MULTI-SCALE MOLECULAR DYNAMICS SIMULATIONS OF MEMBRANE-ASSOCIATED PEPTIDES

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ABSTRACT

Biological membranes function as an essential barrier between living cells and their environments. The membrane associated peptides (MAPs) interact with membrane either to facilitate the molecules exchange between the environments and cytoplasm (e.g. cell-penetrating peptide), or to disturb the membrane (e.g. amyloid peptides and antimicrobial peptides). The structures and activity of these peptides are essential to understand the mechanisms and to screen the drug candidates. Thus, in this dissertation, the structure prediction and screening of MAPs were firstly performed in Chapter II, III, and IV. We developed a structures-screening program base on GBMV implicit-solvent evaluation and a structure population evaluation program by Monte Carlo simulation to search the aggregated structures of amyloid peptide hIAPP with dominant populations. Seven stacking-sandwich models and three the wrapping-cord models were determined, which can also serve as templates to present double- and triple-stranded helical fibrils via peptide elongation, explaining the polymorphism of amyloid oligomers and fibrils. Base on the predicted oligomeric structures, the mechanisms of amyloid toxicity can be studied. We further investigated the dynamic structures, ion conductivity, and membrane interactions of hIAPP pores in the DOPC bilayer using molecular dynamics simulations (Chapter V and VI). Our results suggested that loosely-associated β-structure motifs can be a general feature of toxic, unregulated channels.
The process how MAPs adsorb on membrane and further penetrate across the membrane was further evaluated by the transmembrane potential mean force (PMF). We constructed an effect platform including adaptive biasing force (ABF) method which accelerates the membrane penetration process, umbrella sampling method which effectively generates trans-membrane PMFs, and MARTINI coarse-grained force field to measure the free energy required to transfer the MAPs from bulk water phase to water-membrane interface, and further to bilayer interior (Chapter VII). The results implied that biological activity of antimicrobial peptides appeared to be closely related to their trans-membrane ability indicated by the PMF profiles.

Moreover, due to the complicated components of cell membrane, it is better to simplify the interactions between MAP-membrane to MAP-artificial surfaces. Thus, in the last part of the dissertation, we further presented a series of exploratory molecular dynamics (MD) simulations to study the early adsorption and conformational change of amyloid peptide Aβ oligomers from dimer to hexamer on three different self-assembled monolayers (SAMs) (Chapter VIII). Within the timescale of MD simulations, the conformation, orientation, and adsorption of Aβ oligomers on the SAMs was determined by complex interplay among the size of Aβ oligomers, the surface chemistry of the SAMs, and the structure and dynamics of interfacial waters.
DEDICATION

I dedicate my dissertation to my dear family. A special feeling of gratitude to my loving parents, Yuqin Li and Chunhai Zhao, and my dear wife, Xiaoming Han, their support, encouragement, and constant love have been supporting me throughout my life.

It’s my greatest happiness to have you.
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CHAPTER I

RESEARCH OVERVIEW

1.1. Background

In living cells, membrane proteins/peptides are essential to signal transduction, nutrient utilization, and energy exchange between the cell and environment, e.g. ion channels, and cell penetrating peptides while some peptides can disturb the membrane leading to the membrane disruption and eventually the cell death, e.g. anti-microbial peptides and amyloid peptides. Rather than ion channel or porin, which have complicate tertiary structures, some peptides, which have only ~8-80 residues and simple secondary structures, can modulate the cell membrane for facilitation of cellular uptake of various molecular cargo, or disrupt the cell membrane resulting in the cell death. We name these peptides “membrane associated peptides” or MAPs. Due to their short length, the secondary structures of membrane associated peptides are simple, which can generally categorized to random coil, α helix, β turn/sheets, and the α/β mixed structure. Currently, three common membrane associated peptides are widely studied: the anti-microbial peptides (AMPs), amyloid peptides and cell penetrating peptides (CPPs). Anti-microbial peptides and cell penetrating peptides are usually amphipathic, which contain both highly
positively charged residues, such as Arginine and Lysine, and hydrophobic residues. Differently, there are highly hydrophobic regions/cores in amyloid peptides, making them prone to aggregate to oligomers and further fibrils containing rich β sheet structures. The functions and targets of these membrane associated peptides are dramatically different. Amyloid peptides usually target certain human cells to induce different diseases. For example, Alzheimer’s related Aβ aggregates deposit on the neuron cell membrane, disturbing the neuron cell; Diabetes II related hIAPP oligomers target mainly the β cells. Both AMPs and CPPs are amphipathic, but their functions are totally different. AMPs mainly target bacterial cells, of which the membrane is highly negatively charged. CPPs are short peptides that facilitate cellular uptake of various molecular cargos for human cells, and they never cause damage to human cells. The difference in the functions of the membrane associated peptides might lies in their sequence difference and the distribution of residues of different properties in the 3D space.

There are several factors which influence the penetrating activity of membrane associated peptides. The first important factor is the membrane components. AMPs attack only microbes, like bacteria and virus while never making the cell death of host. The outermost leaflet of the bacterial membranes bilayer is heavily populated by lipids with negatively charged phospholipid headgroups while the outer leaflet of the membranes of plants and animals is composed principally of lipids with no net charge. Besides membrane components, secondary structure (i.e. α-helical and β-sheet) and hydrophobic/cationic residue distribution are two factors that influence peptides-membrane interactions. However, the relationship between these factors and the
real membrane disruption/penetration mechanisms is still unclear. Due to the transient and heterogeneous nature of AMPs, it is extremely difficult to extract their structural information by traditional experimental methods. Thus, in this dissertation, computational methods, e.g. molecular dynamics, Monte Carlo simulation, computational statistics, were performed to study the structure-activity relationship of AMPs. The mechanisms behind these membrane associated peptides may help us design more potent drug candidates, or more efficient drug deliver vectors.

1.2. Fundamental problems

Although various experimental and computational efforts have been established to study the mechanisms and functions of membrane associated peptides, the key elements for the membrane disruption are still unclear. Moreover, the design strategies for drug candidates base on these membrane associated peptides are still limited. To provide the complete understanding of the molecular mechanism of the membrane penetrating or disruption of membrane associated peptides, three fundamental problems and technical challenges need to be tackled:

(1) Missing 3D structures in membrane and the transient and dynamic nature. The biological functions are closely related to the three dimensional structures of bio-molecules. However, we are lack of the three dimensional atomic structures of membrane associated peptides because the current high resolution AFM, SEM, X-ray, and NMR results only provide us the structure of crystals or fibrils, but hard to analyze the most toxic and fast transited heterogeneous small oligomers. Moreover,
the real functional structures of the peptides are membrane associated, which are more difficult to obtain the structures imbedded in membrane, especially the atomic-level details.

(2) Components of biological membrane are various. During the evolution, the lipid components have large variety in different species and different kinds of cells in the same species. For example, the lipids of human cells are usually neutral, like PC lipids, while the lipids of bacterial cells are highly negatively charged, like PG lipids. The components will also shift in different bacterial species, which result in different activities. Besides lipids, the membrane proteins are even more complicated. Thus, a general membrane models are missing to represent the key properties of the membrane.

(3) No efficient screening methods to evaluate the membrane penetrating ability.

Membrane associated peptides are proved to be targets (e.g. amyloid peptides), molecules delivery vectors (CPPs) or the drug agents (e.g. AMPs), thus their membrane penetrating ability has close relationship with their either toxicity or activity. As a result, many efforts have been done to evaluate the membrane association activity. However, due to the huge amounts of membrane associated peptides, it’s a time consuming, low efficiency, and expensive work to screen tens of thousands of peptides by experimental methods. Moreover, due to the different measurement techniques used in experiments, it is difficult to compare the membrane penetrating ability of different kinds of membrane associated peptides, which make it impossible to unveil the common features among the peptides.
1.3. Projects goals

The main purpose of the projects is to establish the platform to perform the membrane associated peptide structure prediction and the interactions between membrane associated peptides and various membranes using a circulatory multiple-step computational and experimental strategies, including an integrated mathematical model, all-atom molecular dynamic (MD) simulation, and experimental approaches.

1.3.1. Structure prediction and screening

The structures of membrane associated peptides are essential to study the interactions with membrane. Among all the three main types of membrane associated peptides, amyloid peptides are prone to aggregate to form polymorphic oligomers or fibrils, while AMPs and CPPs are less aggregatable due to the rich positive charges. Thus, amyloid peptides form more complicated structures than AMPs and CPPs. For amyloids, due to their polymorphoric structures, the home built “PEP-PACKING” scripts\textsuperscript{10-12}, are used to pre-screen the possible aggregates of amyloids, and the structures are further put to all atom MD simulation to test their structural stability (Chapter II, Chapter III, and Chapter IV). For membrane structures, CHARMM-GUI membrane builder\textsuperscript{13} was used to construct the all-atom membrane model for the mechanism study (Chapter V and Chapter VI). For AMPs and CPPs, we use MEMSAT program\textsuperscript{14, 15}, which is specifically developed for predicting the 3D structure of membrane proteins, to predict 3D atomic structures of Bac2A-based peptides (Chapter VII). The MARTINI membrane builder was used to construct the coarse-grained model for the screening
study. For Aβ oligomeric structures on SAMs surfaces (Chapter VIII), the initial monomer coordinate of Aβ_{17-42} peptide was taken and averaged from 10 NMR structures (PDB code 2BEG), derived from quenched hydrogen/deuterium-exchange NMR. The predicted structures will be used to study the interactions with membrane in the following tasks.

1.3.2. Mechanism study of membrane associated peptides

MAPs can insert into the cell membranes to form “toroidal” or “barrel-stave” transmembrane pores. The toroidal and barrel-stave structures are fundamentally different: in the toroidal pore, MAPs induce a local curvature of the lipid bilayer to form a highly curved pore, while in the barrel-stave pore, MAPs align vertically and parallel with respect to each other to form a circular pore. Apart from the transmembrane pore models, MAPs act as “detergents” to distract lipids from the membrane (“detergent model”) or intensively adsorb onto and completely/partially insert into the membrane to induce changes in membrane permeability and integrity (“carpet” model). Combination of these models have also been proposed. All of these models above could cause membrane disruption, leakage of cytoplasmic contents, and cell death. Experimentally, leakage of essential components of cell was observed, while the leakage mechanism in detail is still not well-established. Base on the morphology of the pore structure from AFM and EM, series potential pore structures which have similar dimension with those from experiments were constructed. And the ion leakage process was studied (Chapter V). MAPs always have polymorphic structures, while experiments only provide limited information. Thus, different ion leakage pores with various packing patterns were
constructed, and double ion leakage pores were constructed to mimic the polymorphic nature (Chapter VI).

1.3.3. Statistical classification of membrane associated peptides

In this dissertation, we utilized QSAR as the first level screening method to search potential membrane associated peptides (AMPs, Chapter VII). The QSAR program will be used to quickly and systematically construct, optimize, and rank a series of candidate peptides with different levels of membrane penetrating activity according to Factor Analysis Scales of Generalized Amino acid Information (FASGAI) which represents the structural features of the peptides and Linear Discriminant Analysis (LDA) which classifies the dependent of the samples\(^\text{18}\). This model will be testified by resubstitution test and subsampling test, separately. Moreover, support vector machine (SVM) method\(^\text{19}\) was also applied to select sequences with high potent activity as an alternative screening method.

1.3.4. Evaluate trans-membrane activity of membrane associated peptides using potential mean force

The peptides screened in section 1.3.3 will be subsequently subject to the coarsed-grained MD simulation to examine their trans-membrane potential by potential mean force (PMFs). To facilitate peptide penetration process across the bilayer, an external force was applied to either N-terminal or C-terminal bead to pull the peptide across the bilayer with a constant velocity. During the peptide penetration process, PMFs
were calculated using the umbrella sampling protocol\textsuperscript{20} as a function of the distance between the peptides and the lipid bilayer. To test the peptide activity, we use POPE:POPG 3:1 lipid bilayers to mimic the negatively charged bacterial membrane and pure POPC lipid bilayers to mimic the neutral mamalian membrane, and more particularly to mimic the human blood cell membrane for hemolytic effects. The computationally designed peptides with strong trans-membrane ability will be further tested experimentally for their activity and toxicity.

1.3.5. Adsorption, aggregation and orientation behaviours of membrane associated peptides

All the works above are base on the final state of the MAP aggregates, while how MAPs monomers or lower-oligomers associate, aggregate and adsorb on membrane dynamically are still unknow. Artificial surfaces, e.g. self-assembled monolayers (SAMs), are well-packed and simple surfaces, of which the head groups can be modified to mimic different membrane components, e.g. hydrophobic, hydrophilic and charged components. Thus, series of MAP oligomers on the aggregation pathway were studied on the SAMs to figure out the binding affinity, seed structure, and binding orientation (Chapter VIII).

1.4. Perspectives

Base on all the projects above, the long term objective of this dissertation is that the effective computational methods can be applied to not only membrane associated peptides, but also the other kinds of structure based drugs, such as small molecular drugs
and proteins. In a broader context, the effective and low-cost platform integrated with structure prediction, mechanism study, transmembrane activity evaluation, and drug design will decrease the cost of early drug test and benefit the whole society.
CHAPTER II

STRUCTURAL POLYMORPHISM OF HUMAN ISLET AMYLOID POLYPEPTIDE (IAPP) OLIGOMERS HIGHLIGHTS THE IMPORTANCE OF INTERFACIAL RESIDUES

2.1. Introduction

Human islet amyloid polypeptide (hIAPP, also known as amylin), a 37-residue hormone peptide, is produced and stored in pancreatic islet β-cells and co-secreted with insulin \(^{21}\). A number of \textit{in vitro} and \textit{in vivo} studies have shown that the spontaneous aggregation of hIAPP peptides into β-sheet-rich amyloid fibrils in patients with non-insulin-dependent (type II) diabetes has deleterious effects on the β-cells with severe consequences for insulin production. Mice do not ordinarily suffer from type II diabetes, although mouse IAPP differs from human IAPP in only 6 of 37 residues. But, transgenic mouse models that express human IAPP develop fibrillar deposits and exhibit signs of diabetes \(^{22}\). This suggests that hIAPP is pathologically associated with β-cell toxicity in type II diabetes. Similar aggregation of misfolded amyloid proteins into amyloid fibrils has also been found in other neurodegenerative diseases including Alzheimer’s, Parkinson’s, and Huntington’s. All these amyloid fibrils display a high content of
cross-β-sheet structure regardless of their sequences and native structures, where β-strands within a β-sheet or β-layer are packed perpendicular to the long fibril axis and linked by backbone and sidechain hydrogen bonds parallel to the same fibril axis.

The full-length hIAPP peptide is composed of multiple function regions, including an N-terminal region (residues 1-19) that involves in membrane binding, a primary amyloidogenic region (residues 20-29), and a C-terminal region (residues 30-37) that enhances amyloid formation. Mounting evidence suggests that some soluble hIAPP oligomers rather than mature hIAPP fibrils are cytotoxic species to islet β-cells. For example, a number of in vivo studies reported that increased β-cell apoptosis poorly correlated with the levels of insoluble hIAPP fibrils and monomers, but not soluble oligomers. Studies of transgenic rat and mice models also confirmed that β-cell death and impaired insulin secretion occurred before the appearance of extracellular islet amyloid deposits. Consistently, several antibodies of hIAPP only specifically bind to soluble hIAPP oligomers, but not to amyloid fibrils and monomers.

Recently, Tycko’s lab proposed two possible molecular structures of full-length hIAPP protofibrils using solid-state NMR method. Both structures were composed of two layers of hIAPP1-37 with a 2-fold symmetry along the fibril axis and with the C-terminal β-strands to form the interface between two layers. Similar to the Tycko’s model, Wiltzius et al. proposed another similar atomic model of full-length hIAPP1-37 fibrils by combining two crystal structures of hIAPP fragments of 21NNFGAIL and 28SSTNVG, which consisted of two layers laterally associated by the C-terminal.
β-strands with a 2-fold symmetry along the fibril axis, and each hIAPP1-37 peptide possessed a “U-bend” structure. But, the Tycko’s and Wiltzius’s models differ in the details of interfacial sidechain packing at the cross section. The Wiltzius’s model has a steric zipper formed by the interfacial residues of SSTNVG, while the Tycko’s models do not interdigitate sidechains very tightly to form a zipper. More importantly, several structural studies of full-length hIAPP fibrils have suggested that mature fibrils may contain two, three, four, and up to five layers based on mass per length (MPL) analysis. Goldsbury et al. 32, 33 and Kajava et al. 34 reported that full-length hIAPP fibrils predominately contained three-layered protofibril, coexisting with the second highest population of two-layered protofibril and other minor populations of four and five-layered protofibrils. Tycko et al. 29, however, reported that amylin fibrils mainly contained two cross-β-layers, and a minority fraction of other fibril morphologies (one, three, four, and five layers).

MPL data raise the questions if such multiple-layer protofibrils exist, there must exist a number of packing possibilities to laterally associate different layers via different interfaces. Apart from a few atomic structures of two-layered hIAPP oligomers with a two-fold symmetry 29,30,35,36, detailed structural information for multiple-layered hIAPP oligomers (> 2 layers) with different sizes, association interfaces, and symmetries along the lateral direction are not available to date. Here, we employed an in-house peptide-packing program and explicit solvent all-atom molecular dynamics (MD) simulations, which have been used to model oligomeric structures of Aβ peptides (linear, annular, triangular, and micelle) 37-41, to search, optimize, and determine a series of
two-layer and three-layer hIAPP oligomers with various sizes, symmetries, and lateral interfaces, with particular attention to the effects of different layer-to-layer interfaces (i.e. C-terminal–C-terminal, C-terminal–N-terminal, and N-terminal–N-terminal interfaces) on the structural stability and conformational dynamics of hIAPP linear oligomers. Three two-layer oligomers in a two-fold symmetry and four asymmetrical three-layer oligomers were determined and used as possible subunit architectures of hIAPP fibrils. Simulation results showed that all stable oligomers were well hydrated in a global structure by optimizing peptide solvation energy, yet dehydrated at the layer-to-layer interfaces to some extent by maximizing peptide-peptide interactions at the interface. Comparison of binding energies between two adjacent layers associated through different interfaces, along with computational mutagenesis of residues of Asn31Ala and Phe15Ala at the interface, further revealed that interfacial residue interactions were driven by delicate balance among van der Waals (VDW), electrostatic, and solvation interactions with different intermolecular contributions. Knowledge of structural details of multiple-layered hIAPP oligomers derived from this work may provide valuable insights into the design of specific inhibitors for preventing type II diabetes.

2.2. Materials and Methods

2.2.1. hIAPP models.

Initial monomer coordinate of hIAPP$_{1-37}$ peptide was extracted and averaged from 10 solid-state NMR-based structures from Tycko’s lab $^{29}$. Each hIAPP$_{1-37}$ peptide had a β-strand─loop─β-strand (U-bend) fold. Intra-molecular disulfide bond between Cys2 and
Cys7 was formed to stabilize the structure at the N-termini. The N- and C-termini were blocked by \( \text{NH}_3^+ \) and \( \text{COO}^- \) groups, respectively.

To build a two-layer hIAPP oligomer, an hIAPP\(_{1-37}\) pentamer was first constructed by packing hIAPP\(_{1-37}\) monomers on top of each other in a parallel and register manner, with an initial peptide-peptide separation distance of \( \approx 4.7 \ \text{Å} \), corresponding to the experimental data \(^{29}\). Within the pentamer, no translation was applied to one peptide relative to the other. Single hIAPP pentamer has two different \( \beta \)-sheets (i.e. C-terminal \( \beta \)-sheet and N-terminal \( \beta \)-sheet), allowing to laterally associate with the other hIAPP oligomer via C-terminal─C-terminal (CC) interface, N-terminal─C-terminal (NC) interface or N-terminal─N-terminal (NN) interface. Thus, the second IAPP\(_{1-37}\) pentamer was duplicated, rotated, and translated with respect to the first pentamer to form a two-layer hIAPP decamer with CC, NC or NN interface in an antiparallel fashion (Figure 2.1).

