre-pore complex into the membrane. Evidence from studies on cholesterol dependent cytolysins (CDCs, β toxin family) exist for both pathways, and partially oligomerized arcs occurring during pore formation have been observed⁵ in support of the growing pore model. The presence of arcs implies the existence of a proteo-lipid complex which consists of partially oligomerized proteins that form the arc, and lipids that complete the transmembrane channel by forming a toroidal lipid edge.^{6,7} Subsequently, membrane-bound protomers and proteo-lipid arcs self assemble or oligomerize to form the membrane-inserted pore complex. The dominant secondary structure of the transmembrane domains of PFT pores enable their classification into two classes: α -PFTs and β -PFTs. In α -PFTs such as Cytolysin A (ClyA) from *Escherichia coli*,⁸ the protein is comprised mainly of α -helices which insert and assemble in the membrane to create a hydrophilic pore interior. In the case of β -PFTs such as α -hemolysin (AHL) from *Staphylococcus aureus*, the dominant motifs are β sheets which form the membrane-inserted β -barrel stabilized by inter-strand hydrogen bonding, and hence, β -PFTs have greater structural stability when compared with α toxins.^{9,10}

Membrane proteins have been extensively simulated using both atomistic as well as coarse-grained molecular dynamics (MD) simulations.^{11–17} However, while many anti-microbial peptides have been extensively simulated, there are comparatively fewer MD simulations of pore forming toxins, either as water soluble monomers or transmembrane oligomeric pores. Previous studies have reported simulations of the β -PFT transmembrane pore - AHL, and have investigated the solvent and ion transport properties of the pore lumen, and biomolecule (ssDNA and protein) translocation through the nanopore, and functionality of truncated mutant pores.^{18–22} However, few simulations of α PFTs (and ClyA in particular) have been reported in literature.^{23–25} Atomistic simulations of these large heterogeneous systems can elucidate important molecular phenomena such as pore forming mechanisms and membrane-protein interactions by simulating length scales of tens of nm (a single membrane-pore system) for up to hundreds of nanoseconds (ns). However coarse-grained simulations of membrane-proteins (using popular force-fields such MARTINI^{15,26,27} for membranes and proteins) can be used to explore significantly longer time and length scales needed to study mesoscale phenomena such as pore diffusion on the membrane, pore aggregation and inter-pore interactions, curvature generation on large membranes by multiple pores, and membrane domain interactions with pores.

Hence, it is vital to assess the ability force-fields such as MARTINI to accurately depict membrane-inserted PFT pores. Therefore, in this manuscript, we carry out 20 μ s MARTINI coarse-grained simulations of the α toxin, ClyA and the β toxin, AHL in their membrane-inserted pore states, and compare structural attributes with corresponding 100 ns long all-atom simulations. Unlike all-atom simulations, we find that the MARTINI force-field is unable to capture inter-protomer interactions sufficiently, leading to large structural distortions in both pores, especially in the important transmembrane domains. Improvement of MARTINI parameters for these large multimeric membrane-inserted complexes (not explored in this study) may open up opportunities for studying pore related phenomena at mesoscales, which are currently challenging to accomplish through all atom simulations and experiments.

2. Simulation methodology

MARTINI simulations of the ClyA and AHL pores were performed using the latest tested version of the MARTINI force field with elastic dynamic network (Elnedyn 2.2) implemented in GROMACS 4.6.4.^{28–31} The ClyA and AHL pore crystal structures (PDB ID 2WCD and 7AHL respectively) were modeled using the parameters and protocols from the MARTINI force-field with elastic dynamic network and its extension to proteins. Before converting the all-atom structures into MARTINI representation, missing residues in ClyA (N-terminal residues 1–7 and the C-terminal residues 293–303) were modeled using the I-TASSER web-server,^{32,33} and residues with missing coordinates in AHL (Arg 66 and Lys 70 in chain A, Lys 30 and Lys 240 in chain D, Lys 283 in chain F and Lys 30 in chain G) were reconstructed using the “psfgen” module of VMD as reported in a previous study that simulated the AHL pore.¹⁸ Standard amino acid mapping of the ClyA pore was combined with fixed secondary structure classification of the protein backbone from the PDB-structure, as determined using the DSSP algorithm (version 2.0.4),³⁴ and an elastic network with a force constant of 500 kJ mol⁻¹ nm⁻² was used. It is important to note that only an intra-protomer elastic network was applied to beads within each protomer subunit in the pore, and no elastic bonds between inter-protomer beads were applied. Modifying the elastic dynamic network to match the mean and spread of the RMSD histograms obtained from the MARTINI simulations with the atomistic simulations is possible;³⁵ however we did not pursue this aspect here.