To obtain possible stable two-layer hIAPP oligomer by screening vast variability of conformational ensemble at the early stage of aggregation process, the inter-layer distance between two pentamers along the \( y \) axis and the inter-layer translation along the \( x \) axis (i.e. along the \( \beta \)-strand direction) were considered as critical factors to affect sidechain optimization and sheet association responsible for structural stability and packing energy (total energy) of the IAPP oligomers.
Figure 2.1. Packing energy profiles as a function of inter-layer translation ($dx$) and inter-layer distance ($dy$) in both $x$ and $y$ directions for two-layer hIAPP oligomers with three distinct (a) CC, (b) NC, and (c) NN interface. Distances of $d_x$ and $d_y$ are varied by translation of one of the two-layers relative to the other along the strand axis ($x$ axis) and the axis perpendicular to both fibril and strand axles ($y$ axis). Five lowest-energy structures, i.e. the $\text{N}_U\text{CC} \cap \text{N}_N$ -30 and the $\text{N}_U\text{CC} \cap \text{N}_N$ -6 models for CC interface, $\text{C}_U\text{NC} \cap \text{N}_N$ -2 model for NC interface, and $\text{C}_U\text{NN} \cap \text{C}_N$ -16 and $\text{C}_U\text{NN} \cap \text{C}_N$ -21 models for NN interface, are selected from total 780 candidates and identified by arrows.

As shown in Figure 2.1, the origin of the coordinates was specified to COM of the bottom layer, and for each CC, NN or NC interface packing, the inter-layer distance ($d_y$) between two pentamers were varied by every 0.5 Å from 8.5 to 9.5 along the $y$ axis, while the inter-layer translation ($d_x$) along the $x$ axis (i.e. one layer was translated with respect to the other along the β-strand direction) were varied by every 1 Å from 0 to 50 Å for the CC interface packing, from 0 to 50 Å for the NC interface packing, and from 0 to 30 Å for the NN interface packing (the less scanning distance of NN interface is due to
the relative short length of N-terminal β strand). Using this structural scanning method, total 300, 300, and 180 candidate structures were generated for CC, NC, and NN interfaces. Two lowest-energy structures from the CC packing with $d_y=9.1$ Å, one from the NC packing with $d_y=8.6$ Å, and two from the NN packing with $d_y=9.1$ Å were selected and subsequently subjected to explicit-solvent MD simulations for assessing the suitability of each model. The Tycko’s two-layer model was also studied for comparison. The Tycko’s model consists of two anti-parallel β-sheets via the CC interface with the overall width of ~50 Å and initial inter-layer separation of 8.6 Å.

Given stable two-layer hIAPP oligomers with distinct interface (i.e. CC, NN, or NC interface) are determined by the MD simulations, a three-layer hIAPP oligomer was directly constructed by stacking third hIAPP pentamer on top of the stable two-layer hIAPP oligomer in an antiparallel way. The third pentamer was placed at the same position as one of three interfaces in the two-layer models, i.e. with the same inter-layer distance and translation relative to the two-layer hIAPP oligomer. There are four possible three-layer models by considering different combinations of two interfaces.

Previous studies have pointed out that Asn ladder and π-stacking play an important role in stabilizing amyloid oligomers/fibrils. The wild-type sequence in the two-layer models was mutated to examine the layer-to-layer association at the interface, i.e. Phe15 was mutated to Ala at the NN interface and Asn31 was mutated to Ala at the CC interface. All initial mutant structures were built by replacing sidechains of the targeted residues without changing the backbone conformations and sidechain orientations. The structures of designed mutant were first minimized for 500 steps using
Table 2.1. Details of hIAPP oligomers including one, two, and three layers

<table>
<thead>
<tr>
<th>System</th>
<th>Interface Residues</th>
<th>Cross-section Area (Å²)</th>
<th>Time (ns) and Runs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tycko pentamer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tycko</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$N_{\text{CC}}$, $N_{\text{CN}}$</td>
<td>$^1$Ala25 $^1$Leu27 $^1$Ser29 $^1$Asn31 $^1$Asn35 $^2$Tyr37 vs. $^2$Phe23 $^2$Ala25 $^2$Leu27 $^2$Ser29 $^2$Asn31 $^2$Asn35 $^2$Tyr37</td>
<td>60.3×40.1</td>
<td>30, 2</td>
</tr>
<tr>
<td>$N_{\text{CN}}$, $N_{\text{CC}}$</td>
<td>$^1$Ser29 $^1$Asn31 $^1$Asn35 $^2$Tyr37 vs. $^2$Arg11 $^2$Ala13 $^2$Phe15 $^2$Val17 $^2$Ser19</td>
<td>57.3×35.8</td>
<td>30, 2</td>
</tr>
<tr>
<td>$N_{\text{CN}}$, $N_{\text{CC}}$-N31A</td>
<td>$^1$Ala25 $^1$Leu27 $^1$Ser29 $^1$Ala31 $^1$Asn35 $^2$Tyr37 vs. $^2$Ala25 $^2$Leu27 $^2$Ser29 $^2$Ala31 $^2$Asn35 $^2$Tyr37</td>
<td>63.2×39.6</td>
<td>30, 2</td>
</tr>
<tr>
<td>$c_{\text{NN}}$, $c_{\text{CN}}$</td>
<td>$^1$Ala13, $^1$Phe15, $^1$Val17 vs. $^2$Ala13, $^2$Phe15, $^2$Val17</td>
<td>78.9×40.3</td>
<td>30, 2</td>
</tr>
<tr>
<td>$N_{\text{CN}}$, $N_{\text{CC}}$-F15A</td>
<td>$^1$Ala13, $^1$Ala15, $^1$Val17 vs. $^2$Ala13, $^2$Ala15, $^2$Val17</td>
<td>83.9×33.5</td>
<td>30, 2</td>
</tr>
<tr>
<td>$c_{\text{NN}}$, $c_{\text{CN}}$</td>
<td>$^1$Ala13, $^1$Phe15, $^1$Val17 vs. $^2$Ala13, $^2$Phe15, $^2$Val17; $^2$Ser29 $^2$Asn31 $^2$Asn35 $^2$Tyr37 vs. $^3$Arg11 $^3$Ala13 $^3$Phe15 $^3$Val17 $^3$Ser19</td>
<td>79.0×63.8</td>
<td>30, 2</td>
</tr>
<tr>
<td>$N_{\text{CN}}$, $N_{\text{CC}}$-U</td>
<td>$^2$Ser29 $^2$Asn31 $^2$Asn35 $^2$Tyr37 vs. $^2$Arg11 $^2$Ala13 $^2$Phe15 $^2$Val17 $^2$Ser19; $^2$Ala25 $^2$Leu27 $^2$Ser29 $^2$Asn31 $^2$Asn35 $^2$Tyr37</td>
<td>63.3×59.9</td>
<td>30, 2</td>
</tr>
<tr>
<td>$c_{\text{NN}}$, $c_{\text{CN}}$</td>
<td>$^1$Ala13, $^1$Phe15, $^1$Val17 vs. $^2$Ala13, $^2$Phe15, $^2$Val17; $^2$Ser29 $^2$Asn31 $^2$Asn35 $^2$Tyr37 vs. $^3$Ala25 $^3$Leu27 $^3$Ser29 $^3$Asn31 $^3$Asn35 $^3$Tyr37</td>
<td>85.2×58.3</td>
<td>30, 2</td>
</tr>
<tr>
<td>$c_{\text{NN}}$, $c_{\text{CN}}$-U</td>
<td>$^2$Ser29 $^2$Asn31 $^2$Asn35 $^2$Tyr37 vs. $^2$Arg11 $^2$Ala13 $^2$Phe15 $^2$Val17 $^2$Ser19; $^2$Ser29 $^2$Asn31 $^2$Asn35 $^2$Tyr37 vs. $^3$Arg11 $^3$Ala13 $^3$Phe15 $^3$Val17 $^3$Ser19</td>
<td>56.9×66.5</td>
<td>30, 2</td>
</tr>
</tbody>
</table>
2.2.2. MD protocol.

All MD simulations were performed using the NAMD software package with CHARMM27 force field for peptides and TIP3P for waters. Each oligomer was solvated in a TIP3P water box with a margin of at least 15 Å from any edge of the water box to any peptide atom. Any water molecule within 2.6 Å of the peptide was removed. Each system was then neutralized by adding Cl\(^-\) and Na\(^+\) ions to mimic ~200 mM ionic strength. The resulting systems were subject to 5000 steps of steepest decent minimization with peptide backbone atoms harmonically constrained, followed by additional 5000 steps of conjugate gradient minimization with all atoms allowed to move. Short 1-ns MD simulations were performed to equilibrate systems by constraining the backbones of oligomers. The production MD simulations were performed using an isothermal-isochoric ensemble (NPT, T=300K and P=1 atm) under periodic boundary conditions with the minimum image convention. The Langevin piston method with a decay period of 100 fs and a damping time of 50 fs was used to maintain a constant pressure of 1 atm, while the Langevin thermostat method with a damping coefficient of 1 ps\(^{-1}\) was used to control the temperature at 300 K. All covalent bonds involving hydrogen were constrained by the RATTLE method so that 2-fs timestep was used in the velocity Verlet integration. Van der Waals (VDW) interactions were calculated by the switch function with a twin-range cutoff at 12 and 14 Å. Long-range electrostatic interactions were calculated using the force-shifted method with a 14 Å cutoff. Structures were saved every 2 ps for analysis. All analyses were performed using tools within the CHARMM, VMD, and code developed in-house.
2.3. Results and discussion

For clarity and convenience, we borrowed the notation used in the Aβ simulations to define the hIAPP models. We denote a single cross-β-layer as \( N_U C \) where U represents a parallel β-strand-loop-β-strand assembly, N for the N-terminal β-sheet, and C for the C-terminal β-sheet. For a two-cross-β-layer, there are three typical models of \( N_U C \cap \), \( C_U N \cap C \), and \( C_U N \cap N \) where U and \( \cap \) represent antiparallel assembly, double letters of CC, NN, and NC represent distinct interface between neighboring β-sheets, and single letter of N or C represent the N-terminal or C-terminal β-sheet exposing to bulk water. Similarly, there are four asymmetrical three-cross-β-layer models of \( C_U N \cap C_U N \), \( N_U C \cap C_U N \), \( C_U N \cap C_U N \), \( N_U C \cap C_U N \), \( N_U C \cap C_U N \), and each having two interfaces. All β-layers were aligned alternatively in an antiparallel arrangement.

2.3.1. Structural stability of single-layer hIAPP oligomers

Since single-layer hIAPP pentamer is a basic building block to construct two-layer and three-layer models, we first examined the structural stability and conformational dynamics of single-layer pentamer by stacking five repeated hIAPP monomers on top of each other with an interpeptide distance between two neighboring peptides of 4.7 Å via 30 ns simulations at 300 K. Figure 2.4a showed that the backbone root-mean-square deviation (RMSD) quickly approached to a plateau of \( \approx \) 5.2 Å within 6 ns and then stabilized around this value for the remainder of the simulations. Visual inspection of MD trajectories clearly showed that overall single-layer structure was very stable without peptide dissociation during the 30 ns simulations, although the edge residues at the
N-/C-terminals displayed structural fluctuation to some extent leading to the twisted β-sheets. The residue-based root-mean-square fluctuation (RMSF) of the backbones also confirmed that residues at the turn region (residues 18-23) had much lower fluctuations than residues at the edges (residues 1-5, 33-37) (data not shown). Taken together, the results indicate that the relatively large structural fluctuation mainly originated from the two edges of the cross-β-layer, but the secondary structure of the strand-loop-strand motif was well preserved. In addition, the simulated single-layer pentamer was compared to the NMR structure by removing one cross-β-layer from the Tycko’s two-layer models. In both single-layer models with different starting structures, superimposition of final structures displayed remarkably structural similarity in overall size, well conserved U-bend conformation, and twisted β-sheets (Figure 2.2d). Such a similar structural conformation in both simulation and crystal models further suggests that the single-layer hIAPP oligomer is a reasonable representation during the hIAPP aggregation and can be served as a stable build block for fibril polymorphism.

2.3.2. Conformational search for two-layer hIAPP oligomers

Similar to a number of amyloid-forming peptides such as Aβ, human CA150 \(^{48}\), and β2-microglobulin \(^{49}\) peptides, the hIAPP peptide also has a U-bend shape consisting of two antiparallel β-strands connected by one loop, β-strand (Lys1-Val17)—loop (His18-Leu27)—β-strand (Ser28-Tyr37), as shown in Figure 2.2. The U-bend conformation allows β-sheets to be associated in the lateral direction via different layer-to-layer interfaces to enrich fibril polymorphism.
Figure 2.2. MD snapshots of two-layer hIAPP oligomers with three distinct interface packings of (a) $N_{UC} \cap N$, (b) $C_{UC} \cap N$, and (c) $C_{NN} \cap C$ at 30 ns. Color codes are: backbone (white), polar residues (green), non-polar residues (orange), positively charged residues (red), and negatively charged residues (blue). (d) Structural superimposition of simulated one-layer and two-layer $N_{UC} \cap N$ model (light blue) with the Tycko’s models (orange) derived from the NMR data.
To find the most favorable packing arrangement between two cross-β-layers, an in-house peptide program is used to systematically search possible packing combinations by displacing one layer with respect to the other in both $x$ and $y$ directions, yielding 180-300 candidate structures for various interfaces (details in Materials and Methods). Figure 2.1 showed the packing energy profile, calculated by the generalized Born with simple switching method (GBSW)\textsuperscript{50}, as a function of inter-layer translation ($d_x$) and inter-layer distance ($d_y$) in both $x$ and $y$ directions for three distinct CC, NN, and NC interface. During the $d_x$ and $d_y$ variations, inter-layer distance ($d_y$) varied from 8.1 Å to 8.6 Å to 9.1 Å did not yield any significant change in packing energy for all three interface models, while inter-layer translation ($d_x$) can largely mediate packing energy by improving sidechain geometry match. Given inter-layer distance of $d_y=8.6$ or 9.1 Å close to experimental value, the five lowest-energy two-layer structures were identified at $d_x=-6$ Å and $d_x=-30$ Å for the CC interface, $d_x=16$ Å and $d_x=21$ Å for the NN interface, and $d_x=-2$ Å for the NC interface (marked by an arrow in the packing energy profiles). All five models have different interfaces formed by two β-layers at the cross section with a two-fold symmetry. It should be noted that the lowest-packing-energy structures determined by the GBSW method do not necessarily imply the most stable structures in the explicit solvent due to the less accurate value of peptide-water interactions compared with the explicit solvent simulation, thus all five two-layer models were then submitted to all-atom explicit-solvent MD simulations to examine their structural stability for model validation.
2.3.3. Modeling of two-layer hIAPP oligomers with three distinct interfaces

Tycko et al.\textsuperscript{29} have reported that the mass-per-length (MPL) data for a single-layer of hIAPP molecules are approximately 8.1 kDa/nm. Higher MPL values of 19.9, 29.0, and 40.3 kDa/nm extracted from scanning transmission electron microscopy (STEM) images are most likely attributed to lateral association of two and three molecular layers, as well as a minority fraction of four molecular layers. Since the single cross-β-layer is stable, it can be used as a building block to construct a series of two-layer structures with three distinct interfaces of CC, NN, and NC and with two-fold symmetry. As described earlier, for the CC interface model (i.e. the $\cap_{\text{N}}$), two lowest-energy structures were identified and subjected to explicit-solvent MD simulations. Visual inspection of MD trajectories shows that one CC interface structure at $d_{x}=-30$ (referred as the CC$\text{\_30}$ model) displayed marginal stability within 30 ns simulation, presumably due to insufficient interfacial interactions spanning only four polar residues of Ser29, Asn31, Asn35, and Tyr37 (Figure 2.2a). In contrast, the other CC interface model at $d_{x}=-6$ (referred as the CC$\text{\_6}$ model) was very stable with small conformational change relative to the initial structure (RMSD$\approx$3.5 Å). The CC$\text{\_30}$ model will not be discussed thereafter due to structural instability, while the stable CC$\text{\_6}$ model is referred as the $\cap_{\text{N}}$ for convenience. Inspection of the layer-to-layer interface of the $\cap_{\text{N}}$ structure revealed a maximum overlap of sidechain contacts between two adjacent C-terminal β-sheets. The CC interface consisted of five polar sidechains of Ser29, Asn31, Gly33, Asn35, Tyr37 and two hydrophobic sidechains of Ala25 and Leu27. These interfacial residues contributed $\approx$19 hydrogen bonds and $\approx$7 hydrophobic contacts at the
optimized interface (Figure 2.5a) to structural stability via well conserved polar contacts of \( ^1\text{Leu27} - ^{II}\text{Asn35}, \; ^1\text{Ser29} - ^{II}\text{Gly33}, \; ^1\text{Ser29} - ^{II}\text{Asn35}, \; ^1\text{Asn31} - ^{II}\text{Asn35}, \; ^1\text{Asn31} - ^{II}\text{Ser29}, \; ^1\text{Gly33} - ^{II}\text{Ser29}, \; ^1\text{Asn35} - ^{II}\text{Leu27} \) and partially conserved contacts of \( ^1\text{Tyr37} - ^{II}\text{Phe23} \) and \( ^1\text{Phe23} - ^{II}\text{Tyr37} \) (superscripts I and II represent two different cross-\( \beta \)-layers), suggesting that the CC interface is mainly governed by polar interactions.

Figure 2.3. MD snapshots of three-layer hIAPP oligomers with four distinct interface packings of (a) \( c_{\text{NN}} \cap c_{\text{CN}} \cap c_{\text{U}} \), (b) \( n_{\text{UC}} \cap c_{\text{CN}} \cap n_{\text{U}} \), (c) \( c_{\text{NN}} \cap c_{\text{CC}} \cap n_{\text{U}} \) and (d) \( n_{\text{UC}} \cap c_{\text{CN}} \cap c_{\text{UC}} \) at 30 ns. Color codes are: backbone (white), polar residues (green), non-polar residues (orange), positively charged residues (red), and negatively charged residues (blue).
Figure 2.4. Backbone RMSDs of (a) one-layer models, (b) two-layer models, and (c) three-layer models from their energy-minimized structures.

Figure 2.5. Number of native contacts including hydrogen bonds and hydrophobic contacts between two neighboring β-layers for (a) two-layer models and (b) three-layer models. A hydrogen bond is assigned if the distance between donor D and acceptor A is ≤3.6 Å and the angle D-H…A is ≥120. A hydrophobic contact is identified when the mass center distance between a pair of hydrophobic side chains is ≤6.5 Å.

In the $cU_{NN\cap C}$ cross-β-layer organization, two NN interface models at $d_x=16$ and 21 Å were observed at the lowest energy state (Figure 2.1c). The NN interface with $d_x=16$ Å
(referred as the NN-16 model) consisted of a combination of polar residue of Thr9, charged residue of Arg11, and hydrophobic residues of Ala13, Phe15, and Val17, while the NN interface with d_s=21 Å (referred as the NN-21 model) consisted of highly hydrophobic residues of Ala13, Phe15, and Val17. The only difference between these two interface models was a two-residue shift along the axis of N-terminal β-strand. The NN-16 interface spanning five residues from Thr9 to Val17 involved initial intermolecular contacts of \text{I}Arg11--\text{II}Val17, \text{I}Ala13--\text{II}Phe15, \text{I}Phe15--\text{II}Phe15, \text{I}Phe15--\text{II}Arg11, \text{I}Phe15--\text{II}Phe13, and \text{I}Val17--\text{II}Arg11, while the NN-21 interface strongly involved hydrophobic contacts of \text{I}Val17--\text{II}Ala13, \text{I}Phe15--\text{II}Phe15, and \text{I}Ala13--\text{II}Val17. It is interesting to observe from 30-ns MD trajectories that the NN-16 model experienced a dramatic displacement of one cross-β-layer relative to the other by shifting ~two-residue distance of ~11 Å along the axis of N-terminal β-strand, yielding a different structural assembly with a new interface remarkably similar to the final conformation of the NN-21 model. Superimposition of final conformations of the NN-16 and the NN-21 models at 30-ns yielded ~5.2 Å difference in structural deviation, suggesting that the NN-21 model is more energetically favorable conformation. The structural rearrangement from the NN-16 model to the NN-21 model is to eliminate incompatible VDW interactions of small loop formed by a disulfide bond between Cys2 and Cys7 at the N-terminals of one layer with Ser19 and Ser20 of the mating layer. In the NN-21 model, although the overlap region of interfacial residues at the NN interface was small, solely hydrophobic stretches among Ala13, Phe15, and Val17 were strong enough to hold two layers together via ~15 hydrophobic contacts (Figure 2.5a). Especially two
Phe15 ladders from each layer were closely packed against each other at the center of the interface, providing additional π-π stacking interactions to enhance the stability. Meanwhile, Arg11 residues were largely exposed to waters to attenuate electrostatic repulsion within the cross-β-layer. Taken together, the NN-21 model displayed high stability as evidenced by 30-ns MD trajectories without layer dissociation, relatively constant layer-to-layer distance of 9.1 Å, and well conserved secondary structures. Consistently, the NN-21 model is referred as the C\textsubscript{U}\textsubscript{NN}∩C for convenience and will be further discussed thereafter.