The pores were inserted into a homogeneous 20 $\times$ 20 nm² MARTINI DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) bilayers, and the lipids overlapping with the protein were deleted. Appropriate amount of solvent (CG water) was added along with 0.15 M Na⁺ and Cl⁻ ions to

create a charge neutral system (similar to all-atom simulations of ClyA and AHL membrane-pore systems described elsewhere³⁶). Anti-freeze particles were not added, and no freezing of the CG water was observed in both simulations. Upon energy minimization, production simulations were run at 310 K with separate temperature coupling for the solvent, lipids and protein using the stochastic rescaling scheme³⁷ ($\tau = 1$ ps) and the Parrinello-Rahman³⁸ semi-isotropic coupling at 1 bar ($\tau = 12$ ps, $\kappa_{xy} = \kappa_z = 1.0 \times 10^{-5} \text{bar}^{-1}$). A conservative timestep of 20 fs was used. Electrostatic interactions were smoothly shifted from 0-12 Å and Lennard-Jones interaction from 9-12 Å. A default temporal scaling factor of 4 was used to scale the time evolution of all the quantities calculated from the MARTINI coarse-grained simulations. This scaling factor is system dependent and is thought to arise because of intrinsically smoother energy landscapes in the coarse-grained system which results in enhanced sampling and faster dynamics. Protocols for the 100 ns long all-atom simulations of the ClyA and AHL pores, whose results are compared with quantities calculated from the MARTINI simulations of the same pores in this article, are described in another structural study.^{24,36}

The inner pore radius along the highly non-uniform pore lumen of both ClyA and AHL pores were calculated using the following algorithm. We extract the coordinates of all the protein atoms from both all-atom as well as MARTINI simulations, and fit the pore structure in each snapshot to the initial pore snapshot (to remove translation and rotation). For the all-atom pore simulations, only the C_α atoms of each amino acid are considered. From the pore coordinates, we generate the Solvent Accessible (SA) surface by using the Double Cubic Lattice Method with a probe of size 0 nm. The van der Waal radius of the C_α atoms in the all-atom snapshots is considered to be 0.17 nm,³⁹ and that for all the MARTINI beads (including 'S' type beads) is considered to be 0.26 nm.⁴⁰ Subsequently, the entire pore volume is divided into 5 Å slices along the vertical z direction. The radius of a given slice is calculated as follows. Each slice is subdivided into smaller $9^\circ \theta$ bins, and the radius vector with the lowest magnitude among all the radius vectors in that bin (calculated from the central axis to each point on the SA surface) is taken as the radius of that particular bin. The radius of the 5 Å thick slice is then calculated as the average of all the 40θ bins, which then yields a single value per slice.

3. Results and Discussion

3.1 Structural stability of the ClyA, AHL pores in a DMPC membrane from MARTINI simulations

Oligomeric membrane channels often have global domain motions on longer time scales since their tertiary and quaternary structures are primarily stabilized by non-bonded interactions. The long timescale stability of ClyA and AHL pore complexes were tested

Figure 1. Final snapshots from MARTINI simulations illustrating the side and top views of the final structures for (A) ClyA and (B) AHL. Solvent is excluded, and membrane phosphate beads are colored green for clarity.

through 22 and 20 μs long coarse-grained MARTINI simulations of the respective pores inserted in a DMPC membrane as shown in Figure 1. Though the MARTINI force-field imposes stringent constraints on the protein secondary structure, the relative motion of individual domains are unrestricted. Hence, these simulations can be used to assess structural stability of the pore complex. Figures 1 and 2 illustrate final snapshots for both ClyA and AHL pore complexes from MARTINI and all-atom simulations respectively (initial snapshots of all-atom simulations are also shown). In the MARTINI simulations, the quaternary structure of the pores is well-preserved in the extra-cellular regions, but exhibit distortions in the transmembrane domains (elaborated below). However, the all-atom simulations of the pores show that the final conformations are very similar to the crystal structures. Membrane undulations are also clearly observed in both all-atom and MARTINI simulations, with both pores causing local positive curvatures toward the extra-cellular sides of the membrane.

The time evolution of the radius of gyration (R_g) of the pores is illustrated in Figure 3. The R_g which is an indicator of the geometric stability of the transmembrane protein complexes, is stable and convergent from 100 ns all-atom simulations (insets) as well as coarse-grained MARTINI simulations (illustrated in Figure 3 for ClyA and AHL respectively). The R_g values for ClyA (Figure 3A) also reveal larger fluctuations when compared with the values obtained for AHL. This is consistent

Figure 2. Initial (energy minimized crystal structure) and final snapshots (100 ns) from all-atom simulations of (A) the ClyA pore, and (B) the AHL pore are illustrated (side and top views). Solvent and ions are excluded, transmembrane pore domains are colored mauve, extra-cellular pore domains are colored red (helix α G and the C-terminus comprising residues 272-303 colored green for ClyA), and membrane phosphate beads are colored orange for clarity.

Figure 3. Radius of gyration, R_g for ClyA (A) and AHL (B) from MARTINI simulations. Data from all-atom simulations are shown in the insets.

with the smaller root mean square deviations (RMSDs) of AHL from its initial structure (discussed in the following section).

3.2 Stiffness landscapes of ClyA and AHL

The root mean square deviation (RMSD) histograms, $h(r)$ of the ClyA and AHL pores obtained from the all-atom and MARTINI simulations are illustrated in Figure 4 for comparison. The corresponding RMSD histograms show the mean value and the spread of the distributions sampled by both pores (Figure 4). The spread in the histograms is inversely correlated to the structural rigidity of the pore complex, with a higher mean implying greater deviation from the conformation of the energy minimized initial structure. The all-atom RMSD histograms of the ClyA and AHL pores have a mean value of ~ 0.36 nm and ~ 0.18 nm

respectively from the energy minimized initial structures. In comparison, the corresponding values from the MARTINI simulations are ~ 0.65 nm and ~ 0.35 nm respectively.

As a measure of the stiffness landscapes as reflected in the RMSD histograms, we define the quantity,

$$S(r) = -\ln h(r) \quad (1)$$

The stiffness landscapes, $S(r)$ reveal some interesting differences. AHL which is an example of a β toxin, has a much narrower landscape when compared with the ClyA which is an example of an α toxin. This relatively stiffer membrane pore complex associated with the β toxin is consistent with the overall stability attributed to the β toxins due to the inter-strand hydrogen bonding imparted to the membrane-inserted β sheets. Hence β toxins are in general more amenable to characterization. As a consequence, many more β -PFT pore

Figure 4. The RMSD histograms, $h(r)$ from both all-atom and MARTINI simulations for ClyA (A) and AHL (C). The corresponding stiffness landscapes (B) and (D) respectively are defined as $-\ln h(r)$ with arbitrary y-axis units. The stiffness landscapes indicate that the ClyA pore samples similar profiles in both the all-atom and the MARTINI simulations. However, the relative broadness of the MARTINI landscape is an indicator of the softer potentials due to coarse-graining.

crystal structures^{41–45} have been solved compared to α -PFTs,^{8,46} whose membrane-inserted domains consist of amphipathic α helices leading to weaker inter-protomer contacts.^{9,47,48}