Unlike both N\textsubscript{U}C\textsubscript{C}∩N and C\textsubscript{U}\textsubscript{NN}∩C models, the C\textsubscript{U}C\textsubscript{N}∩C structure consisted of a heterogeneous interface by packing Ser29, Asn31, Asn35, and Tyr37 from the C-terminal β-strands of one layer against Arg11, Ala13, Phe15, Val17, and Ser19 from the N-terminal β-strands of the mating layer, with a maximum overlap of interfacial residues spanning 39 Å. A small disulfide-linked loop between Cys2 and Cys7 was protruded from the end of N-terminal β-strands, yielding an uneven surface packing against the C-terminal β-sheets of the mating layer. Unexpectedly, MD simulations did not observe significant changes with respect to initial structure in overall structural deviation of RMSD=4.0 Å, separation distance of 10.9 Å between two layers, and local secondary structure (Table 3.2 & Figure 2.4b). Examination of the interface details revealed ~7 hydrogen bonding interactions, 2 hydrophobic contacts, and some VDW interactions via shape complementarity between two layers (Figure 2.5a), providing atomic basis for the enhanced interactions.
<table>
<thead>
<tr>
<th>System</th>
<th>RMSD (Å)</th>
<th>Rg (Å)</th>
<th>Layer-to-layer distance</th>
<th>Sc‡</th>
<th>Twist* (degree)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tycko 1 Tycko pentamer</td>
<td>4.9±0.2</td>
<td>17.9±0.1</td>
<td>-</td>
<td>-</td>
<td>9.2±1.7</td>
</tr>
<tr>
<td>Tycko</td>
<td>4.6±0.1</td>
<td>21.2±0.1</td>
<td>8.5±0.1</td>
<td>0.65±0.04</td>
<td>10.0±1.8</td>
</tr>
<tr>
<td>NUC CN</td>
<td>3.6±0.2</td>
<td>21.3±0.1</td>
<td>8.2±0.1</td>
<td>0.74±0.04</td>
<td>8.0±0.9</td>
</tr>
<tr>
<td>NUC CN</td>
<td>4.2±0.2</td>
<td>21.5±0.1</td>
<td>10.9±0.2</td>
<td>0.61±0.04</td>
<td>6.4±1.1</td>
</tr>
<tr>
<td>cU CN</td>
<td>6.6±0.2</td>
<td>24.3±0.2</td>
<td>9.9±0.2</td>
<td>0.63±0.05</td>
<td>2.4±0.7</td>
</tr>
<tr>
<td>NUC CN=N31 A cU CN=F15 A</td>
<td>3.3±0.1</td>
<td>21.4±0.1</td>
<td>7.1±0.1</td>
<td>0.68±0.02</td>
<td>5.3±1.1</td>
</tr>
<tr>
<td>cU CN</td>
<td>7.2±0.2</td>
<td>24.7±0.1</td>
<td>8.1±0.1</td>
<td>0.64±0.10</td>
<td>4.7±0.8</td>
</tr>
<tr>
<td>cU CNU CN</td>
<td>6.3±0.3</td>
<td>28.1±0.1</td>
<td>9.6±0.1</td>
<td>0.73±0.03</td>
<td>8.2±1.0</td>
</tr>
<tr>
<td>NUC CNU CN</td>
<td>3.1±0.1</td>
<td>25.4±0.1</td>
<td>10.1±0.2</td>
<td>0.62±0.05</td>
<td>3.7±1.0</td>
</tr>
<tr>
<td>cU CNU CN</td>
<td>5.5±0.1</td>
<td>28.5±0.1</td>
<td>9.7±0.1</td>
<td>0.72±0.04</td>
<td>11.3±1.8</td>
</tr>
<tr>
<td>NUC CNU CN</td>
<td>5.7±0.4</td>
<td>26.5±0.2</td>
<td>11.9±0.2</td>
<td>0.58±0.07</td>
<td>8.5±1.1</td>
</tr>
</tbody>
</table>

‡All data were averaged over the last 10 ns.

# The shape complementarity (Sc) is used to measure the geometric surface complementarity of protein-protein interfaces between two adjacent layers, where 1.0 represents a perfect match between the interfaces, while 0.0 represents two unrelated interfaces. Sc is calculated by using the program SC of CCP4 with default parameters 51.

* Twist angle is measured by averaging over the angles between two vectors connecting the first Cα atom to the last Cα atom in the same C-terminal or N-terminal β-strand region within a β-layer. Each angle represents the averaged twist of β-strands in the same β-layer.
2.3.4. Structural comparison of different two-layer interface models

Three proposed two-layer hIAPP oligomers have three distinct interfaces, but all display high structural stability, demonstrating that different surface packings stabilized by different forces can lead to different supramolecular structures and fibril polymorphism in a rugged energy states. The $c_{NN} \cap C$ oligomer is mainly stabilized by hydrophobic interactions, whereas both $N_{CC} \cap N$ and $N_{CN} \cap C$ oligomers are controlled by mixed polar and hydrophobic interactions with different contributions (detailed analysis of interfacial residue interactions among two-layer models will be discussed later, along with those interactions in three-layer models).

On the other hand, from a structural point of view, comparison of these three models also reveals some interesting similarities. First, all interfaces exhibited the geometrical match of sidechains to some extent, characterized by a shape complementarity parameter ($Sc$) where $Sc=1.0$ represents a perfect match between two adjacent surfaces, where 0.0 represents two unrelated surfaces $^{52}$. $Sc$ values of the $N_{CC} \cap N$, $c_{NN} \cap C$, and $N_{CN} \cap C$ were 0.74, 0.63, and 0.61, respectively, indicating that the sidechains that protrude from two facing antiparallel $\beta$-layers were more comfortable in interacting with each other without large unfavorable steric effects. Second, all interfaces were largely solvent inaccessible, as quantified by solvent accessible surface area (SASA). As shown in Table 2.2, in the $N_{CC} \cap N$ structure, as compared to the SASA values of two exterior $\beta$-sheets exposed to solvent (SASA=2015 $\AA^2$ and 2101 $\AA^2$), the SASA of the interface was significantly reduced to 740.8 $\AA^2$. Similar trends were also observed for the $c_{NN} \cap C$ and $N_{CN} \cap C$ structures. It should be noted that unlike completely dehydrated neighboring
peptides within the same β-layer, visual inspection of three kinds of interfaces (\(N \cup CC \cap N\), \(c \cup NN \cap C\), and \(N \cup CN \cap C\)) for two layers models from MD trajectories revealed some confined waters at the interfaces, which had interactions with inward-pointing interfacial sidechains via hydrogen bonds.

Table 2.3. Solvent accessible surface area (SASA) for interfacial residues and exposed residues, respectively.

<table>
<thead>
<tr>
<th>Models</th>
<th>SASA of Interfacial Residues ((\AA^2))</th>
<th>SASA of Exposed Residues ((\AA^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tycko</td>
<td>932.4±43.5</td>
<td>2305.0±47.6</td>
</tr>
<tr>
<td>(N \cup CC \cap N)</td>
<td>740.8±18.6</td>
<td>2015.6±42.5</td>
</tr>
<tr>
<td>(N \cup CN \cap C)</td>
<td>940.7±53.0</td>
<td>2166.0±67.9</td>
</tr>
<tr>
<td>(c \cup NN \cap C)</td>
<td>244.8±13.9</td>
<td>3368.4±71.4</td>
</tr>
<tr>
<td>(c \cup NN \cap CN\cup C)</td>
<td>249.3±13.0</td>
<td>3278.4±61.0</td>
</tr>
<tr>
<td>(N \cup CN \cap CC \cup N)</td>
<td>917.3±25.5</td>
<td>1656.9±34.3</td>
</tr>
<tr>
<td>(c \cup NN \cap CC \cup N)</td>
<td>936.0±33.5</td>
<td>2150.3±72.8</td>
</tr>
<tr>
<td>(N \cup CN \cap CN \cup C)</td>
<td>777.9±19.4</td>
<td>2507.5±52.6</td>
</tr>
<tr>
<td>(c \cup NN \cap CC \cup N)</td>
<td>225.2±1.0</td>
<td>3388.0±52.7</td>
</tr>
<tr>
<td>(N \cup CN \cap CN \cup C)</td>
<td>766.2±14.1</td>
<td>2180.8±45.2</td>
</tr>
<tr>
<td>(c \cup NN \cap CC \cup N)</td>
<td>880.8±27.0</td>
<td>2184.0±82.5</td>
</tr>
<tr>
<td>(N \cup CN \cap CN \cup C)</td>
<td>957.4±29.2</td>
<td>1705.8±40.5</td>
</tr>
</tbody>
</table>

It probably does not necessary to contain a complete dry interface between two β-layers since the desolvation of interfacial waters would impose a large energy and entropic penalty on these very early aggregates, while existence of these confined water may assist in maintaining the interface. Similar less hydrated interfaces between two- and three-layers of Aβ oligomers were also observed by other simulations \(^{40,47,53}\). Third, all
two-layer models developed certain degrees of twists (i.e. from 2.4 to 8.0°) between peptides of the same layer during 30-ns structural relaxation (Table 2), although all models were initially constructed as perfect flat β-layers. The degree of twists was suppressed for the β-sheets at the interface, as compared to those β-sheets exposed to solvent. This fact suggests that the structural twisting results from the competition between peptide-peptide and peptide-water interactions. Intertwinement of two or more β-layers twisted around each other into a long-range twist protofibril/fibril with a certain helical periodicity is a common structural architecture of protofibril/fibril observed by the cryo-EM and AFM images.

2.3.5. Structural stability of three-layer hIAPP oligomers

Based on stable one-layer and two-layer hIAPP oligomeric structures, we modeled three-layer hIAPP oligomers to mimic higher-order aggregates by laterally stacking one-layer pentamer on top of two-layer decamer via different combinations of the interfaces, yielding total four distinct three-layer structures of \( c_U \cap c_N U_C \), \( n_U c_N \cap c_C U_N \), \( c_U \cap c_C U_N \), and \( n_U c_N \cap c_C U_C \) each with two interfaces. Each interface presented in the three-layer models has been established earlier in the two-layer models. Figure 2.3 showed the final structures for four simulated three-layer models. It can be seen that throughout each 30 ns MD simulations, except for \( n_U c_N \cap c_C U_C \) model, other three-layer models were able to retain overall structural integrity and local secondary structure, with the RMSDs of 6.3 Å for \( c_U \cap c_N U_C \), 3.1 Å for \( n_U c_N \cap c_C U_N \), and 5.5Å for \( c_U \cap c_C U_N \).
(Figure 2.4c), relatively high $S_c$ values of 0.63─0.73, and stable layer-to-layer distances of 8.1─10.2 Å (Table 2), depending on the interface involved.

Special attention should be paid to the $NU_{\text{CN}} \cap _{\text{CN}} U_C$ structure involving two NC interfaces. Initial docking of alternative N-terminal and C-terminal β-sheets from different layers placed the disulfide-linked loop of the N-terminals in contacts with Asn22 and Phe23 residues of a U-bend loop (i.e. residues 18-27 connecting two β-strands), with averaged separation distance of ~8.3 Å between disulfide-linked loop and U-bend loop. As MD simulations progress, the U-bend loops of the upper layers and the disulfide-linked loops of the lower β-layers gradually moved away from the corresponding disulfide-linked loops and U-bend loops of the middle β-layers, respectively, leading to large separation distance of 26.5 Å and 22.8 Å between these two loops. Consequently, three layers were tilted and adjusted into a “zigzag” organization by only accommodating tail residues of Gly33-Tyr37 of the C-terminal in contacts with loop residues of Ser9-Phe15 of the N-terminal. Due to significant loss of hydrogen bonds and hydrophobic contacts between layers (Figure 2.5b), it is not expected that the proposed the $NU_{\text{CN}} \cap _{\text{CN}} U_C$ model would be survived in the longer simulation unless a new CN interface will be developed by avoiding the disulfide-linked loops to be appeared at the interface.

Although three-layer models had two interfaces, inspection of interfacial interactions revealed similar contributions of hydrogen bonds and hydrophobic contacts across the layers to those interactions in the two-layer structures. Regardless of number of β-layers, any CC interface involved in the $NU_{\text{CN}} \cap _{\text{CC}} U_N$, the $CU_{\text{NN}} \cap _{\text{CC}} U_N$, and $NU_{\text{CC}} \cap _{\text{N}}$
models had dominant polar interactions via the formation of extensive hydrogen bonding network among Ser29, Asn31, Gly33, Asn35, and Tyr37. Conversely, the NN interface in the \( c U_{NN} \cap C U_{C} \), the \( c U_{NN} \cap C C U_{N} \), and the \( c U_{NN} \cap C \) models mainly involved hydrophobic contacts of Ala13, Phe15, and Val17 across the layer. The CN interface presented rather weak polar and hydrophobic interactions at the interface between two adjacent layers, consistent with the loss of contacts between disulfide-linked loops and U-bend loops. Combining the information of the relative structural deviation (RMSD), geometrical characterization (layer-to-layer distance and Sc), visual inspection (MD trajectories), and interfacial interactions (hydrogen bonds and hydrophobic contacts), it is clear that the overall structural organization and stability of hIAPP three-layer models are driven by heterogeneous interactions including VDW interactions, hydrophobic interactions, and hydrogen bonds, strongly depending on details of sidechain packing at interfaces. Similar interface properties between three-layer models and two-layer models also imply that the stability of interface is important for the multi-layer packing of peptides.

### 2.3.6. Peptide-peptide and peptide-water interactions

To rationalize the stabilizing forces underlying the \( \beta \)-layer association via different interfaces, total interaction energies and the decomposed energy contributions (i.e. VDW and electrostatic interactions) between any two neighboring layers were calculated and averaged from the last 10-ns simulations. For the two-layer models, averaged peptide-peptide interaction energies were -346.9 kcal/mol for the \( N U_{CC} \cap N \), -98.4 kcal/mol for the \( c U_{NN} \cap C \), and -132.0 kcal/mol for the \( N U_{CN} \cap C \), respectively (Figure 2.6a),
suggesting that the $\text{N}_{\text{UCC}} \cap \text{N}_\text{N}$ is the most favorable conformation, consistent with earlier structural analysis. Decomposition of total interaction energy into VDW and electrostatic components further revealed that the $\text{N}_{\text{UCC}} \cap \text{N}_\text{N}$ was stabilized by both comparable VDW and electrostatic interactions, while the $\text{c}_{\text{U}} \cap \text{N}_\text{C}$ and the $\text{N}_{\text{U}} \cap \text{C}_\text{C}$ were mainly stabilized by VDW interactions. Large differences in energy contribution confirm that (i) different sidechain packings at the interface lead to different VDW and electrostatic contributions to each case and (ii) VDW interactions clearly play a dominant role in peptide-peptide interactions, contributing over 53.9%, 117.0%, and 86.5% to peptide association at the CC, NN, and NC interface, respectively.

Figure 2.6. Layer-to-layer interaction energies and the decomposed VDW and electrostatic energy contributions to (a) two-layer and (b) three-layer models. All energies were averaged over the last 10 ns simulation.
Figure 2.7. Averaged overall solvation energies of (a) two-layer and (b) three-layer models over the last 10-ns simulations.

Figure 2.7 showed the mean interaction energies between oligomer and interfacial waters where interfacial waters were identified if they were within 6 Å of heavy atoms of oligomer. It can be seen that regardless of different oligomeric models, overall solvation energies consistently showed strong favorable interactions between peptides and water molecules (Figure 2.7a), indicating that exterior residues on the surface are well hydrated, providing an important driving force for stabilizing amyloid aggregation in agreement with recent experimental\cite{54} and computational works\cite{55}.

For the three-layer models, overall packing energy were -13351.7 kcal/mol (the $c_{NNN} \cap c_{NNC} \cap c_{NCC}$), -13310.4 kcal/mol (the $n_{CNC} \cap n_{CCN} \cap n_{CNC}$), -13456.4 kcal/mol the ($c_{NNN} \cap c_{NNC} \cap c_{NCC}$), and -13293.1 (the $n_{CNC} \cap n_{CNC} \cap n_{CNC}$), respectively. The $c_{NNN} \cap c_{NNC} \cap c_{NCC}$ has the most favorable peptide-peptide interactions, while the $n_{CNC} \cap n_{CNC} \cap n_{CNC}$ has the least peptide-peptide interactions. These data were qualitatively consistent with the previous structural analysis of these models. Comparison of peptide-peptide and peptide-water interactions among
various hIAPP oligomers revealed similar interaction patterns and energy contributions at the same interface regardless of number of layers (Figure 2.6 and 2.7). Specifically, favorable interfacial interactions between two β-layers are in the order of CC>NN>NC. Similar to native protein folding, most of amyloidogenic peptides require some minimal hydrophobic interactions to drive peptide aggregation to form a dehydrated or less hydrated interior core/interface by expulsing waters from peptides. Expulsion of waters from peptides is the first and obligatory step to facilitate peptide association and subsequent fibrillization by reducing free energy barrier arising from dehydration entropic effects\textsuperscript{54}. Once the stable aggregates form, highly hydrated water layer around the aggregate surface helps to prevent peptide disassociation.

2.3.7. Effect of mutations at the interfacial residues on the stability of hIAPP oligomers

Previous studies\textsuperscript{42,43} reported that Asn ladder and π-stacking play an important role in stabilizing amyloid oligomers/fibrils and our data also showed that the interfacial interactions are very important for structural stability of hIAPP oligomers. Here, we replaced Asn\textsubscript{31} at the CC interface and Phe\textsubscript{15} at the NN interface by Ala to examine the role of Asn sidechain hydrogen bond and Phe π-stacking on the structural stability and layer-to-layer association of the two most energetically favorable CC and NN interfaces. Each mutant was studied in a two-layer decamer. Both Asn\textsubscript{31} and Phe\textsubscript{15} were deeply buried in the middle regions of the CC and the NN interfaces, respectively.
Figure 2.4b showed that the backbone RMSDs of both mutants were comparable to those of the corresponding wild-types. Visual inspection of MD trajectories also showed that both mutants were well maintained and did not change much from their initial structures. For the Asn31Ala mutant, when the hydrophilic Asn was replaced by the hydrophobic Ala at the CC interface, hydrogen bonds of Asn31 with Asn35 and Ser29 between the layers and hydrogen bonds of Asn31 with its adjacent peptides within the layers were eliminated, but the loss of hydrogen bonds was partially compensated by increased hydrophobic contacts induced by Ala (Figure 2.5a). Competition between the loss of hydrogen bonds and the gain of hydrophobic contacts finally leads to the less favorable peptide-peptide interactions at the interface, with a significant loss of electrostatic interactions by 98.8 kcal/mol (Figure 2.6a). Conversely, substitution of large Phe15 with small Ala eliminated not only favorable π-π stacking, but also unfavorable steric effects between Phe15 and its neighboring residues. The net effect was that two β-layers move towards each other by ~1 Å, inducing more hydrophobic contacts among Ala13, Ala15, and Val17 from 13 to 35. Energy comparison between the wild-type and the Phe15Ala further supports data in structural analysis that the Phe15Ala mutant was energetically more favorable than the wild-type, with a large gain in electrostatic interactions by 115.5 kcal/mol (Figure 2.6a). Wiltzius et al. 31 also reported that mutants of Phe15Lys, Phe15Ala, and Phe15Asp accelerated fibril formation as compared to wild-type. Computational mutagenesis studies showed that substitution of Asn31 or Phe15 with Ala has a limited effect on the overall structural stability of the interface,
suggesting that (i) both mutant sequences were highly adaptable to maintain stable interfaces and (ii) all residues at the interface contribute cooperatively to the structures.