The stiffer RMSD landscapes for AHL are observed in both the all-atom as well as MARTINI simulations. The relative broadness of the MARTINI stiffness landscape is an indicator of softer potentials used in a coarse-grained representation. As discussed earlier, the increased deformation of the pores on longer timescales accessible to coarse-grained simulations remains a possibility, but other factors such as the softer potentials and the reduction of molecular friction in the MARTINI coarse-grained force-field are more likely to contribute. Interestingly the mean values of the RMSD histogram peaks obtained from the MARTINI simulations are approximately twice the values obtained from the all-atom simulations. A second feature of the MARTINI landscapes are the longer tails toward the smaller RMSD values (when compared with the all-atom landscape) indicating that the high frequency modes or smaller displacements are partially sampled.

The elastic dynamic network which is used to preserve the secondary structure in the MARTINI force-field is applied within the protomer sub-unit, and the inter-protomer contacts in the pore are treated using the MARTINI potentials. From a detailed analysis of inter-protomer interactions, we find that polar interactions arising due to specific inter-protomer salt bridges,

hydrogen bonds, and van der Waal interactions play significant roles in stabilizing the inter-protomer interfaces in both α -PFT and β -PFT pores.³⁶ These inter-protomer contacts not only stabilize the multimeric pore assembly, but are directed to assist in forming the cylindrical shape of both the transmembrane and cytosolic domains during oligomerization. Interactions between these residues may not be captured accurately in the MARTINI representation. Protein-protein interactions in MARTINI must also compensate for factors such as decreased molecular friction in coarse-grained simulations that leads to larger RMSDs, and the absence of adequate protein-solvent interactions. In MARTINI simulations of viral capsid proteins,³⁵ the elastic network was modified based on data from atomistic MD simulations such that the RMSDs from the all-atom simulations matched those from the MARTINI simulations. In our study, distortions in specific regions such as the transmembrane domains in our simulations and previous observations³⁶ suggest that specific inter-protomer contacts, especially in the transmembrane domain which is exposed to the membrane lipids as well as the aqueous water channel in the pore lumen, have to be carefully re-calibrated (perhaps by including them in the elastic dynamic network).

3.3 Structural distortions are observed in the transmembrane domains of both pores

The overall length of the ClyA pore is 13 nm, and the pore diameter of the extra-cellular (or aqueous) side is 7 nm with the cytosolic pore opening in the transmembrane region being 4 nm in diameter. In contrast, the corresponding dimensions for AHL are 10 nm, 4.6 nm and 1.6 nm respectively. Hence the AHL pore lumen in the transmembrane region is ~ 3 times smaller than that observed for ClyA. In the 20 μ s long MARTINI simulations, although structural deviations from the initial crystal structures are observed, the pores mostly remain intact during this timescale (see Figures 1 and 3). In both ClyA and AHL MARTINI simulations, we find that the transmembrane pore opening has noticeable distortions resulting in deviations from their respective initial structures (illustrated in Figure 5). In the case of AHL which has a smaller pore interior when compared with ClyA, we observe a constricted pore lumen in the membrane-inserted region where β sheets interact and arrange to form the β barrel (see Figure 5 A). The formation of this constriction is illustrated by comparing the inner radius profiles of AHL at the start of simulation and at the end (see Figure 5 B). This constriction was not observed in the corresponding all-atom simulations of AHL, as shown in Figure 2 B. The transmembrane β -barrel of AHL is primarily stabilized by electrostatic interactions

Figure 5. A comparison of simulation snapshots at the start (0 μs , pore structures are colored cyan) and end (20 μs for AHL and 21.6 μs for ClyA respectively, pore structures are colored red) of the MARTINI simulations are illustrated to highlight the structural distortions in the transmembrane domains of both pores. Front and side views of (A) the AHL pore and (B) the ClyA pore are shown. The MARTINI beads are represented as van der Waal beads.

such as hydrogen bonds between the anti-parallel inter-protomer beta strands. Polar amino acids such as serine and threonine, which are responsible for hydrogen bonding, are not charged in the MARTINI representation. Hence, the under-representation of hydrogen bonding in the MARTINI force field could lead to loss of mechanical stability in the transmembrane β -barrel of AHL, which gets distorted in the MARTINI simulations.