2.3.8. Comparison with experimental hIAPP models

Similar to other amyloidogenic peptides, hIAPP peptides can interact in various ways to form the basic cross-β structure leading to high polymorphs in the 3D oligomers/fibrils organization. Tycko and co-workers\(^{29}\) have proposed quaternary structure of hIAPP oligomers comprising of two layers of hIAPP pentamers associated by a CC interface (referred as \(\text{NU} \cap \text{N-tycko}\)) based on solid-state NMR. Meanwhile, MPL data by STEM have shown that basic structural unit in hIAPP fibrils contains two, three, or four layers of hIAPP molecules winded around together, providing the clues on the existence of (i) other interfaces alternative to the CC interface and (ii) asymmetrical structures alternative to two-fold symmetrical structures for laterally associating two or more layers. By superimposing the Tycko’s model with our simulated two-layer hIAPP oligomer with the CC interface (i.e. \(\text{NU} \cap \text{N}\)), it can be clearly seen that two structures were almost identical with very small structural difference (Figure 2.2d). This fact also demonstrates that our conformational search strategy in peptide-packing program is likely to capture the most energetically favorable structure similar to experimentally derived model. All stable two-layer and three-layer models have separation distance of 8.1-11.1 Å between two adjacent layers, which represent a typical distance of 8.5-10.5 Å between layers in the crystal state. The averaged cross-sectional areas were 61.5×42.8 Å\(^2\) for \(\text{NU} \cap \text{N}\), 57.3×35.8 Å\(^2\) for \(\text{NU} \cap \text{C}\), 78.9×40.3 Å\(^2\) for \(\text{CU} \cap \text{C}\), 79.0×63.8 Å\(^2\) for
cU_{NN} \cap C_{U}, 63.3 \times 59.9 \text{ Å}^2 \text{ for } N_{U} \cap C_{C} \cap U_{N}, 85.2 \times 58.3 \text{ Å}^2 \text{ for } cU_{NN} \cap C_{C} \cap U_{N}, \text{ and } 56.9 \times 66.5 \text{ Å}^2 \text{ for } N_{U} \cap C_{C} \cap U_{C}, \text{ respectively (Table 2.1). Recent MPL data}^{29, 33, 56} \text{ showed that hIAPP fibrils have heights about 45-50 Å for two-layer protofilaments and about 40-100 Å for three protofilaments intertwined around each other, compatible with the simulation data.}

![MD snapshots of mutated hIAPP models](image)

Figure 2.8. MD snapshots of mutated hIAPP models of (a) \( N_{U} \cap C_{C} \cap N_{-}N_{31}A \) and (b) \( cU_{NN} \cap C_{C} \cap F_{15}A \) at 30 ns. Color codes are: backbone (white), polar residues (green), non-polar residues (orange), positively charged residues (red), and negatively charged residues (blue). Mutated residues are represented by cyan balls.

### 2.3.9. Comparison with other amyloidogenic oligomers

It is interesting to compare various amyloidogenic structures to reveal different scenarios in the oligomeric aggregation/disaggregation process. Similar to the hIAPP peptide, Aβ peptides are highly polymorphic, displaying a wide range of structural morphologies including micelle\(^{39}\), annular\(^{37, 57}\), triangular\(^{38, 58}\), globulomer, and linear structures. Here we only focus on Aβ linear structures for comparison. MPL data indicated that Aβ fibrils primarily consisted of two or three cross-β layers\(^{59}\). Our
previous structural studies of Aβ17-42 and Aβ9-40 peptides \cite{40,41} showed that Aβ peptides can associate two β-layers through either the CC interface (interfacial residues of Ile31, Gly33, Met35, Gly37, Val39, and Ile41) or the NN interface (interfacial residues of Lys16, Val18, Phe20, and Glu22) (Figure 2.9a-b).

![Figure 2.9](image)

Figure 2.9. Structural comparison of different two-layer models formed by Aβ and K3 peptides, which have similar U-bend motif to hIAPP peptide. Both Aβ oligomers of (a) Aβ-\text{N}_{CC}\cap\text{N} and (b) Aβ-\text{C}_{NN}\cap\text{C} are stable, while only one K3 oligomer of (c) K3-\text{C}_{NN}\cap\text{C} is stable and the other two oligomers of (d) K3-\text{N}_{CC}\cap\text{N}, (e) K3-\text{C}_{NC}\cap\text{N} are unstable. Color codes are: backbone (white), polar residues (green), non-polar residues (orange), positively charged residues (red), and negatively charged residues (blue).
The CC interface was essentially stabilized by hydrophobic and van der Waals (shape-complementarity via M35-M35 contacts) intermolecular interactions, while the NN interface was stabilized by hydrophobic and electrostatic interactions. Both simulated structures of Aβ-N\(\cap\)N and Aβ-C\(\cap\)C are compatible with 2D \(^{13}\)C NMR experiments \(^{60}\) and computational models \(^{47}\) with a structural consensus in the presence of contact pairs of Ile31-Gly37 and Met35-Gly33. Since both CC and NN interfaces exhibited high structural stability, this indicates that Aβ peptides can laterally form multiple layers through either the CC or the NN interface. Recently, Wu et al. \(^{47}\) proposed two molecular structures of three-fold asymmetric Aβ fibrils with three cross-β-layers under quiescent and agitated conditions. Both structures had similar structural organization of C\(\cap\)N\(\cap\)C\(\cap\)N with a polar NN interface and a hydrophobic CC interface and both displayed high structural stability, although sidechain orientation at the C-terminal β-strands was different.

It is interesting to note that although K3 peptides linked to dialysis-related amyloid can theoretically associate different layers via distinct interface similar to Aβ and hIAPP peptides, MD simulations \(^{49}\) showed that K3 peptide only had one stable hydrophobic NN interface (i.e. K3\(\cap\)N\(\cap\)C\(\cap\)N) consisting of highly hydrophobic patches of Leu4, Cys6, and Val8, while other two-layer models with the CC or the NC interface suffered from large conformational changes and layer dissociation (Figure 2.9c-e). Structural instability of the K3-N\(\cap\)C\(\cap\)N and K3-N\(\cap\)C\(\cap\)N were largely due to strong repulsive electrostatic interactions induced by three negatively charged residues of Asp15, Glu17, and Asp19 closely located at the C-terminal β-strand. Unstable CC and NC interfaces also exclude
the possibility of multiple layers. Structural comparison of hIAPP, Aβ, and K3 peptides reveals that although different primary amyloidogenic sequences could fold into similar monomeric motifs (the β-strand-turn-β-strand motif), when assembling these motifs into highly ordered aggregates, final stable amyloid structures can be dramatically different in size, interface, and morphology due to different side-chain packings at the interfaces, suggesting that different amyloid oligomeric structures correspond to different polymorphic states in a rugged amyloid landscape, depending on both sequences and experimental conditions.

After the discovery of fibril structures of hIAPP by Tycko and coworkers, solution structures (conformations) of hIAPP monomer were further explored by experimental techniques and molecular simulation methods. It is reported that there are totally three main conformations groups of aqueous-state structures of hIAPP monomer, obtained either by simulations or experiments, including β hairpin conformation, α helical conformation and unstructured coil. β-hairpin conformation group shows the best stability and is believed to be important for the fibrillation of hIAPP, base on the results that β-hairpin conformations were found for ratIAPP neither in simulations nor experiments and showed the high similarity with Tycko’s model of hIAPP fibrils. Also, it reported that folded (α-helical segment in residues 7-17) and random-coil conformations of ratIAPP co-exist in solution. Thus, β-hairpin conformation can be a possible amyloidogenic precursor to form one-layer hIAPP by the transition from intra-interactions in hIAPP monomer to inter-interactions in hIAPP one-layer structure. And our simulation, which is base on the above mechanism, shows the important
interactions and residues blocks that play an important role in the transition from intra-layer interactions to inter-layer interactions (lateral association).

Based on both experimental and computational findings, Figure 2.10 shows a general postulated hIAPP aggregation mechanism, which may also apply to other amylogenic peptides such as Aβ.

Figure 2.10. Hypothetical mechanism for hIAPP aggregation in bulk solution at different aggregated stages of (a) monomers, (b) one cross-β-layer, (c) two cross-β-layer, (d) three cross-β-layer, and (e) mature fibrils, based on our stacking MD simulations and other experimental studies. Detailed explanations for aggregation pathways are given in the main text.

First, hIAPP monomers quickly aggregate into amorphous assemblies driven by hydrophobic interaction, followed by slow structural reorganization and transition into the ordered β-sheet-rich structures driven by hydrogen bonds (a→b) 65. 66. These β-sheet-rich single-layers are then laterally associated via different interfaces to form
large oligomers consisting of two cross-β-layers (b→c) or three cross-β-layers (b→d). Alternatively, three cross-β-layers can also be formed by a two body assembly (2 layer+1 layer) when competing with a three body assembly (1 layer +1 layer+1 layer) (c→d). Finally, all these two-layer, and three-layer oligomers will gradually evolve into mature fibrils by monomer addition along the fibril axis (c,d→e)\textsuperscript{67,68}.

Our mechanistic hypothesis can explain the polymorphism in Tycko’s experiment. Double-layer model is corresponding to the protofibrils of striated ribbons with MPL around 16.2kDa/nm in Tycko’s experiment while 3-layer model is corresponding to the polymorphism with MPL equals to 30kDa/nm. And two double-layers, which are the protofibrils, will associated with each other to form higher order fibrils. Thus, base on the experimental data and simulation results, our mechanistic hypothesis is one possible scenario of hIAPP lateral aggregation as striated ribbons and is an extension to the axial aggregation mechanism.

2.4 Conclusions

The aggregation of hIAPP peptides into either small soluble oligomers or final fibrils are highly polymorphism similar to other amyloidogenic peptides. Recent MPL and STEM data have revealed that hIAPP fibrils consist of minimal either two or three cross-β layers laterally associated together. But, atomic details of these structural morphologies and organizations have not yet been investigated. Previous experimental studies of amyloid structures\textsuperscript{29,60,69} only focused on symmetrical models (i.e. two-fold or three-fold), not paid attention to asymmetrical models. Here, we used an in-house
developed peptide-packing program coupled with explicit-solvent MD simulations to model a series of linear hIAPP oligomers with different β-layer, symmetry, and interface for gaining insights into polymorphic architectures of hIAPP at the early stage of aggregation. Structural comparison of three symmetrical two-layer structures of $N_U \cap N_C$, $N_U \cap N_C \cap N_U$ and $C_U \cap C_N \cap C_U$ and four asymmetrical three-layer structures of $C_U \cap N_C \cap U_N$, $N_U \cap C_N \cap N_C \cap U_N$, and $C_U \cap N_C \cap C_N \cap U_N$ reveals three distinct interfaces of CC, NN, and NC, with favorable interfacial interactions in the order of CC>NN>NC. The association of multiple hIAPP β-layers via the combination of three interfaces can indeed produce stable oligomers with distinct structural morphologies. Overall structural stability of these modeled oligomers is controlled by both peptide-peptide interactions at the dehydrated interface and peptide-water interactions at the outer surface, but stabilizing forces (i.e. VDW, electrostatic, and solvation energies) contribute differently to each structure, highlighting the multiple packing possibilities of importance of sidechain packings at the interface.

It should be noted that amyloid landscape presents a wide range of aggregated conformational states with different populations, depending on peptide sequence and experimental condition. Our hIAPP models are likely to represent only a very small percentage among the ensemble, but carefully selected models from a systematical structure search procedure could represent the most likely organization. Other structures with relative populations are also possible based on energy landscape (Figure 2.1). Further experimental studies are necessary to examine our simulated molecular structures of hIAPP oligomers.
CHAPTER III
HETEROGENEOUS TRIANGULAR STRUCTURES OF HUMAN ISLET AMYLOID POLYPEPTIDE (AMYLIN) WITH INTERNAL HYDROPHOBIC CAVITY AND EXTERNAL WRAPPING MORPHOLOGY REVEAL THE POLYMORPHIC NATURE OF AMYLOID FIBRILS

3.1. Introduction

Human islet amyloid polypeptide (hIAPP, or amylin) is a 37-residue hormone peptide synthesized and co-secreted with insulin in the pancreatic β-cells and released from secretory granules\textsuperscript{21}. A number of \textit{in vitro} and \textit{in vivo} experiments have shown that the accumulation of hIAPP deposits in the pancreas is a pathological hallmark of type II (non-insulin-dependent) diabetes\textsuperscript{22,70,71}. Although the underlying mechanism about how hIAPP aggregates cause inevitable β-cell degeneration remains unclear, it is well recognized that metastable, heterogeneous hIAPP prefibrillar oligomers are mainly responsible for β-cell dysfunction and death via the disruption of cell membrane integrity\textsuperscript{72}, while mature hIAPP fibrils may serve as a peptide reservoir in equilibrium with different aggregates\textsuperscript{73,74}. Similarly, other amyloidgenic oligomers from ~20 different peptides/proteins including amyloid-β (Aβ) in Alzheimer’ disease and
α-synuclein in Parkinson’ disease have also demonstrated their high toxicity to impair the function and physiology of different cells and tissues\textsuperscript{75}. Such a common view suggests a direct correlation between amyloid oligomers and amyloid toxicity.

Structural determination of both hIAPP oligomers and fibrils at the atomic level still presents a great challenge for current techniques in structural biology, primarily because of structural heterogeneity and insoluble nature for fibrils and small size, structural heterogeneity, short-lived nature, and highly sensitive to environmental conditions for oligomers. Several groups\textsuperscript{29, 31, 33, 34} have conducted detailed structural studies of the full-length hIAPP fibrils using solid-state NMR, AFM, transmission electron microscopy (TEM), and scanning TEM (STEM) techniques. They observed that mature hIAPP fibrils mainly contain two and three cross-β-layers and some fibrils may contain up to five cross-β-layers based on mass per length (MPL) analysis. Two cross-β-layers can simply stack on top of each other to form a quasi-two-fold symmetrical fibril. With increment of number of cross-β-layers, lateral association of three or more β-sheet-layers on top of each other like a “sandwich” structure becomes less populated due to the twisting of β-sheet-layer and other structural defects from asymmetrical β-sheet-layer organization\textsuperscript{10, 47}. Considering MPL data and twist nature of fibrils, it is also likely to wind multiple cross-β-layers around the fibril axis to form a three-, four-, or five-fold fibril organization rather than to stack on top of each other like a sandwich. These multiple-fold fibril structures usually have a central core formed by β-sheets from each layer to enhance overall stability.
Figure 3.1. Total packing energies of 3-fold hIAPP oligomers as a function of simultaneously rotating each β-layer along its fibril axis. Five lowest-energy structures of (a) wt50, (b) wt120 (C-WT), (c) wt155 (T-WT), (d) wt240 (N-WT), and (e) wt300 are selected from total 72 candidates and identified at 50°, 120°, 155°, 240°, and 300°, respectively. In each model, three β-layers are packed against each other via distinct interface. Color codes are: backbone (white), polar residues (green), non-polar residues (orange), positively charged residues (red), and negatively charged residues (blue).
But, these symmetrical structures can vary considerably in terms of peptide packing at the cross-section to reflect a highly polymorphic nature of amyloids in a rugged energy landscape\textsuperscript{76}. Additionally, since NMR-derived hIAPP\textsubscript{1-37} structure has a very similar U-bend conformation to Aβ\textsubscript{1-40} peptide, and three-fold and five-fold Aβ fibrils have been recently observed and characterized by both experiments and simulations\textsuperscript{38, 58, 77}, it is conceivable that hIAPP peptide can also adopt similar multi-fold symmetrical structures.

Here, we model a series of three-fold hIAPP 15-mers by systematically searching different packing possibilities for three β-sheet-layers (each layer consists of a hIAPP pentamer) using an in-house peptide-packing program and explicit-solvent molecular dynamics (MD) simulations, which have been proved to be effective in determining oligomeric structures of Aβ peptides (linear, annular, triangular, and micelle)\textsuperscript{37-39, 41}. Three stable 3-fold hIAPP oligomers (i.e. N-WT, C-WT, and T-WT) are determined from 72 structural candidates (Figure 3.1). Although all stable hIAPP oligomers consist of three cross-β-layers winding around a hollow, triangular-like, β-sheet-rich core with similar structural symmetry and β-strand-loop-β-strand monomeric conformation, detailed structural organizations resulting from three cross-β-layer association are completely different at the cross-section of central core formed by different hydrophobic fragments of hIAPP peptide. These hydrophobic fragments have been demonstrated by their ability to form amyloid fibril independently \textit{in vitro}\textsuperscript{30, 33, 73, 78-82}. Overall structural stability of these 3-fold triangular hIAPP oligomers depends on the balance between peptide-peptide interactions and peptide-water interactions. More importantly, these triangular models could serve as core architecture of triple-stranded helical fibrils via
monomer addition at the edge of each β-layer to form heterogeneous fibrils with different twists, widths, and pitches, providing a possible molecular basis of amyloid oligomer/fibril polymorphism.

3.2. Materials and methods

3.2.1. hIAPP models.

Initial monomeric structure of hIAPP\textsubscript{1-37} peptide was extracted and averaged from 10 solid-state NMR-derived structures from Tycko’s lab\textsuperscript{29}. Each hIAPP\textsubscript{1-37} monomer had a β-strand─loop─β-strand (U-bend) fold consisting of two antiparallel β-strands connected by one loop, β-strand (Lys1-Val17)─loop (His18-Leu27)─β-strand (Ser28-Tyr37). Intra-molecular disulfide bond between Cys2 and Cys7 was formed to stabilize the structure at the N-termini. The N- and C-termini were blocked by NH\textsubscript{3}\textsuperscript{+} and COO\textsuperscript{−} groups, respectively. A single-layer hIAPP\textsubscript{1-37} pentamer was first constructed by packing hIAPP\textsubscript{1-37} monomers on top of each other in a parallel and register manner, with an initial peptide-peptide separation distance of \(\sim 4.7\ \text{Å}\), corresponding to the experimental data\textsuperscript{29}. Within the pentamer, no translation was applied to one peptide relative to the other.

To further build a triangular hIAPP complex, the center of mass of the hIAPP pentamer was first positioned at the origin of the Cartesian coordinate with its fibril axis parallel to the \(z\) axis. The pentamer was then replicated and rotated along the \(z\) axis at every 60\(^{\circ}\) to form a triangular 15-mer. Then, each pentamer in the 15-mer was simultaneously rotated along its fibril axis at the center of mass by every 5\(^{\circ}\) interval to
generate total 72 three-fold oligomeric candidates, which present a wide variety of structural morphologies with different geometry at the cross-section (size, shape, and width) and contact interface between adjacent β-layers. Finally, each candidate was optimized by a short 1-ns MD simulation with generalized born of a simple switching function (GBSW) implicit solvent model\(^{50}\). Five lowest-energy candidates (referred as C-WT, N-WT, T-WT, wt50 and wt300 in Figure 3.1) were selected from 72 candidates and subsequently subject to explicit-solvent 40-ns MD simulations for examining their structural and energetic aspects at the early stage of aggregation process.