Similar to AHL, the iris-like transmembrane α -barrel of ClyA, which is composed of the partly solvent-exposed and partly membrane-stabilized N-terminus and helix αA1 (residues 1 to 34), is mostly stabilized by polar inter-protomer interactions such as hydrogen bonds and salt-bridge networks. This region loses its structural integrity in the MARTINI simulations thus resulting in helix αA1 retracting into the pore lumen (see illustration in Figure 5 B). Such structural distortions are not seen in the all-atom simulations of ClyA (see Figure 2). While these distortions in the transmembrane domains of both pores could be a result of sampling over longer time scales in the MARTINI simulations, they are detrimental to leakage and lytic activity by these pores and the stability of the pore complex. Hence, a more obvious and biologically relevant inference is that the default MARTINI force-field and elastic dynamic network parameters do not accurately represent intra-protein interactions in

these complex oligomeric assemblies. Thus, careful reparameterization of this region by modifying the elastic dynamic network is necessary to achieve structural stability similar to that observed in the atomistic MD simulations (see Figure 1 vs. Figure 2). Reassessing membrane-protein and protein-solvent interactions in the MARTINI force-field may be another dimension to be explored towards accurately simulating these pore complexes over large timescales.

3.4 Inner radius of the pore lumen of ClyA and AHL

The structural distortions observed in both pores (discussed above) are quantified in this section by computing the inner radius profiles of the lumen of both ClyA and AHL pores. Comparison of the complete radius profiles along the inner axis of the pore (z coordinate) for the initial snapshots (0 μs and 0 ns for MARTINI and all-atom simulations respectively) and the final snapshots (20 μs and 100 ns for MARTINI and all-atom simulations respectively) are illustrated in Figure 6. It can be observed that the all-atom simulations show almost no change in the inner pore geometries over 100 ns, while the MARTINI simulations show large deviations in the transmembrane domains (shaded green) and some deviations in the extra-cellular domains. As illustrated in Figure 5, the β -barrel of AHL is severely constricted in

Figure 6. Comparative inner pore radius profiles (see Simulation Methodology for computation) from the energy minimized starting structures (cyan line, same color scheme as Figure 5) and final simulated structures (red line, same color scheme as Figure 5) of the MARTINI and all-atom simulations are shown for both AHL and ClyA pores. The radius is plotted along the inner radial axis, with $z = 0$ corresponding to the value of the axis at the cytosolic pore opening. The 4 nm green region approximately indicates the transmembrane domains of both pores, and is shown as a guide to the eye. The minimum inner pore radius of both pores from the final snapshots (red line) of the MARTINI and all-atom simulations are shown as purple dashed lines.

the MARTINI simulations, whereas the all-atom simulations show almost no deviation from the initial energy minimized crystal structure (Figure 2 B). In the case of ClyA MARTINI simulations, both constriction and expansion occur in the transmembrane α -barrel, and this can be attributed to the distortion of residues 1 to 34 as explained above and shown in Figure 5. Similar to AHL, no such distortions are observed in the ClyA all-atom simulations which closely resemble the initial energy minimized crystal structure (Figure 2 A).