### 3.2.2. Mutant models.

Numerous studies have showed that π-stacking and hydrogen bonds play an important role in stabilizing amyloid oligomers/fibrils\(^{42,43}\). Three mutants of Phe15Ala in the N-WT model, Phe23Ala in the T-WT model, and Asn21Ala in the C-WT were used to examine the effects of hydrophobic π-stacking interactions at the central core (the former two mutants) and hydrogen bonds (the last mutant) on overall stability of hIAPP oligomers. All initial mutant structures were built by replacing sidechains of the targeted residues without changing the backbone conformations and sidechain orientations. The structures of designed mutant were first minimized for 500 steps using the steepest decent algorithm with the backbone of the protein restrained before being subjected to the following 30-ns MD simulations. All models were summarized in the Table 3.1.
Table 3.1. Summary of simulation systems with characteristic structural organizations.

<table>
<thead>
<tr>
<th>System</th>
<th>β-layer organization</th>
<th>Cross-Section Size (Å²)</th>
<th>Core-Forming Sequence</th>
<th>Number of Atoms</th>
<th>Energy (kcal/mol)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt50</td>
<td>tail-next-to-strand</td>
<td>88.5×73.1</td>
<td>S29TNVGSNTY37</td>
<td>75,242</td>
<td>-12604.5 ± 39.4</td>
<td>6.1</td>
</tr>
<tr>
<td>C-WT</td>
<td>turn-next-to-tail</td>
<td>76.8×67.7</td>
<td>F23GAILSSTNVG33</td>
<td>69,749</td>
<td>-13026.1 ± 34.0</td>
<td>31.8</td>
</tr>
<tr>
<td>T-WT</td>
<td>turn-next-to-strand</td>
<td>85.5×85.1</td>
<td>F23GAILSS29</td>
<td>86,204</td>
<td>-12809.6 ± 39.0</td>
<td>30.4</td>
</tr>
<tr>
<td>N-WT</td>
<td>turn-next-to-tail</td>
<td>76.0×74.9</td>
<td>A13NFLVHS19</td>
<td>72,207</td>
<td>-12655.6 ± 31.1</td>
<td>19.6</td>
</tr>
<tr>
<td>wt300</td>
<td>tail-next-to-strand</td>
<td>79.5×76.3</td>
<td>K6CNTATCATQRL A13</td>
<td>65,209</td>
<td>-12610.2 ± 36.9</td>
<td>12.0</td>
</tr>
<tr>
<td>C-N21A</td>
<td>turn-next-to-tail</td>
<td>91.5×75.1</td>
<td>F23GAILSSTNVG33</td>
<td>70,172</td>
<td>-11547.6 ± 35.3</td>
<td>-</td>
</tr>
<tr>
<td>T-F23A</td>
<td>turn-next-to-strand</td>
<td>83.3×84.0</td>
<td>A13GAILSS29</td>
<td>86,570</td>
<td>-12967.6 ± 47.0</td>
<td>-</td>
</tr>
<tr>
<td>N-F15A</td>
<td>turn-next-to-tail</td>
<td>74.9×65.9</td>
<td>A13NFLVHS19</td>
<td>72,090</td>
<td>-12485.5 ± 32.7</td>
<td>-</td>
</tr>
</tbody>
</table>

*All energies were averaged using structures from the last 10 ns of each simulation.

Mutated residues are bordered.

3.2.3. Explicit-solvent MD protocol.

All MD simulations were performed using the NAMD software with CHARMM27 force field for peptides and TIP3P for water. Each 15-mer was solvated in a TIP3P water box with a margin of at least 15 Å from any edge of the water box to any peptide atom. Any water molecule within 2.6 Å of the peptide was removed. Each system was neutralized by adding Cl⁻ and Na⁺ ions to mimic ~200 mM ionic strength. The resulting systems were subject to 5000 steps of steepest decent minimization with peptide backbone atoms harmonically constrained, followed by additional 5000 steps of conjugate gradient minimization with all atoms allowed to move. Short 1-ns MD simulations were performed to equilibrate systems by constraining the backbones of
oligomers. The production MD simulations were performed using an isothermal-isochoric ensemble (NPT, T=330K and P=1 atm) under periodic boundary conditions with the minimum image convention. The Langevin piston method with a decay period of 100 fs and a damping time of 50 fs was used to maintain a constant pressure of 1 atm, while the Langevin thermostat method with a damping coefficient of 1 ps⁻¹ was used to control the temperature at 330 K. All covalent bonds involving hydrogen were constrained by the RATTLE method so that 2-fs timestep was used in the velocity Verlet integration. Van der Waals (VDW) interactions were calculated by the switch function with a twin-range cutoff at 12 and 14 Å. Long-range electrostatic interactions were calculated using the force-shifted method with a 14 Å cutoff. Each modeled system was run twice with the same initial coordinates but different initial velocities to verify statistical accuracy. Structures were saved every 2 ps for analysis. All analyses were performed using tools within the CHARMM, VMD⁴⁶, and in-house FORTRAN and PERL codes.

3.3. Results

3.3.1. Conformational search for three-fold triangular hIAPP oligomers

Similar to a number of amyloid-forming peptides such as Aβ₄₀/₄₂⁶⁰, human CA150⁴⁸, and K3 peptide from β₂-microglobulin⁴⁹, the hIAPP peptide also has a U-bend shape allowing β-sheet subunits to be associated via different orientations and interfaces to enrich fibril polymorphism. A total of 72 hIAPP 3-fold triangle models were constructed by simultaneously self-rotating each cross-β-layer by every 5° along the fibril axis, followed by the packing-energy evaluation using the GBSW method. Figure 3.1
shows a packing energy profile as a function of cross-β-layer self-rotation for all 72 structures. Five structures with the lowest packing energies were identified at 50° (wt50), 120° (C-WT), 155° (T-WT), 240° (N-WT), and 300° (wt300) and classified into turn-next-to-tail, turn-next-to-strand, and tail-next-to-strand organizations. For convenience, unstable structure (demonstrated by subsequent MD simulations) is notated by the sequence type and the self-rotation degree of each cross-β-layer (e.g. wt50 and wt300), while stable structure is notated by the β-strands forming a core and the sequence type (e.g. C-WT, T-WT, and N-WT). Overall, all triangular models displayed a quasi-3D-symmetry by accommodating three U-shaped β-layers with different orientation and packing at the cross section. The C-WT (wt120) model had a relative large central hydrophobic core formed by C-terminal residues of Phe23, Ala25, Leu27, Ser29, and Asn31, displaying a turn-next-to-tail organization where residues Ser19-Phe23 at the turn region of one β-layer have close contacts with residues Asn31-Tyr37 at the tail of C-terminal of the other β-layer. The T-WT (wt155) model had a much small and compact hydrophobic core at the cross section, formed by the similar but less hydrophobic residues of Phe23, Ala25, Leu27, and Ser29 at the C-terminals. But, three cross-β-layers were assembled into the turn-next-to-strand organization, i.e. turn residues of Phe15-Ser19 of one β-layer were packed against central residues of Leu27, Ser29, and Asn31 at the C-terminal β-strand of the other β-layer. In the N-WT model (wt240), the triangular cross section was mainly formed by N-terminal hydrophobic residues of Arg11, Ala13, Phe15, and Val17 with a turn-next-to-tail organization largely involved hydrophilic interactions between edge residues of Lys1-Arg11 at the N-terminals and
turn residues of His18-Phe23. Additionally, the wt50 model had a hydrophilic central core mainly formed by C-terminal inward-pointing residues of Ser29, Asn31, Gly33, Asn35 and Tyr37 and a tail-next-to-strand organization involving weak inter-layer contacts between edge residues of Tyr37 and middle residues of Ser29 and Asn31 at the N-terminal β-strand. The wt300 model contained 6 positively residues of Lys1 and Arg11 in the central core, imposing strong repulsive forces on disturbing an initial turn-next-to-tail organization. Based on distinct structural features in the cross section and β-sheet-layer organization, five different triangular models exhibited different inner and outer areas of the cross-section for C-WT (inner 30.6×27.9 Å$^2$ vs. outer 76.8×67.7 Å$^2$), N-WT (21.4×17.2 Å$^2$ vs. 76.0×74.9 Å$^2$), T-WT (13.5×9.3 Å$^2$ vs. 85.5×85.1 Å$^2$), wt50 (22.9×17.8 Å$^2$ vs. 88.5×73.1 Å$^2$), and wt300 (29.6×23.5 Å$^2$ vs. 79.5×76.3 Å$^2$) (Table 1 and Figure 3.2). It should be noted that the lowest-packing-energy structures determined by the GBSW method do not necessarily imply the most stable structures in the explicit solvent due to the lack of explicit peptide-water interactions, thus all five triangular models were further examined for their structural stability by subsequent all-atom explicit-solvent MD simulations.
Figure 3.2. Representative hIAPP triangular structures of (a) C-WT, (b) T-WT, and (c) N-WT models averaged from the last 10-ns MD trajectories. Phe23 and Phe15 residues are highlighted in yellow at the central hydrophobic cores of the T-WT and N-WT models, respectively. Each packing scheme highlights the critical residues at the juxtoposed region or central core. Color codes are the same as in Figure 3.1.

(a) C-N21A   (b) T-F23A   (c) N-F15A

Figure 3.3. Representative hIAPP triangular mutants of (a) C-N21A, (b) T-F23A, and (c) N-F15A models averaged from the last 10-ns MD trajectories.
3.3.2. Stable hIAPP triangular structures exhibit heterogeneous β-layer association, reflecting a polymorphic nature of hIAPP fibrils

Visual inspection of 40-ns MD trajectories showed that the C-WT, N-WT, and T-WT structures were able to maintain the overall triangular integrity of β-sheet-layers. The N-WT structure exhibited slightly larger root-mean-square deviations (RMSD) of 7.6 Å than the C-WT (RMSD=4.8 Å) and the T WT (RMSD=6.4 Å), probably due to more flexible residues at the tail-next-to-turn region (Figure 3.2c & 3.4a).

Figure 3.4. Backbone RMSD of hIAPP triangular oligomers for (a) five wild-type and (b) three mutated structures with respect to their initial energy-minimized structures during MD simulations.

To further confirm the geometrical organization of three β-layers, the relative orientation (d\text{angle}) and distance (d\text{layer}) between adjacent β-sheet-layers were calculated to characterize layer-to-layer association (Figure 3.5).
Figure 3.5. Geometrical characterization of the β-layer organization and association of five hIAPP oligomers by relative orientation and distance between neighboring β-layers for (a) C-WT, (b) T-WT, (c) N-WT, (d) C-N21A, (e) T-F23A, and (f) N-F15A structures. Inter-layer distance is calculated by averaging the mass center distance of selected key residues of Ala25, Leu27 and Ser29 in the C-WT and C-N21A, Phe23, Ala25, and Leu27 in the T-WT and T-F23A, and Ala13, Phe15, Val17, and Ser19 in the N-WT and N-F15A between two adjacent core-forming β-strands. Relative orientation is calculated by a
scalar product between two vectors connecting the first Cα atom to the last Cα atom in the middle strand of adjacent β-layers.

It can be seen that in all three models, three interlayer distances were well maintained at their initial values of 23.2 Å, 23.9 Å, 19.6 Å for C-WT, 19.1 Å, 18.8 Å, 18.4 Å for N-WT, and 14.8 Å, 15.3 Å, 14.9 Å for T-WT and three interlayer orientations remain approximately to 58.8o, 72.3o, 49.8o for C-WT, 58.0o, 60.9o, 62.8o for N-WT, and 62.9o, 53.1o, 61.3o for T-WT, indicating that all structures have an almost equilateral triangular shape. A close inspection of interfaces between β-layers of three stable models reveals that each triangular model consists of different hydrophobic cores at the center and different contacts at the juxtaposed intersection, packed by different residue fragments with different packing distances and orientations. In the C-WT model (Figure 3.2a), highly hydrophobic side chains of Phe23-Ala25-Leu27-Ser29-Asn31 protruded inwards to form a triangular hydrophobic core of $76.1 \times 72.3 \times 73.2$ Å. Meanwhile, the edge of C-terminals of one β-layer developed a minor groove to accommodate the turn of the adjacent β-layer by gaining significant hydrogen bonds between Asn31-Gly33-Asn35-Tyr37 at the C-terminals and Ser19-Asn21 at the turn region. Especially, three Asn21I, Asn31II, and Asn35II ladders (I and II represent two different β-layers) at the turn-next-to-tail regions contributed intensive intra- and inter-peptide hydrogen bonds to stabilize the C-WT structure. Consistently, Figure 3.6 also showed dominant inter-peptide hydrogen bonds in the C-WT structure.
Figure 3.6. Number of native contacts including hydrogen bonds and hydrophobic contacts between two neighboring β-layers for all hIAPP triangular models. A hydrogen bond is defined if the distance between donor D and acceptor A is ≤3.6 Å and the angle of D-H…A ≥120. A hydrophobic contact is identified when the mass center distance between a pair of hydrophobic side chains is ≤6.5 Å.

In the T-WT model, the core-forming residues were the same but two residues less than those in the C-WT model, i.e. three hydrophobic C-terminal residues of Phe23-Ala25-Leu27 protruded inwards to form a small and compact hydrophobic core. Particularly, three Phe23 residues were closely packed against each other at the central core, providing additional π-π interactions to enhance the stability (Figure 3.2b). But, unlike the turn-next-to-tail organization in the C-WT model, the T-WT model arranged the turn residues of Ser20 and Asn21 to make contacts with central residues of Ser29 and Asn31 at the C-terminals, displaying a turn-next-to-strand β-layer organization. The gain of strong hydrophobic interactions at the compact core can greatly compensate the loss of hydrogen bonds at the turn-next-to-strand intersection (Figure 3.6). In contrast to both
C-WT and T-WT models whose N-terminal β-strands were exposed to bulk solvent, the N-WT model exposed C-terminal β-strands to solvent, while arranged four N-terminal residues of Arg11-Ala13-Phe15-Val17 to form a hydrophobic core. The turn-next-to-tail interface largely involved the interactions of Arg11\textsuperscript{I}-Ser19\textsuperscript{II}, Thr9\textsuperscript{I}-Ser19\textsuperscript{II}, Lys1\textsuperscript{I}-Asn21\textsuperscript{II}, and Lys1\textsuperscript{I}-Phe23\textsuperscript{II}, leading to these interactions largely protected from solvent.

The additional two models of wt50 and wt300 displayed large structural deviation from their initial conformations. In the wt50 model, residues 30-37 of C-terminals formed a hydrophilic core, while N-terminal β-strands were completely exposed to solvent. Three tail-next-to-strand regions largely involved hydrophilic contacts between Asn35 and Tyr37 at the tails and Leu27, Ser29, and Asn31 at the middle β-strands. Within 40-ns MD simulations, whole structure gradually expanded by moving the tails of one β-layer towards the turns of the other β-layers. Since this structural transition did not occur symmetrically, one β-layer was repelled from triangular organization, leading to the other two β-layers to be associated in a sandwich way via C-terminal-C-terminal interface, similar to our double-layer model\textsuperscript{10} and Tycko’s model\textsuperscript{29}. Structural instability of the wt50 model could be due to insufficient hydrophobic and hydrophilic interactions at the central core and tail-next-to-strand regions. In the wt300 model, the core-forming residues 1-15 consisted of three pairs of positively charged residues of Lys1\textsuperscript{I} and Arg11\textsuperscript{II}, which provide strong electrostatic repulsions to push β-layers away from each other and cause structural instability.
Structural comparison of three stable models (C-WT, T-WT, and N-WT) and two unstable models (wt50 and wt300) reveals the importance of hydrophobic interactions at the central core. Although these hydrophobic cores exhibit large variations in the core-forming fragments and their packing organizations, the core-forming fragments of F23GAILSSTN31 from the C-WT, F23GAILSS29 from the T-WT, and A13NFLVHS19 from the N-WT provide strong but different hydrophobic interactions to stabilize structures. Interestingly, the core-forming sequences from the C-WT and the T-WT models have been demonstrated as critical amyloidogenic regions (i.e. S20NNFGAILSS29, N22FGAIL27, and S20NNFGAIL27) capable of forming fibrils in vitro that are indistinguishable from fibrils formed by the full-length hIAPP30, 73, 79. Moreover, the core-forming fragment of R11LANFLVHS19 from the N-WT also contains two hexapeptide fragments of L12ANFLV17 and F15LVHSS20, which can independently form fibrils and accelerate fibrillogenesis of full-length hIAPP in vitro33, 80, 81. Apart from hydrophobic interactions at the central core, other interactions at the juxtaposed regions, especially for inter-layer hydrogen bonds, also provide critical forces to retain structural integrity. But, both hydrophobic interactions and hydrogen bonds contribute differently to each model, depending on distinct β-layer packing orientation, distance, and fragment in each model (Figure 3.6).
3.3.3. Mutations at central hydrophobic cores or juxtaposed region disfavor β-layer interactions

Stable hIAPP triangular structures (C-WT, T-WT, and N-WT) have implied several critical residues involved in the β-layer associations: Asn21 at the juxtaposed region of C-WT, Phe23 at the central core of T-WT, and Phe15 at the central core of N-WT. Thus, we designed three mutants of Asn21Ala in C-WT (referred as C-N21A) to probe the role of Asn sidechain hydrogen bonds, as well as the Phe15Ala in N-WT (i.e. N-F15A) and Phe23Ala in T-WT (i.e. T-F23A) to examine the effects of hydrophobic and π-stacking interactions on the stability of the triangular structures. In the C-WT model, since Asn21 residues at the turn regions just sit in between Asn31 and Asn35 of the mating β-layer at the juxtaposed region forming an intensive hydrogen bond network, the Asn21Ala mutation largely eliminated both intra-layer hydrogen bonds in Asn ladders and inter-layer hydrogen bonds with Asn31 and Asn35, resulting in one β-layer reoriented by ~12° and slightly pushed away from its original position (Figure 3.3a).

Although triangular shape was still present, the C-N21A structure is expected to be vulnerable to structural stability in a long run due to the lack of hydrogen bonds of Asn21 nearby. In the N-WT model, the Phe15 residues of all three β-layers were packed against each other and buried in a triangular hydrophobic core. The substitution of large hydrophobic Phe15 by small and less hydrophobic Ala not only destroyed the π-stacking and hydrophobic cluster in the central core, but also greatly weakened VDW interactions with its neighboring hydrophobic residues of Ala13, Leu16, and Val17, resulting in total
nonbonded energy loss of \( \sim 135.5 \) kcal/mol (Table 3.2) and a large RMSD of 12 Å (Figure 3.4b).

Table 3.2. Total nonbonded interaction energies and the decomposed VDW and electrostatic energy contributions between \( \beta \)-layers. All energies were summed by the interactions of three pairs of the \( \beta \)-layers and averaged from the last 10-ns of each simulation.

<table>
<thead>
<tr>
<th>System</th>
<th>( E_{\text{total}} )</th>
<th>( E_{\text{Electrostatic}} )</th>
<th>( E_{\text{VDW}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt50</td>
<td>-424.5 ± 34.9</td>
<td>-209.1 ± 28.0</td>
<td>-215.4 ± 11.5</td>
</tr>
<tr>
<td>C-WT</td>
<td>-574.2 ± 24.8</td>
<td>-321.7 ± 16.8</td>
<td>-252.5 ± 7.4</td>
</tr>
<tr>
<td>T-WT</td>
<td>-542.7 ± 28.6</td>
<td>-265.2 ± 22.3</td>
<td>-277.5 ± 12.8</td>
</tr>
<tr>
<td>N-WT</td>
<td>-557.3 ± 34.5</td>
<td>-284.5 ± 28.3</td>
<td>-272.8 ± 11.7</td>
</tr>
<tr>
<td>wt300</td>
<td>-41.2 ± 65.5</td>
<td>138.6 ± 66.3</td>
<td>-179.8 ± 6.9</td>
</tr>
<tr>
<td>C-N21A</td>
<td>-570.4 ± 19.0</td>
<td>-292.6 ± 19.1</td>
<td>-277.8 ± 6.2</td>
</tr>
<tr>
<td>T-F23A</td>
<td>-488.7 ± 32.3</td>
<td>-246.1 ± 33.8</td>
<td>-242.6 ± 5.9</td>
</tr>
<tr>
<td>N-F15A</td>
<td>-421.8 ± 30.3</td>
<td>-156.3 ± 23.8</td>
<td>-265.5 ± 9.9</td>
</tr>
<tr>
<td>C-rat</td>
<td>-479.2 ± 45.5</td>
<td>-259.9 ± 47.6</td>
<td>-219.3 ± 8.1</td>
</tr>
<tr>
<td>T-rat</td>
<td>-454.1 ± 26.9</td>
<td>-215.6 ± 27.0</td>
<td>-238.5 ± 7.0</td>
</tr>
<tr>
<td>N-rat</td>
<td>-458.1 ± 29.3</td>
<td>-200.9 ± 29.7</td>
<td>-257.1 ± 6.6</td>
</tr>
</tbody>
</table>

As a consequence three \( \beta \)-layers tended to dissociate with each other and triangular integrity was lost (Figure 3.3c), as indicated by increased interlayer distances of 24.0, 28.9, 32.7 Å and interlayer angles away from 60° (Figure 3.5f). On the other hand, the Phe23Ala mutation at the central core in the T-WT did not alter the original triangular organization, three \( \beta \)-layers were still associated together, and layer-to-layer interactions (-488.7 kcal/mol) were less favorable to those in the wild type (-542.7 kcal/mol). This
fact could be due to the small and compact core of $13.5 \times 9.3$ Å in the T-WT, upon Phe23Ala mutation, the loss of Phe23 π-stacking and cluster can be partially compensated by hydrophobic interactions of Ala23 with nearby hydrophobic residues. But, Phe23Ala mutant has notably larger disordered structure on the stacked multilayer N$_{22}$FGAIL$_{29}$ oligomers as compared to wild type$^{83,84}$. Thus, mutation results indicate that the changes in interaction pattern at the mutation sites would affect overall stability and β-layer organization to some extent among different models, depending on the position and identity of residues, as well as the packing of β-layers.

### 3.3.4. Peptide-peptide interactions contribute to β-layer association differently

To provide a more quantitative identification of driving forces underlying β-layer association, total nonbonded interactions between any two neighboring layers (i.e. summation of the interactions of three pairs of the β-layers) were evaluated and compared from the last 10-ns simulations (Table 3.2). Overall, the stable structure has the most favorable layer-to-layer interactions of -574.2 kcal/mol for the C-WT, -542.7 kcal/mol for the T-WT, and -557.3 kcal/mol for the N-WT, the marginally stable wt50 structure has the less favorable layer-to-layer interaction of -424.5 kcal/mol, and the disassociated wt300 structure has the least favorable interaction of -41.2 kcal/mol (Table 3.2). Decomposition of total layer-to-layer interaction energy into VDW and electrostatic components further shows that all stable structures were stabilized by comparable VDW and electrostatic interactions. On the other hand, although all three stable structures had a central hydrophobic core within a triangular architecture, sidechain interactions at the
interfaces were completely different. In the C-WT structure, due to the relative large central core of 76.8 Å in width and 67.7 Å in height, association forces mainly came from hydrogen bonds at the turn-next-to-tail region, especially from the interactions of three Asn21, Asn31, and Asn35 ladders with their neighboring residues (Figure 3.6). The T-WT and N-WT had comparable hydrogen bonds and hydrophobic contacts between β-layers, but the number of native contacts in the T-WT was much higher than the N-WT. Comparison of native contacts (Figure 3.6) with layer-to-layer interactions (Table 3.2) shows that the T-WT structure has more short-range interactions from native contacts, but less favorable layer-to-layer interactions than the C-WT and the N-WT structures, suggesting the large contribution of short-range interactions to the T-WT structures. This fact was also supported by large favorable long-range electrostatic energies of -321.7 kcal/mol in the C-WT structure and -284.5 kcal/mol in the N-WT structure, as compared to -265.2 kcal/mol in the T-WT structure.

All three mutants (i.e. C-N21A, N-F15A, and T-F23A) displayed the less favorable layer-to-layer interactions than the corresponding wild types (i.e. C-WT, N-WT, and T-WT) (Table 3.2), reflecting the loss of some favorable native contacts. Especially, the N-F15A mutations almost eliminated all hydrophobic contacts at the central core (Figure 3.6) and caused one β-layer to move away from the other two layers (Figure 3.3c), emphasizing the importance of the Phe15 cluster in β-layer association. On the other hand, the T-F23A mutant only lost 2.8 out of 28.2 hydrophobic contacts and 11.0 out of 33.9 hydrogen bonds as compared to the T-WT structure, thus the remaining interactions arose from the compact central core and the juxtaposed interface were still sufficient.
enough to maintain overall structure. Overall, heterogeneous structures lead to heterogeneous interactions between adjacent β-layers, suggesting that peptides have different ways to be assembled together. Such heterogeneous interactions were quantitatively consistent with the previous structural analysis, strongly depending on the core-forming peptide fragment, packing distance/orientation between adjacent β-sheets, side chain contact pattern at the juxtaposed interface, and possibly conformational changes of peptide.

3.3.5. External surface hydration and internal water wire stabilize hIAPP oligomers

Water may play a significant role in mediating the structure and kinetics of prefibrillar oligomers and mature fibrils at different assembly stages. Surface accessible solvent area (SASA) was calculated to quantify the hydration of each residue (Figure 3.7). It can be seen that (i) outward-pointing residues to bulk water or central core (black bar) generally had larger SASA values than inward-pointing residues (red bar), (ii) external surface residues of 1-18 and 35 in the C-WT structure, 1-18 and 33-37 in the T-WT structure, and 23-37 in the N-WT structure had larger SASA value than core-forming residues of F$_{23}$GAILSSTNVG$_{33}$ in the WT structure, F$_{23}$GAILLS$_{29}$ in the T-WT structure, and A$_{13}$NFLVHS$_{19}$ in the N-WT structure; and (iii) for the C-WT and N-WT structures with a relative large central pore and similar turn-next-to tail organization, the SASA values of core-forming residues displayed a bell-shaped solvent protection curve, with a single peak appearing at the central residues of Leu27 in the
C-WT and Phe15 in the N-WT, while residues at the turn-next-to-tail region were well protected from solvent.

Figure 3.7. Solvent accessible surface area (SASA) of individual residue in (a) C-WT, (b) T-WT, and (c) N-WT triangular models. All data were averaged using structures from the last 10-ns of each simulation.

For the more compact T-WT structure, except for Phe23 all other core-forming residues had small SASA values. SASA data suggest that all central cores had less water accessibility, but inspection of MD trajectories showed that a number of water molecules were confined in the central core region, forming a hydrogen bond network with backbones and sidechains of the core-forming residues. MD snapshots in Figure 3.8
showed that internal water molecules formed a water wire in a compact core of the T-WT, a water pore in the C-WT, and a diffusive structure in the N-WT.

(a) C-WT

(b) T-WT

(c) N-WT

Figure 3.8. Water channels in the central hydrophobic core of (a) C-WT, (b) T-WT, and (c) N-WT structures. Both top and side views are shown for each structure.
To further provide insights into the dynamics and structure of confined water molecules in the central core, self-diffusion coefficient (SDC) and residence time (τ) of confined water molecules are calculated to quantify the affinity between confined water and the sidechain of core-forming residues (Table 3.3 and Figure 3.9).

Figure 3.9. Residence time, measured by the autocorrelation function \( C_R(t) = Ae^{-t/\tau} \), of water molecules in the 4.5 Å thick hydration layer of central residues (CD of Ile27 for C-WT, CZ of Phe23 for T-WT, and CZ of Phe15 for N-WT) in the hydrophobic central core.

The self-diffusion coefficient is obtained from the mean square displacements (MSD) in the Einstein relation\(^{85, 86}\): \[ D = \lim_{t \to \infty} \frac{1}{6t} \left\langle \left[ r_i(t) - r_i(0) \right]^2 \right\rangle \] where \( \left\langle \left[ r_i(t) - r_i(0) \right]^2 \right\rangle \) is the MSD, \( r_i(t) \) is the time-dependent coordinates of atom \( i \) and \( r_i(0) \) is the initial position of atom \( i \). Residence time (\( \tau_s \)) is used to characterize the hydration capability of a
surface by measuring how long water molecules stay in a specified region. The $\tau_s$ is approximately obtained by fitting a correlation function $C_R(t) = \frac{1}{N_W} \sum_{j \in W} \langle P_{R_j}(0) P_{R_j}(t) \rangle / \langle P_{R_j}(0) \rangle^2$ by a single exponential function: $C_R(t) = A \exp(-t/\tau_s)$ where $P_{R_j}$ is a binary function that takes the value of 1 if the $j$th water molecule stays in the layer of thickness $R$ for a time $t$ without leaving the specified region during this interval and is equal to zero otherwise.

The confined water molecules were selected by a separation distance of 4.5 Å within the topmost heavy atoms of core-forming residues, but water molecules within 4.5 Å of edge peptides were not included (details in Table 3.3).

Table 3.3. Self-diffusion coefficient (D) and residence time ($\tau_s$) of water molecules in the central core of hIAPP oligomers a, in comparison with those values in bulk solution.

<table>
<thead>
<tr>
<th>System</th>
<th>D ($10^{-5} \text{cm}^2/\text{s}$)</th>
<th>$\tau_s$ (ps)</th>
<th>Hbonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-WT</td>
<td>1.172</td>
<td>123.9</td>
<td>136.9</td>
</tr>
<tr>
<td>T-WT</td>
<td>0.525</td>
<td>407.0</td>
<td>46.6</td>
</tr>
<tr>
<td>N-WT</td>
<td>0.971</td>
<td>196.8</td>
<td>59.2</td>
</tr>
<tr>
<td>Bulk</td>
<td>4.9</td>
<td>12.2</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Interfacial waters are defined by a separation distance of 4.5 Å within the heavy atoms of selected central core residues of Phe23, Ala25, Leu27, Ser29, and Asn31 in the C-WT, Phe23, Ala25, and Leu27 in the T-WT, and Arg11, Ala13, Phe15, Val17, and Ser19 in the N-WT.
As shown in Table 3.3 and Figure 3.9, these confined water molecules had much smaller SDC values (1.172×10^{-5}\,\text{cm}^2/\text{s} in C-WT, 0.525×10^{-5}\,\text{cm}^2/\text{s} in T-WT, and 0.971 \times 10^{-5}\,\text{cm}^2/\text{s} in N-WT) and larger residence time (123.9 \,\text{ps} in C-WT, 407.0 \,\text{ps} in T-WT, and 196.8 in N-WT) than bulk water (SDC=4.9×10^{-5}\,\text{cm}^2/\text{s} and \tau=12.2 \,\text{ps}), implying that the confined waters in the central core form hydrogen bonds with nearby residues to constraint their mobility depending on the size and sidechains of the central core.

Solvation energy including water interactions with residues at both central pore and external surface is important for the protein structure and its biological function. We thus calculated the mean interaction energies between hIAPP oligomer and interfacial waters where interfacial waters were identified if they were within 6.5 \,\text{Å} of heavy atoms of oligomers (i.e. ~2 water layers). The mean solvation energy was -12040.9 \,\text{kcal/mol} for the C-WT, -9809.4 \,\text{kcal/mol} for the N-WT, and -12358.2 \,\text{kcal/mol} for the T-WT, respectively. Clearly, peptide-water interactions were much more favorable than peptide-peptide interactions, suggesting that once stable hIAPP oligomers form, highly hydrated water layer around external surface and inner core help to prevent peptide disassociation. Recent 2D infrared spectroscopy and MD simulation\textsuperscript{55} also showed that amyloid fibrils contain mobile water molecules between the intersheet region. These confined water molecules serve as a glue and lubricant to facilitate proper packing of the hydrophobic surfaces in the final stages of assembly\textsuperscript{55, 87}. 

3.4. Discussion

3.4.1. Stacking sandwich models vs. Wrapping cord models

Amyloid oligomers and fibrils are highly polymorphic depending on both sequences and environmental conditions. MPL data\textsuperscript{29, 32, 34} have demonstrated that full-length hIAPP fibrils could contain up to 5 cross-β-layers or protofilaments with different sizes and morphologies, suggesting that polymorphic structures of amyloid oligomerization and fibrilization may proceed through different plausible routes. Multiple β-layers can laterally stack on top of each other to form a sandwich-like structure. In the stacking sandwich model, adjacent β-layers are more likely to form a steric zipper by interdigitating sidechains to create a dry interface\textsuperscript{35, 42, 56}. Structural conformation and stability are mainly determined by the interlayer interactions between overlapped interfacial residues. Our previous MD simulations of hIAPP peptides have identified three double-layer and four triple-layer sandwich structures with distinct interface associated by C-terminal β-sheet and C-terminal β-sheet (CC), N-terminal β-sheet and N-terminal β-sheet (NN), and C-terminal β-sheet and N-terminal β-sheet (CN) between adjacent β-layers\textsuperscript{10}. These seven sandwich models display high structural stability within 30-ns MD simulations and are compatible with experimental data in overall size, cross-section area, and molecular weight. But, when more than three layers are stacked together, the sandwich structure is likely to be less populated because the twisting of β-layer, asymmetrical stacking, heterogeneous interface, and other structural defects may prohibit layer-to-layer stacking\textsuperscript{47}. Alternatively, multiple β-layers can also wind around a core or axis to form a quasi-symmetrical “phone-cord” fibril with certain periodicity.
Unlike one-dimension conformation search by translating one layer relative to the other along the β-strand direction in the sandwich model, the wrapping cord model can readily accommodate more than 3 layers via a two dimension conformation search by rotating and translating the β-layers to adopt different favorable packings, which can greatly enrich the polymorphism of fibrils. Moreover, since the wrapping-cord model usually has a central core, instead of a closely packed dry interface in the sandwich model, association forces are very diverse and contributed by different polar or nonpolar sidechain interactions at both core region and juxtaposed region, as well as solvation interactions between confined water molecules and the core residues and between interfacial waters and outward-pointing sidechains. Such heterogeneous interactions in the wrapping-cord model can also explain the generic polymorphism of amyloid fibrils. Although there is no detailed molecular structure for such wrapping-cord model with a hollow core in the hIAPP fibrils to date, similar quasi-3-fold triangular structures formed by the Aβ peptides were observed and characterized by both solid-state NMR and our and other MD simulations. Moreover, wrapping-cord model can be also used to self-assemble native protein building blocks into nanostructures with a range of shapes, surfaces, and chemical properties.

3.4.2. Computational models vs. experimental data

AFM images of twisted hIAPP fibrils containing up to five protofilaments displayed ~40-100 Å in height. Assuming an ideal cross-β-structure in hIAPP fibrils, NMR data showed that the sandwich structure containing two β-layers had ~40 Å in
height, and our previous MD simulations\textsuperscript{10} showed that two-layer and three-layer sandwich structures had \( \sim 40 \) and \( \sim 60 \) Å in height, respectively. It is thus reasonable to estimate that the heights of the sandwich structures containing three, four, or five \( \beta \)-layers are about 60, 80, and 100 Å, respectively, consistent with AFM and MPL data. Alternatively, computational alignment of the NMR-derived U-bend three or four \( \beta \)-layers into 3-fold triangular-like or 4-fold parallelogrammic-like structures around a central core/axis displayed relative large and diverse heights of \( \sim 70-86 \) Å and \( \sim 100-110 \) Å for three-layer and four-layer wrapping-cord structures, respectively. It can be seen that the wrapping-cord model can readily achieve the structural polymorphism of hIAPP fibrils without imposing large entropy penalty. Altogether, it is likely for hIAPP peptides with similar U-bend conformation to A\( \beta \) peptide to be assembled into heterogeneous architectures via different \( \beta \)-layer organizations.

Three stable hIAPP triangular structures were used as templates to construct hIAPP fibrils via peptide elongation (Figure 3.10), in which peptides were added at the edge of each \( \beta \)-layer and rotated by 2.8°, 4.7°, and 11.8° along its fibril growth axis to obtain twisted C-WT-, T-WT-, and N-WT-based hIAPP fibrils.
Figure 3.10. Three hIAPP fibril models formed by three protofilaments intertwining around each other and constructed via peptide elongation on (a) C-WT, (b) T-WT, (c) and N-WT structures. For a complete 360° turn, the pitches of the helical suprastructures of the C-WT-, T-WT-, and N-WT-based fibrils are 610.7 Å, 363.8 Å, and 144.9 Å, respectively. The individual protofilaments are coded in different colors.

Table 3.4. Inter-strand twist angle within the same β-layer averaged from the last 10-ns MD simulations

<table>
<thead>
<tr>
<th>System</th>
<th>Inter-Strand Twist (degree) ( ^\circ )</th>
<th>Pitch (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1-S2</td>
<td>S2-S3</td>
</tr>
<tr>
<td>C-WT</td>
<td>3.5±0.7</td>
<td>2.3±0.6</td>
</tr>
<tr>
<td>T-WT</td>
<td>4.7±0.8</td>
<td>3.4±0.8</td>
</tr>
<tr>
<td>N-WT</td>
<td>14.9±1.0</td>
<td>9.3±1.3</td>
</tr>
</tbody>
</table>

\( ^\circ \) Inner-strand twist angle is measured by averaging over the angles between two vectors connecting the first Cα atom to the last Cα atom in the same C-terminal or N-terminal β-strand region within a β-layer. Each angle represents the averaged twist of β-strands in the same β-layer.
Based on the stepwise left-handed twist of successive peptides in each β-layer (Table 3.4) and characteristic 4.75 Å spacing between β-strands in ideal, planar β-sheets, it can be extrapolated that a complete 360° twist of each β-layer require 128 peptides/layer, 76 peptides/layer, and 30 peptides/layer, with a pitch of the helical suprastructure of 610.7 Å, 363.8 Å, and 144.9 Å in the C-WT, T-WT, and N-WT fibrils, respectively. Given the hIAPP fibril containing three protofilaments (3.91 kDa per full length monomer), our predicted MPL values were ~24.8 kDa/nm for all three fibril models, while experimentally estimated MPL values were 30.4 kDa/nm by Goldsbury et al.\textsuperscript{32, 33} and 26.5 kDa/nm by Kajava et al.\textsuperscript{34} with approximately ±3 kDa/nm in uncertainty. Small discrepancies in MPL data could be explained by that (i) simulation structures were built by all in-register parallel β-sheets, while experimental structures could be built up of a mixture of parallel and antiparallel β-sheets\textsuperscript{92} and (ii) fibrils with different lateral association of protofilaments (e.g. intersheet spacing between protofilaments) and with different morphologies (e.g. twisted or striated ribbon) can also lead to different MPL values. Overall, our hIAPP triangular models are in reasonable agreement with experimental observations.

3.4.3. Peptide-peptide interactions vs. peptide-water interactions

Similar to native protein folding, most of amyloidogetic peptides (except for GNNQQNY) require some minimal hydrophobic interactions to drive peptide aggregation to form a dehydrated or less hydrated interior core/interface by expulsing waters from peptides. At the early stage of oligomer formation, expulsion of waters from
peptides is the first and obligatory step to facilitate peptide association and subsequent fibrillization by reducing free energy barrier arising from dehydration entropic effects\textsuperscript{54}. Once the stable aggregates form, highly hydrated water layer around the aggregate’s surface helps to prevent peptide disassociation. The stability of hIAPP oligomers is a result of the delicate balance between maximization of favorable peptide-peptide interactions at the interfaces and optimization of solvation energy with globular structure. In our triangular models, the peptide-peptide interactions favor in the order of C-WT (-574.2 kcal/mol) > N-WT (-557.3 kcal/mol) > T-WT (-542.7 kcal/mol), while solvent energy favors in different order of T-WT (-12358.2 kcal/mol) > C-WT (-12040.9 kcal/mol) > N-WT (-9809.4 kcal/mol), suggesting these interactions cooperatively stabilized the hIAPP triangular structures. In combination of overall packing energies including bonded, nonbonded, and solvation energy with the relative stabilities, the C-WT and the T-WT were the two most populated structures among the ensemble examined (Table 3.1). The N-WT model is not highly populated, but it still represented \( \sim 19.6\% \) of the ensemble. It should be noted that the overall energies were calculated on the basis of the last 10-ns MD simulations, thus comparable overall energies for the N-WT and the wt300 models do not necessarily imply that the wt300 has comparable structural stability to the N-WT. Instead, visual inspection of MD trajectory showed that peptide dissociation in the wt300 model helped to gain large favorable solvent energy, suggesting that final dissociated structure is more likely conformation as compared to initial well-packed conformation. These results further indicate that stable and unstable
hIAPP aggregates can coexist in a rugged energy landscape with a range of different populations.