The radius of the cytosolic and extracellular pore openings from MARTINI and all-atom simulations for both ClyA and AHL pores are plotted as a function of time in Figure 7 A. For AHL, the energy minimized initial structures from the all-atom and MARTINI

simulations have cytosolic pore radii of 1.28 nm and 1.12 nm respectively, and extracellular pore radii of 1.80 nm and 1.78 nm respectively. In all-atom AHL simulations, radii values averaged from 20 equally shaped snapshots between 80–100 ns are 1.23 ± 0.05 nm and 1.95 ± 0.09 nm for the cytosolic and extracellular openings respectively, and do not deviate much from the crystal structure. This is in contrast to the AHL MARTINI simulations, where radii values averaged from 100 equally shaped snapshots between 16–20 μ s are 0.44 ± 0.11 nm and 2.51 ± 0.18 nm for the cytosolic and extracellular openings respectively. Thus, the cytosolic opening of the transmembrane β -barrel is significantly smaller while the extracellular opening is widened. Similarly in ClyA, pore radii of the cytosolic

Figure 7. (A) Time evolution of the radius of the cytosolic pore opening (green line) and the extracellular pore opening (orange line) from the MARTINI and all-atom simulations are shown for both AHL and ClyA pores. Data from 500 snapshots spaced at 40 ns intervals, and 100 snapshots spaced at 1 ns intervals are used for radius computation from MARTINI and all-atom simulations respectively. (B) Time evolution of the minimum inner pore radius from MARTINI and all-atom simulations are shown for both AHL and ClyA pores. Trends from the MARTINI simulations show greater fluctuations than the all-atom simulations.

and extracellular openings from 80-100 ns of the all-atom simulations are 1.81 ± 0.05 nm and 3.74 ± 0.04 nm respectively, and don't vary significantly from values of 2.47 nm and 3.92 nm obtained from the initial structure. The pore radii of the cytosolic and extracellular openings from MARTINI simulations are 3.57 ± 0.37 nm and 4.08 ± 0.12 nm respectively. The radius of the cytosolic pore opening in the transmembrane domain thus varies significantly from 1.63 nm observed in the initial structure (see time trend in Figure 7 A), while the radius of the extracellular pore opening is similar to 3.64 nm obtained from the initial structure.

In Figure 6, the location of the most constricted part of the pore is indicated using purple dashed lines for the final snapshots (red line profiles) from both MARTINI and all-atom simulations. The minimum radius corresponding to the most constricted region inside the pore lumen (R_{min}) of both ClyA and AHL pores decides the maximum size of the molecules that can permeate through the pore. Hence, R_{min} decides the leakage flux through the pore and the type of molecule that can pass through, and hence is important for the pore lytic activity. For AHL, the minimum inner pore radius in the final snapshot is located at $z = 1.5$ nm (middle of the transmembrane β -barrel) for both MARTINI and all-atom simulations. Similarly for ClyA, the minimum inner pore radius in the final snapshot is located at $z = 2$ nm (iris-like transmembrane α -barrel) in the MARTINI simulations, and at $z = 0$ nm (cytosolic pore opening) in the all-atom simulations. The time evolution of the minimum pore radius of both ClyA and AHL pores from all-atom and MARTINI simulations are illustrated in Figure 7 B. It can be seen that AHL is severely constricted in the MARTINI simulations, with an average value of 0.26 ± 0.01 nm (from 100 equally shaped snapshots between 16–20 μ s) compared to an initial value of 0.59 nm. In contrast, AHL is unchanged in the all-atom simulations with an average value of 1.06 ± 0.01 nm (from 20 equally shaped snapshots between 80-100 ns) compared to an initial value of 1.07 nm. Both MARTINI and all-atom simulations of ClyA show slight constrictions with average values of 1.50 ± 0.09 nm and 1.81 ± 0.05 nm respectively compared to initial values of 1.63 nm and 2.00 nm. In addition, the all-atom trends show much smaller fluctuations than the MARTINI trends for both ClyA and AHL.

3.5 Membrane inserted arcs and the proteo-lipid complex

Unlike β toxins, the transmembrane domains of α toxins are formed by a bundle of α helices. The membrane-inserted bundle of helices must necessarily be compati-

ble with the hydrophobic interior of the lipid membrane. The pore formation pathways of α toxins compared to β toxins is rather poorly understood. The two main pore formation paradigms are the prepore pathway and the membrane-inserted “growing pore” oligomerization pathway. The prepore pathway is a widely accepted pore formation paradigm for β toxins, where the monomers assemble on the membrane surface to form a “pre-pore” complex, which subsequently inserts into the membrane to form a functional and lytic pore. However recent time resolved atomic force microscopy indicates that the pre-pore paradigm as a general mechanism is still under debate⁴⁹ especially for α -PFTs. In the membrane-inserted growing pore mechanism, the monomers first insert into the membrane and subsequently oligomerize to form the functional pore. In this pathway, oligomerization intermediates must necessarily be formed by partially assembled protomers and lipids forming a stable proteo-lipid complex or transmembrane arcs.⁷

We investigate the structure of possible proteo-lipid ClyA arcs by carrying out MARTINI simulations where we create an initial structure consisting of 6-mer to 10-mer ClyA arcs from the original dodecameric crystal structure. This oligomer is inserted into three different types of membranes (DMPC - 1,2-Dimyristoyl-sn-glycero-3-phosphorylcholine, POPC - 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, and DPPC - 1,2-dipalmitoyl-sn-glycero-3-phosphocholine). These three membranes are used because of their different physico-chemical properties. DMPC has saturated acyl chains and is experimentally in the liquid crystalline (L- α) phase at 310 K, DPPC also has saturated acyl chains and is experimentally in the gel (L- β) phase at 310 K, and POPC has one saturated and one unsaturated acyl chain, and is experimentally in the liquid crystalline (L- α) phase at 310 K. In the simulated systems, lipids that overlap with the arcs are removed to minimize repulsive contacts. The relaxed structure consists of the oligomeric arc-like complex with lipids contained within the arc in their native membrane structure. These initial structures are illustrated in Figure 8 at 0 ns.

Figure 8 illustrates snapshots of the 7 to 10-mer oligomers in different lipid environments at the end of 800 ns long MARTINI simulations. The configurations reveal significant distortions of the protein arcs from the initial structures indicating a fair degree of flexibility in these membrane-inserted partially oligomerized complexes. There is a greater tendency for the arcs to close as the number of protomers in the arcs increase. In particular, this is observed in the 10-mer arc where pore-like closed structures are formed in DPPC and POPC (illustrated in the time trends of the center-of-mass distance between edge protomers, Figure 9 C, F,

Figure 8. Top view of final snapshots at 800 ns from MARTINI simulations of different *n*-mer arcs (7 to 10-mer chosen for representations) in three different membrane environments (DMPC - sea green, DPPC - purple, POPC - dark blue, phosphate beads in all membranes colored fluorescent green) using MARTINI simulations. Significant distortions are observed in the arcs when compared with their initial structures (top row). Solvent has been removed for the sake of clarity.

I). This phenomenon can either be due to inadequacies in the MARTINI force-field (explained previously for the full pores) that results in an arc backbone that can undergo large distortions, or it could be a genuine biological phenomenon where larger arcs that are on the verge of forming a dodecamer close to form smaller pores. It must be noted that reports of ClyA pores of varying sizes (8-mer,⁵⁰⁻⁵² 12-mer,⁸ 13-mer,⁵³ 12-mer to 14-mer⁵⁴) exist in literature, and smaller pores could hypothetically be formed by arc closure. However, 300 ns long all-atom simulations of the 6-mer and 10-mer arcs in a DMPC membrane do not show any tendency to close,³⁶ and hence we cannot conclusively comment on this phenomenon at present.