### 3.4.4. hIAPP triangular models vs. Aβ triangular models

It is interesting to compare various amyloidogenic structures to reveal similar or different scenarios in the oligomeric aggregation-disaggregation process. Similar to the hIAPP peptide, Aβ peptides are highly polymorphic, displaying a wide range of structural morphologies including micelle\(^{39}\), annular\(^{37,57}\), triangular\(^{38,58}\), globulomer\(^{59,93}\), and linear structures\(^{29}\). Here we only focus on Aβ triangular structures for comparison. MPL data showed that Aβ fibrils primarily consisted of two or three cross-β layers. Tycko and co-workers determined the atomic structures of 3-fold Aβ fibrils by NMR, EM, and MPL\(^{58}\) (referred as Aβ-C-WT model). Later, we determine alternative triangular structure of 3-fold Aβ oligomer (referred as Aβ-N-WT model)\(^{38}\). Comparison of Aβ triangular models with hIAPP triangular models showed some interesting similarities and discrepancies. First, all 3-fold Aβ and hIAPP models have a hydrophobic core, but different peripheral interactions at the juxtaposed region. The Aβ-C-WT model showed that the hydrophobic C-terminal β-strands (residues Lys28-Val40) formed a central core while the hydrophilic N-terminal β-strands (residues Gly9-Glu22) oriented to the bulk solvent, displaying a loop-next-to-tail arrangement with relative weak inter-sheet salt bridges between Glu11 and Lys28. The Aβ-N-WT structure had a similar hydrophobic core at the triangular cross section formed by the central hydrophobic cluster (CHC) residues Leu17-Ala21 (LVFFA) in the N-terminal, whereas hydrophobic C-terminal
β-strands were exposed to solvent, displaying a loop-next-to-strand association, in which more protected VDW interactions of turn residues (Glu22-Lys28) with strand residues of (Gln15-Leu17) and intersheet salt-bridges between Lys16 and Glu22 were involved to enhance the overall structural stability. Thus, comparison between Aβ and hIAPP triangular structures highlights the importance of hydrophobic interactions in stabilizing amyloid oligomers, and the peripheral interactions such as hydrogen bonds and salt bridges also provide additional driving forces to stabilize the structures effectively. Moreover, the amphiphilic nature of Aβ and hIAPP peptides in combination with different β-layer organizations provide different driving forces to stabilize different polymorphic structures. In both Aβ triangular models, electrostatic interactions contribute ~81% and ~70% of peptide-peptide interactions to the Aβ-C-WT and Aβ-N-WT structures, while in all hIAPP triangular models, VDW and electrostatic interactions contribute comparably to the whole structures.

### 3.4.5. Human IAPP triangular models vs. rat IAPP triangular models

It is well known that rat and mice do not suffer from type II diabetes because rat IAPP (rIAPP) is not able to form β-sheet amyloid aggregates, although rIAPP differs from hIAPP in only 6 of 37 residues at His18, Leu23, Pro25, Val26, Pro28, and Pro29. Thus, it is interesting to compare hIAPP with rIAPP sequence. Here, we mutated three stable triangular structures of hIAPP (i.e. C-WT, N-WT, and T-WT) to the rIAPP sequence (denoted as C-rat, N-rat, and T-rat) and performed additional 30-ns MD simulations to study the conformational change and β-sheet association of rIAPP.
triangular oligomers. As compared to the human IAPP models, all rat IAPP models experienced relative large RMSDs of 7.4 Å for the C-rat, 8.7 Å for T-rat, and 11.1 Å for N-rat.

MD trajectories also showed that three β-sheets were largely disassociated in the N-rat structure, tended to disassociate in the C-rat structure, and well maintained in the initial organization in the T-rat structure (Figure 3.11).

The averaged sheet-to-sheet interactions were -479.2 kcal/mol for the C-rat, -458.1 kcal/mol for the N-rat, and -454.1 kcal/mol for the T-rat, which were all disfavored as compared to the corresponding hIAPP models (Table 3.2). It should be noted that since rIAPP mainly adopted α-helical conformation in the N-region and random coil in the C-terminal region in solution, rIAPP peptides might not be able to assemble into our proposed triangular structures with preformed β-hairpin and β-sheet conformation. On the
other hand, mutations of 6 residues from hIAPP to rIAPP indeed have large impact on disrupting or disfavoring the formation of triangular structures.

3.4.6. A simple model to estimate the maximum number of symmetrical folds in the fibrils

Based on specific structural information of hIAPP and Aβ triangular structures and general amphiphilic nature of amyloid peptides with a β-strand-loop-β-strand motif such as human CA15048, and β2-microglobulin94, we proposed a simple model to estimate the maximum number of symmetrical folds in amyloid fibrils. We have demonstrated the importance of hydrophobic core in the Aβ and hIAPP triangular structures. To form the hydrophobic core, the identity, position, and number of hydrophobic residues are all critical.

Figure 3.12. Schematic estimation of the maximum number of folds (N) in amyloid fibrils, where D is hydrophobic interaction distance between hydrophobic central residue and the origin of central core; Rres is the radius of hydrophobic central residue; and L is the distance between hydrophobic central residue and the terminals or turn.
As shown in Figure 3.12, the size ($R_{\text{res}}$) and location of the central hydrophobic residue and its distances to either terminals or turn (i.e. near either terminals or turn is qualified, $L$) and to the origin of central core ($D$) were used to estimate the maximum number of folds ($N$) in a complete $360^\circ$ turn by $N \leq \frac{360}{2 \cdot \arctan \left[ \frac{L}{(R_{\text{res}} + D)} \right]}$. If distance of $L$ is too large, it prefers to form 2-fold structure. Similarly, distance $D$ is better within 7 Å, an effective cutoff distance for long-range electrostatics interactions, to ensure all inward-pointing sidechains to have sufficient interactions to main the central core structure. Based on this simple model, for example, in the triangular model of Aβ-C-WT model (i.e. Tycko’s model), Met35 at the C-terminal β-strand was one of core-forming residue, and eight residues away from C-terminal by ~17.3 Å, the maximum number of folds was 3.5 (Table 3.5), suggesting that only three protofilaments are allowed to form Aβ fibrils with a hydrophobic core formed by C-terminal residues.

On the other hand, considering the Phe20 residue at the N-terminal residues is one of core-forming residues, four protofilaments are likely to wind together to form Aβ-N-WT fibrils with different size and morphology from Aβ-C-WT fibrils. It should be noted that this model is very crude without considering many structural and chemistry properties of amyloid peptides, especially for the core-forming residue sequences, but it may provide a quick estimation for possible polymorphisms of amyloid fibrils.
Table 3.5. Maximum symmetric folds in amyloid fibrils estimated by

\[ N \leq \frac{360}{2 \cdot \arctan \left[ L / (R_{\text{res}} + D) \right]} \]

where hydrophobic interaction distance D is set to 7 Å, \( R_{\text{res}} \) is the radius of central hydrophobic residue, and L is the distance between central hydrophobic residue and the terminals or turn.

<table>
<thead>
<tr>
<th>Amyloid peptide</th>
<th>Core-forming β-strand</th>
<th>Central hydrophobic Residue</th>
<th>L (Å) and # of residues between central hydrophobic residues and terminals or turn</th>
<th>( R_{\text{res}} ) (Å)</th>
<th>Max. # of Folds (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha \beta )</td>
<td>C-terminal</td>
<td>Met35</td>
<td>17.3, 8</td>
<td>6.63</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>N-terminal</td>
<td>Phe20</td>
<td>13.9, 5</td>
<td>7.75</td>
<td>4.2</td>
</tr>
<tr>
<td>hIAPP</td>
<td>N-terminal</td>
<td>Phe15</td>
<td>14.8, 5</td>
<td>7.75</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>C-terminal</td>
<td>Phe23</td>
<td>7.00, 3</td>
<td>7.75</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>C-terminal</td>
<td>Leu27</td>
<td>18.8, 7</td>
<td>5.2</td>
<td>3.1</td>
</tr>
</tbody>
</table>

3.4.7. hIAPP aggregation mechanism

Base on the stacking sandwich and wrapping-cord models of hIAPP, Figure 3.13 shows a postulated kinetic mechanism of hIAPP aggregation. Soluble monomeric hIAPP peptides exist in a mixture of \( \alpha \)-helical, extended \( \beta \)-hairpin, or random coil in solution\(^{95-98} \). Driven by nonspecific hydrophobic forces, hIAPP monomers begin to form amorphous assemblies via initial slow nucleation (a \( \rightarrow \) b), although exact kinetic pathways and sizes of nucleation seeds are still largely unknown. These amorphous assemblies then undergo slow structural reorganization and transition into single cross-\( \beta \)-layer driven by specific hydrogen bonds (b \( \rightarrow \) c)\(^{65,99} \).
Figure 3.13. Hypothetical mechanism of hIAPP aggregation in solution via different oligomeric intermediates of (a) monomers, (b) amorphous assemblies, (c) single cross-β-layer, double cross-β-layers of (d) $N_{CC} \cap N$ and (e) $C_{NN} \cap C$, (f) triple cross-β-layers, triangular oligomers of (g) N-WT, (h) T-WT, and (i) C-WT, and (j) mature fibrils, based on our MD simulation and other experimental data. Detailed description of hIAPP aggregation pathways are given in the main text.
Based on our previous MD simulations of hIAPP sandwich models\textsuperscript{10}, these single cross-β-layers can grow longer via peptide elongation or thicker via laterally association on the top of each other to form two-cross-β-layer \( \cap \) structure (i.e. Tycko’s model) with the C-terminal-C-terminal-associated (CC) interface (c→d) and the C-terminal-N-terminal-associated (NN) interface (c→e) where \( U \) and \( \cap \) represent antiparallel assembly, double letters of CC, NN, or NC represent distinct interface between neighboring β-sheets, and single letter of N or C represent the N-terminal or C-terminal β-sheet exposing to bulk water. Our previous MD simulations\textsuperscript{10} have showed that the \( N U_{CC} \cap_N \) has more favorable layer-to-layer interactions than the \( U_{NN} \cap_C \). So, the \( N U_{CC} \cap_N \) could also be developed into three-layer structures of \( N U_{CC} \cap_{NC} U_N \) and \( N U_{CC} \cap_{NN} U_C \) by a two body assembly (2 layer+1 layer, d→f) due to the high stability of the CC interface. In the \( U_{NN} \cap_C \) structure with relatively weak layer-to-layer interactions, Phe15 contributed negatively to the stability of the hydrophobic NN interface, as evidenced by that the Phe15Ala mutant was energetically more favorable than the wild-type, with a large gain in total layer-to-layer interactions by 106.7 kcal/mol. The \( U_{NN} \cap_C \) structure is likely to accommodate additional β-layer to form a more stable triangular N-WT structure with a hydrophobic core by gaining more favorable layer-to-layer interactions from -98.4/2=-49.2 kcal/mol to -557.3/3=-185.8 kcal/mol normalized by number of layers (e→g). It appears that the steric hindrance of Phe15 sidechains is not energetically favorable in the sandwich \( U_{NN} \cap_C \) model, but when Phe15 residues are clustered in the relative large triangular core, steric hindrance of sidechains is minimized and the π-π packing is enhanced,
contributing more favorable association force to N-WT structure. Alternatively, three single cross-β-layer can also be twisted around one another along a fibril axis to form 3-fold wrapping-cord structures of the C-WT (c→i) and the T-WT (c→h) when competing with the sandwich models. Eventually, all these one-layer, two-layer, and three-layer oligomers can gradually evolve into mature fibrils by monomer addition along the fibril axis (j).

3.5. Conclusions

The capture and characterization of hIAPP oligomeric structures are essential for understanding their biological roles in hIAPP aggregation and toxicity associated with diabetes type II. Based on solid-state NMR, MPL, and EM data, we model a series of 3-fold hIAPP triangular oligomers with different cross-β-layer organizations at the cross section and assess their structural stability and population using an in-house developed peptide-packing program and all-atom MD simulations. Three triangular structures of C-WT, T-WT, and N-WT are determined, all displaying high structural stability with favorable packing energy. Structural and energetic comparisons of these 3-fold triangular models reveal that although all structures are composed of three same in-register cross-β-layers winding around one another to form a hydrophobic core, they adopt completely different cross-β-layer associations (i.e. turn-next-to-tail in C-WT, turn-next-to-strand in the T-WT, and turn-next-to-tail in the N-WT) and hydrophobic cores in sizes and core-forming residues, leading to distinct structural morphologies. Computational mutagenesis studies of C-N21A, T-F23A, and N-F15A show that
disruption of Phe23 and Phe15 packings at the central core of the T-WT and N-WT structures and Asn21 ladder at the juxtaposed region of C-WT structure disfavors the layer-to-layer association, suggesting that sequence modification to destabilize hIAPP structures strongly depends on their detailed peptide packings. Moreover, the proposed three 3-fold hIAPP structures can present the core architecture of the fibrils, with different widths of 8.7-9.9 nm, twists of 2.8°-11.8°, and pitches of 14.5-61.1 nm, in accordance with experimental data. Alternative to the stacking sandwich model, the wrap of multiple cross-β-layers or protofilaments around the central core via different cross-β-layer associations to form helical fibrils provides a molecular basis of amyloid fibril polymorphism. Further high-resolution experiments are also necessary to validate our 3-fold hIAPP models.
CHAPTER IV
COMPARATIVE MOLECULAR DYNAMICS STUDY OF HUMAN IAPP AND RAT IAPP OLIGOMERS

4.1. Introduction

Human islet amyloid polypeptide (hIAPP or amylin), a 37-residue hormone peptide (KCNTATCATQ\textsuperscript{10}RLANFLVHSS\textsuperscript{20}NNFGAILSST\textsuperscript{30}NVGSNTY), is produced by pancreatic islet β-cells and co-secreted with insulin in response to glucose and other secretagogues\textsuperscript{21}. The aggregation of hIAPP peptides into small soluble amyloid oligomers and large insoluble amyloid fibrils, which are deposited and found in the islets of Langerhans of more than 90\% of type II diabetes patients, has been pathologically linked to the death of β-cells, the reduction of insulin production, and the action of insulin\textsuperscript{100}. It is still unclear about which of the hIAPP species, i.e. monomers, oligomers, protofibrils, or fibrils induce toxicity to β-cells, but converging evidence has supported the “amyloid hypothesis” that small hIAPP oligomers are likely to be the most harmful species to β-cells, in common with other amyloidogenic oligomers formed by amyloid-β (Aβ) peptides associated with Alzheimer’s disease\textsuperscript{101} and α-synuclein peptides with Parkinson’s disease\textsuperscript{102}. The toxicity of hIAPP oligomers could be correlated with their interactions with
cell membrane. Such interactions are most likely to damage membrane integrity and functions, resulting in ionic homeostasis, oxidative injury, and altered signaling pathways\textsuperscript{103-107}. Although the exact mechanisms of membrane damage induced by hIAPP oligomers remain controversial, a number of experimental and computational studies have led to several plausible mechanisms for membrane damage, including the formation of ion-permeable oligomeric pores, nonspecific binding of oligomers to cell membrane, or carpet-like membrane dissolution\textsuperscript{75, 103, 108-111}. Due to a significant structural polymorphism of hIAPP oligomers, these different membrane-disruption mechanisms appear not to be mutually exclusive.

The full-length hIAPP\textsubscript{1-37} peptide is composed of multiple function regions\textsuperscript{23}, including an N-terminal region (residues 1-19) that involves membrane binding and insulin binding, a primary amyloidogenic region (residues 20-29), and a C-terminal region (residues 30-37) that involves peptide self-association. Fibrillar structures of full-length hIAPP have been extensively studied by AFM, cryo-electron microscopy, solid-state NMR, and 2D-IR\textsuperscript{22, 29, 31, 33, 92, 112-115}. The hIAPP fibril structures consistently reveal that multiple U-shaped hIAPP monomers longitudinally stack on top of each other to form a parallel in-register β-sheet and each U-shaped hIAPP monomer consists of two antiparallel β-strands connected by a turn. Being more biological relevant species, even hIAPP dimers can impair insulin secretion, resistance, and hyperglucagonemia\textsuperscript{72}. But, high-resolution atomic structures of hIAPP oligomers especially for small dimer to pentamer at the very early stage of amyloid aggregation are still lacking. In addition to their role as neurotoxic species, small oligomers could also serve as templated seeds for growing into amyloid
fibrils. Small size, transit nature, fast aggregation tendency, and heterogeneous conformations of hIAPP oligomers render conventional experimental techniques extremely difficult to capture and characterize their structural features.

A number of computational studies have provided some structural details for hIAPP monomer, oligomers, and template fibrils formed by the full-length or fragment of hIAPP peptide at atomic level\textsuperscript{10, 11, 35, 96, 98, 116-120}. The full-length hIAPP monomers were found to exist as a rather large conformational variability, which could be attributed to intrinsic flexibility of hIAPP peptide and different computational conditions (i.e. force fields, solvation models, sampling algorithms, temperature, and pH). The hIAPP monomers could adopt a wide variety of conformations including unstructured coil\textsuperscript{96, 120}, disordered conformation with transient α-helical structure\textsuperscript{98, 116}, mixed α-helical and short antiparallel β-sheet\textsuperscript{96}, mixed helical and β-sheet structure\textsuperscript{120}, extended antiparallel β-hairpin\textsuperscript{95, 96}, and compact helix-coil structure\textsuperscript{95}. Similarly, experimental studies also show different and even controversial results for hIAPP monomers. A number of studies\textsuperscript{33, 121, 122} reported that hIAPP monomers mainly adopted random structures using CD spectroscopy. Another CD study by Breder et al.\textsuperscript{107} revealed that the full-length hIAPP mainly adopted a random coil conformation at a low ionic strength of 25 µM, while contained a small degree of β-sheet structure at a high ionic strength of 150 mM. However, Yonemoto et al.\textsuperscript{123} and Williamson et al.\textsuperscript{124} revealed the helical conformations with little β-sheet structure for hIAPP monomers using NMR. Even more complex conformations of hIAPP monomers were also characterized. Kayed et al.\textsuperscript{114} found that the co-existence of two distinct conformers with both β-sheet and α-helix structural motifs. Dupuis et al.\textsuperscript{95} combined ion
mobility mass spectrometry and MD simulations to investigate the structure of hIAPP monomers. They concluded that hIAPP monomers adopted two populated conformational families: an extended β-hairpin structure and a compact helix-coil structure.