We also computed the RMSD and R_g histograms for 6 to 10-mer arcs in the three different membrane environments (Figure 9). In general, we observe that the upper and lower bounds sampled by these histograms, are the greatest in the DMPC membrane, followed by POPC and the least for the DPPC membrane. This indicates that flexibility of these proteo-lipidic complexes is connected to lipid hydrophobic thickness and membrane stiffness, as reflected in packing of the hydrocarbon chains of the surrounding lipid molecules in the membrane. Hence, the greatest degree of flexibility (as reflected by the RMSD and R_g) is observed in DMPC which has 14 carbon atoms in both its acyl chains, followed by the unsaturated and asymmetric POPC, which

Figure 9. Structural attributes of the 6 to 10-mer arcs from the 800 ns MARTINI simulations in DMPC, POPC and DPPC bilayers are shown (snapshots for 7 to 10-mer arcs illustrated in Figure 8). The RMSD time traces and corresponding histograms (A, D, G) and the radius of gyration (B, E, H) imply that most arcs in the three membranes are flexible and show large plasticity in their quaternary structures, but none of them show a tendency to dissociate in the simulated timescales. However, some of the arcs have a tendency to close, as illustrated by the center-of-mass distance between the edge protomers (C, F, I).

has a 16 carbon and an 18 carbon acyl chains with a double bond in the 18 carbon chain. The saturated lipid DPPC, which is in the gel phase at 310 K, appears to offer the best packing with 16 carbons atoms in each chain. We point out that these connections with the physico-chemical properties of the lipid membrane are qualitatively captured in the MARTINI representation.

As the simulation proceeds, the lipids initially present in the arc interior (see initial configurations at 0 ns in Figure 8) are pushed out into the surrounding membrane to form a solvated toroidal edge. The final proteo-lipid complex has the following structure; the *n*-mer arc formed by the ClyA protomers form one edge of the pore and lipids arranged in a toroidal conformation in regions where protomers are absent to complete the pore formation. In the toroid, lipids are oriented with their headgroups directed towards the water lumen, thereby completing the hydrophilic lining of the pore. Our simulations reveal that these arc-like oligomeric complexes are stable suggesting that arcs are capable of leaking small ions during the process of oligomerization. All these molecular phenomena and their implications towards ClyA pore formation will be explored using comprehensive all-atom MD simulations in the future.

4. Conclusions

We have carried out coarse-grained molecular dynamics simulations of ClyA and AHL pores in a DMPC lipid membrane with explicit solvent for $\sim 20 \mu\text{s}$. From the RMSDs of both the atomistic and CG simulations, AHL is found to have a more rigid pore complex when compared with the α -PFT, ClyA. This is consistent with the increased stability imparted to AHL by β sheets in the transmembrane pore domain. We observe that both the mean value and spread in the RMSDs obtained from the MARTINI simulations is greater when compared with the all-atom simulations, which is a consequence of the softer potentials and reduced atomic friction in coarse-graining along with nonexistent or incorrectly represented inter-protomer interactions. Further, we have extensively characterized the extent of structural distortions by measuring the inner pore radius of the cytosolic and extracellular pore openings, and the minimum pore radius at the most constricted region of the pore lumen in both all-atom and MARTINI simulations and contrasting them with the initial structures for both ClyA and AHL. It may be possible to tune the values of the spring constants in the elastic dynamic networks to reconcile these differences. In addition, 800 ns MARTINI simulations of different ClyA protein arcs in

the membrane reveal that these arcs which are intrinsically connected with the pore formation pathway, are stable structures capable of ion leakage and inducing lytic activity. The extent of deformation in these arcs correlate with the overall hydrophobic content in the lipid membrane, with the greatest deformation observed in the DMPC bilayer which has the shortest hydrocarbon tail compared to POPC or DPPC. The presence of stable arcs supports the membrane-inserted growing pore model for pore formation which is emerging as the natural pathway for pore formation of α toxins. Finally, we conclude by observing that although MARTINI simulations are well suited to exploring long time scales for complex membrane-protein systems as illustrated in this study, the manner in which the inter-protomer contacts and the elastic dynamic network are treated in large protein assemblies (commonly encountered in PFTs) must be recalibrated to capture the deformation modes in a more realistic manner. Further, the mechanisms by which these additional deformations influence the long time dynamics of the membrane-pore complex must be understood to eventually make quantitative predictions, thereby alleviating the limitations of smaller time scales in the all-atom representation for these large membrane-pore assemblies.

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