For small hIAPP oligomers, Laghaei et al.\textsuperscript{118} reported that a full-length hIAPP dimer displayed a strong expanded β-strand conformation between residues 17–27 and 29–35, which are required to form β-sheet for oligomerization. Dupuis et al.\textsuperscript{119} found that hIAPP dimers form an extended β-hairpin conformation at residues 11-18 and 23-32 using combined ion mobility spectrometry-mass spectrometry and molecular dynamics (MD) simulations. Barz and Urbanc\textsuperscript{125} systematically compared the structure and dynamics of both Aβ\textsubscript{1-40} and Aβ\textsubscript{1-42} dimers and examined the effects of force fields of coarse-grained discrete MD and all-atom MD and water models of SPCE and TIP3P on resulting dimer structures. Simulations showed that dimer formation increased the structural disorder in Aβ\textsubscript{1-42}, but not in Aβ\textsubscript{1-40} conformations. The structural difference between Aβ\textsubscript{1-40} and Aβ\textsubscript{1-42} dimers was not statistically significant using different water models and force fields. These computational studies have shown that hIAPP dimers have a larger conformational variability, and they also achieve certain structural consensus with rich β-structure at residues 23-32. For other high-ordered hIAPP oligomers, MD simulations of the self-assembly process of multiple hIAPP\textsubscript{20-29}\textsuperscript{35, 36, 97} and hIAPP\textsubscript{22-27}\textsuperscript{78, 126, 127} peptides showed different slab-like nucleus with different sidechain packings at the interface, but the assembled β-sheets preferred to adopt antiparallel packing orientations with respect to each other. More interestingly, during the self-assembly process, the dewetting phenomenon was observed between adjacent β-sheets. In our recent study\textsuperscript{10, 11}, we have
modeled and simulated a number of high-ordered hIAPP oligomers, which are generally classified into “stacking-sandwich” and “wrapping-cord” structures depending on β-sheet organization. In the “stacking-sandwich” structures, two or three β-layers are laterally stacked on top of each other in an antiparallel way to form a “sandwich-like” structure with either 2-fold symmetry or asymmetry. The stacking β-layers are mainly associated together by interdigitating overlapping side chains to form a steric zipper. Alternatively, multiple β-layers can wind around a hydrophobic core to form a “phone-cord-like” structure with certain periodicity. Unlike the “stacking-sandwich” models, the “wrapping-cord” structures can naturally accommodate the twist of cross-β-layers without causing severe structural defects, and therefore they can accommodate more cross-β-layers to form a 3-, 4-, or 5-fold fibril organization. Different “stacking-sandwich” and “wrapping-cord” hIAPP oligomeric structures serve as elementary templates to grow into fibrils via peptide elongation, reflecting a general and intrinsic nature of amyloid polymorphism. Similarly, Wang et al.\textsuperscript{113} also proposed some structural models of 2-fold and 3-fold hIAPP protofilaments using 2D-IR spectroscopy and MD simulations, compatible with our stacking-sandwich and wrapping-cord models.

More importantly, rat and mice IAPP sequence (rIAPP, KCNTATCATQ\textsuperscript{10}RLANFLVRSS\textsuperscript{20}NNLGPVPPT\textsuperscript{30}NVGSNTY) differs from the hIAPP in only 6 out of 37 residues, but rats/mice do not develop diabetes-like symptoms even when rIAPP is over expressed. Upon six mutations of His18Arg, Phe23Leu, Ala25Pro, Ile26Val, Ser28Pro, and Ser29Pro in hIAPP, 6 different residues of rIAPP mainly locate at residues 20-29, called as a primary amyloidogenic region. Due to high sequence similarity
and three hydrogen-bond-breakers of proline residues, rIAPP has been used as an inhibitor to prevent amyloid formation by hIAPP as previously demonstrated by Thioflavin-T fluorescence, transmission electron microscopy, and circular dichroism. It is speculated that rIAPP can not aggregate into β-sheet-rich species, which are required for amyloid toxicity. However, a striking finding recently showed that rIAPP peptides can also form its own amyloid β-sheet upon templating with the hIAPP β-sheet, rather than block the β-sheet formation of hIAPP. Although exact biological role of rIAPP serving as inhibitor or catalyzer to prevent or promote the hIAPP β-sheet formation is still unclear, a number of studies have shown that cross-seeding of different but conformational similar peptides may catalyze each other to promote protein aggregation via conformational selection. Thus, structure characterization of rIAPP peptides is critical for a mechanistic understanding of hIAPP fibrillogenesis, toxicity, and inhibition. But, major questions remain elusive: what is the minimal size of the hIAPP seeds and their stability? how sensitive is hIAPP oligomer formation to sequence changes? To better understand the structural features and differences between the human and the rat versions of IAPP, here we use the NMR-derived β-strand-turn-β-strand motif as a building block to construct a series of hIAPP and rIAPP oligomers from dimer to pentamer with parallel peptide organization, and to probe the differences in structural stability, conformational dynamics, and underlying stabilizing forces between hIAPP and rIAPP oligomers using all-atom MD simulations in explicit water. MD simulations show that overall structural stability and β-sheet population of hIAPP oligomers significantly increase with the number of peptides. Small hIAPP oligomers such as trimer, tetramer, and pentamer are highly stable in the
parallel organization at 330 K, suggesting that seeds for hIAPP aggregation can be quite small. Conversely, rIAPP oligomers from dimer to pentamer suffer from large secondary-structure changes by losing β-strand conformation over time. Comparison of structural stability between hIAPP and rIAPP oligomers reveals the importance of inter-peptide hydrogen bonds in maintaining in-register peptide association. This work provides atomic details in hIAPP and rIAPP oligomeric structures, leading to a better understanding of the biological role of hIAPP oligomers in aggregation and toxicity mechanisms.

4.2. Materials and Methods

4.2.1. hIAPP and rIAPP models.

Initial monomer coordinate of hIAPP\textsubscript{1-37} peptide was extracted and averaged from 10 solid-state NMR-based structures as provided by the Tycko’s lab\textsuperscript{29}. Each hIAPP\textsubscript{1-37} monomer had a β-strand-loop-β-strand (U-bend) fold consisting of two antiparallel β-strands connected by one turn, β-strand (Lys1-Val17)-turn (His18-Leu27)-β-strand (Ser28-Tyr37). Intramolecular disulfide bond between Cys2 and Cys7 was formed to stabilize the structure at the N-termini. The N- and C-termini were blocked by NH\textsubscript{3}\textsuperscript{+} and COO\textsuperscript{-} groups, yielding a net charge of +3e at a pH of 7.4. An hIAPP\textsubscript{1-37} oligomer (from dimer to pentamer) was constructed by packing hIAPP\textsubscript{1-37} monomers on top of each other in a parallel and register manner, with an initial peptide-peptide separation distance of ~4.7 Å, corresponding to the experimental data\textsuperscript{29}. All starting structures of the rIAPP\textsubscript{1-37} from monomer to pentamer were built from the corresponding hIAPP\textsubscript{1-37} structure by replacing
sidechains of 6 targeted residues (i.e. His18Arg, Phe23Leu, Ala25Pro, Ile26Val, Ser28Pro, and Ser29Pro), but without changing the backbone conformations and side-chain orientations. The structures of rIAPP1-37 monomer and oligomers were first minimized for 1000 steps using the steepest decent algorithm with the backbone of the protein restrained before being subjected to the following MD simulations. All models were summarized in the Table 4.1.

4.2.2. Explicit-solvent MD simulation.

All MD simulations were performed using the NAMD software package \textsuperscript{44} with the CHARMM27 force field for peptides and the modified TIP3P model for water \textsuperscript{45}. Each oligomer was solvated in a TIP3P water box with a margin of at least 15 Å from any edge of the water box to any peptide atom. Each system was then neutralized by adding Cl\textsuperscript{-} and Na\textsuperscript{+} ions to mimic ~200 mM ionic strength. The resulting systems were subject to 5000 steps of steepest decent minimization with peptide backbone atoms harmonically constrained, followed by additional 5000 steps of conjugate gradient minimization without any constraint. Short 1-ns MD simulations were performed to heat system from 0 K to 330 K by constraining the backbones of oligomers. The production MD simulations were performed using an isothermal-isochoric ensemble (NPT, T=330K and P=1 atm) under periodic boundary conditions. The Langevin piston method with a decay period of 100 fs and a damping time of 50 fs was used to maintain a constant pressure of 1 atm, while the Langevin thermostat method with a damping coefficient of 1 ps\textsuperscript{-1} was used to control the temperature at 330 K. The simulation temperature of 330 K is slightly higher than the
physiological temperature of 310 K and helps to aid in avoiding kinetic traps, which allow us to probe the stabilities and dynamics of rIAPP and hIAPP peptides more quickly in the limited simulation time \(^{41,135}\). All covalent bonds involving hydrogen were constrained by the RATTLE method so that 2-fs timestep was used in the velocity Verlet integration. Van der Waals (VDW) interactions were calculated by the switch function with a twin-range cutoff at 12 and 14 Å. Long-range electrostatic interactions were calculated using the force-shifted method with a 14 Å cutoff. Structures were saved every 2 ps for analysis. All analyses were performed using tools within the CHARMM, VMD \(^{46}\), and code developed in-house.

4.3. Results and Discussion

4.3.1. Structural stability of hIAPP and rIAPP monomers

The hIAPP and rIAPP monomers are the smallest building blocks to self-assemble into high-ordered oligomers. Atomic-resolution structural information for both hIAPP and rIAPP monomers can help to understand the difference in the oligomerization mechanism between hIAPP and rIAPP peptides. Visual inspection of 50-ns MD trajectories clearly revealed that hIAPP monomer essentially lost its initial β-hairpin structure and folded into a mixed conformation of small β-strand at the C-terminus, random coil in the middle, and small α-helix at the N-terminus, leading to a large backbone root-mean-square deviation (RMSD) of 16.3 Å. We next clustered the similar conformations of hIAPP monomer using the cluster plugin in the VMD program with the criteria of Cα-rmsd cutoff of 5.0 Å. Figure 4.1a shows five representative conformations
belonging to the five most populated clusters for hIAPP monomer. Each cluster represents comparable structural population of ~3.3%, summing up to represent ~16.3% of all conformations. It can be seen that five representative hIAPP conformations displayed little transit β-strand and α-helical structures, but a large propensity for turn/random conformations, occupying 51.4%/29.7%, 56.8%/43.2%, 43.2%/37.8%, 37.8%/37.8%, and 43.2%/56.8% in the five clustered conformations, respectively. This structural property is reasonably agreement with experimental data that monomeric hIAPP monomer exists as disordered structures in solution\textsuperscript{33, 114, 121, 122}.

Figure 4.1. The five most populated representative conformations of (a) hIAPP monomer and (b) rIAPP monomer collecting from a cluster analysis with a Cα-rmsd cutoff of 5.0 Å. The population of each cluster is given in the parenthesis. The secondary structure is generated using the STRIDE program implement in VMD. Color scheme for secondary structure: α-helices (purple), extended β-strand (yellow), turn (cyan), and random (white). C-terminus of each conformation is presented by a red bead.
As described in the Introduction, all of these structural studies suggest that hIAPP monomers sample more complex and multiple conformations in solution. These different conformers not only equilibrate between random coil structure, partially helical structure, and β-hairpin structure, but also dynamically balance with other high-ordered aggregates. No exact consensus on secondary structure contents of hIAPP monomers is achieved at this moment due to the complexity of hIAPP. It should also be noted that our conventional MD simulations have rather limited ability to search large conformational space of monomers, and more populated monomeric structures could be obtained using high-efficient sampling techniques such as replica-exchange MD and MC simulations.

Similar to hIAPP monomer, rIAPP monomer also lost its initial β-hairpin conformation and evolved into different clustered conformations with similar populations (Figure 4.1b). The percentages of turn/random conformations were 15.7%/55.3%, 30.5%/54.1%, 13.5%/56.8%, 46.4%/29.7%, and 35.1%/40.5% for the five representative clustered conformations. Reddy et al.\textsuperscript{136} reported two populated conformations of rIAPP monomers in solution: one contains an α-helical conformation comprised of residues 7–17, while the other adopted random coil conformation, in reasonable agreement with our results. Moreover, the bend motif, stabilized by intramolecular contacts between Gly24/Pro25/Val26 and Leu16/Val17/Arg18, were consistently presented in all five rIAPP clusters. It appears that rIAPP monomer displays stronger tendency to maintain the bend conformation at positions 16-26 than hIAPP monomer. Overall, both monomeric hIAPP and rIAPP exhibit little β-propensity due to the lack of stabilizing interactions from adjacent peptides. This in turn suggests that inherent propensity to form β-sheet-rich
aggregates requires additional intermolecular peptide-peptide interactions. It should be noted that unlike replica-exchange MD or Monte Carlo simulations, our conventional MD simulations only sample relative small conformational space, and thus these representative hIAPP and rIAPP clusters are not necessarily excluded other populated conformations.

4.3.2. Structural stability of hIAPP and rIAPP oligomers

The 37-residue hIAPP peptides can polymerize into different heterogeneous oligomers. Although these hIAPP oligomers are structurally diverse and transit, most of them contain β-sheet rich structure and some of them could act as nuclei seeds for subsequent fibrillization. Figure 4.2 shows the final MD snapshots of hIAPP and rIAPP aggregates from monomer to pentamer at 330 K and neutral pH. For hIAPP systems, despite dramatic decrease in RMSD values from monomer (RMSD=16.3 Å) to dimer (RMSD=7.4 Å) (Table 4.1 and Figure 4.3a), the parallel β-strands in hIAPP dimer, particularly C-terminal β-strands, were still largely disturbed. But, two hIAPP peptides of the dimer still retain associated and certain degree of extended β-strand structure.
Figure 4.2. MD snapshots of (a) hIAPP (blue) and (b) rIAPP (cyan) aggregates from monomer to pentamer averaged from the last 10-ns simulations. Six residues at the position of 18, 23, 25, 26, 28 and 29 are shown to present the sequence difference between hIAPP and rIAPP. Color scheme: polar residues (green), nonpolar residues (orange), and negatively charged residues (blue). C-termini are presented by red beads for guiding the eyes, and water and ions are removed for clarity.
Table 4.1. Summary of simulation systems with structural characteristics.

<table>
<thead>
<tr>
<th>System</th>
<th>Backbone RMSD (Å)</th>
<th>Rg (Å)</th>
<th>Normalized SASA* (Å²)</th>
<th>Twist (degree)</th>
<th>Times (ns) and # of runs</th>
</tr>
</thead>
<tbody>
<tr>
<td>hIAPP1</td>
<td>16.3 ± 1.1</td>
<td>14.7 ± 0.8</td>
<td>4014.7 ± 173.4</td>
<td>-</td>
<td>50, 2</td>
</tr>
<tr>
<td>hIAPP2</td>
<td>7.4 ± 0.4</td>
<td>12.5 ± 0.2</td>
<td>2742.2 ± 116.3</td>
<td>27.0 ± 5.6</td>
<td>50, 2</td>
</tr>
<tr>
<td>hIAPP3</td>
<td>5.8 ± 0.2</td>
<td>14.7 ± 0.3</td>
<td>2322.1 ± 52.2</td>
<td>16.0 ± 1.6</td>
<td>50, 2</td>
</tr>
<tr>
<td>hIAPP4</td>
<td>5.3 ± 0.2</td>
<td>15.9 ± 0.2</td>
<td>2183.1 ± 50.3</td>
<td>12.8 ± 1.1</td>
<td>50, 2</td>
</tr>
<tr>
<td>hIAPP5</td>
<td>5.3 ± 0.3</td>
<td>16.5 ± 0.1</td>
<td>1957.1 ± 35.0</td>
<td>9.8 ± 0.9</td>
<td>50, 2</td>
</tr>
<tr>
<td>rIAPP1</td>
<td>11.1 ± 1.0</td>
<td>12.0 ± 0.5</td>
<td>3490.0 ± 140.9</td>
<td>-</td>
<td>50, 2</td>
</tr>
<tr>
<td>rIAPP2</td>
<td>10.8 ± 0.4</td>
<td>14.1 ± 0.3</td>
<td>2866.5 ± 92.6</td>
<td>32.9 ± 9.8</td>
<td>50, 2</td>
</tr>
<tr>
<td>rIAPP3</td>
<td>13.5 ± 0.3</td>
<td>17.9 ± 0.2</td>
<td>2808.9 ± 76.5</td>
<td>20.9 ± 1.2</td>
<td>50, 2</td>
</tr>
<tr>
<td>rIAPP4</td>
<td>9.1 ± 0.2</td>
<td>16.2 ± 0.1</td>
<td>2239.0 ± 54.5</td>
<td>13.4 ± 1.4</td>
<td>50, 2</td>
</tr>
<tr>
<td>rIAPP5</td>
<td>7.4 ± 0.3</td>
<td>17.9 ± 0.1</td>
<td>2144.5 ± 49.0</td>
<td>12.6 ± 1.0</td>
<td>50, 2</td>
</tr>
</tbody>
</table>

All data are averaged from the last 10-ns simulations.

* The total SASA values of hIAPP and rIAPP aggregates are normalized by the number of peptides for comparison.

Figure 4.3. Backbone RMSDs of (a) hIAPP and (b) rIAPP aggregates from monomer to pentamer with respect to their energy-minimized structures.
As the number of peptides continuously increased to the hIAPP trimer, tetramer, and pentamer, they displayed very high structural stability with comparable RMSD values of ~5.3, 5.3, and 5.8 Å, respectively. The parallel in-register β-strands and the U-shaped peptide topology in trimer, tetramer, and pentamer were well maintained, with a typical twist of ~4° between adjacent β-strands. The presence of a twist is consistent with experimental data obtained from NMR of hIAPP fibrils and might represent a prerequisite for the incorporation of oligomers into fibrils. Consistently, hIAPP monomer and dimer had larger residue-based root-mean-square fluctuation (RMSF) than hIAPP trimer, tetramer, and pentamer across all residues (Figure 4.4a).

![Figure 4.4. Residue-based backbone RMSF of (a) hIAPP and (b) rIAPP aggregates from monomer to pentamer with respect to their energy-minimized structures.](image)

It can be seen that overall structural stability of hIAPP oligomers generally increases as the number of peptides. Although the hIAPP monomer and dimer exhibit a rather larger conformational flexibility, single peptide addition from dimer to trimer dramatically enhances the structural integrity and the β-sheet content of hIAPP aggregates, suggesting that small hIAPP trimer could act as a nuclei seed for facilitating
amyloid fibril formation and fibril growth. We also extended MD simulations of hIAPP trimer, tetramer, and pentamer to 80 ns. These oligomers still remained stable with well organized parallel in-register β-sheets (data not shown).

It is important to quantify the role of amino acid sequence in the structure and dynamics of rIAPP aggregates from monomer to pentamer. At first glance, MD trajectories clearly showed that all of rIAPP aggregates were structurally unstable, as also confirmed by large RMSD values of 11.1 Å for monomer, 10.8 Å for dimer, 13.5 Å for trimer, 9.1 Å for tetramer, and 7.4 Å for pentamer (Table 4.1 and Figure 4.3b). In contrast to the well-preserved β-sheets in hIAPP oligomers, the parallel, in-registered β-sheets were largely disrupted in rIAPP dimer to tetramer and less distorted in rIAPP pentamer. Figure 4.4b shows that the rIAPP tetramer and pentamer had very similar RMSF values, but the other rIAPP aggregates (i.e. monomer, dimer, and trimer) exhibited greater local fluctuations across all residues, especially for Arg18, Leu23, Pro25, Val26, Pro28, and Pro29. These six residues locate in the amyloidogenic region of residue 20-29, which plays an important role in fibril formation. As shown in Figure 4.2, the presence of these six residues in all rIAPP oligomers causes the turn and the C-terminal β-strands less stable than the N-terminal β-strands, resulting in the less populated β-conformation for residues 29-37 as compared to residues 1-17. We also performed additional 20-ns MD simulations for each hIAPP aggregate from monomer to pentamer at 310 K. MD trajectories showed that all rIAPP aggregates had unstable structures with disturbed turns at 310 K (data not shown), similar to these rIAPP oligomer structures at 330 K. The disturbed turn region in rIAPP tetramer and pentamer could prevent the rIAPP from
stacking on top of each other to form high-order, in-registered oligomers and fibrils. This prevention effect will become more pronounced as the peptide elongation process due to unfavorable entropy effect. In addition, it is also possible that our preformed rIAPP oligomers could not resemble in vitro and in vivo aggregation of rIAPP, i.e. rIAPP may be not able to fold into the U-bent beta-strand-turn-beta-strand conformation and to form beta-sheet-rich seeds for fibril growth, and both effects would result in the prevention of the formation of rIAPP fibrils. Taken together, large structural instability of the rIAPP aggregates mainly arise from the loss of perfectly parallel packings from the C-terminal β-strands and the large movement of the turn regions.

In our previous study\textsuperscript{11}, based on NMR, mass per length, and electron microscopy, we have selected and determined three stable 3-fold triangular hIAPP 15-mers (referred as C-WT, N-WT, and T-WT) from a total of 72 triangular models by considering different packings among three U-shaped β-sheets. Although these three triangular hIAPP oligomers had different size, periodicity, sheet-to-sheet orientation and interface, and core-forming sequence at the cross section, all of them displayed high structural stability with favorable layer-to-layer interactions. Upon introduction of the rIAPP sequence to three stable hIAPP structures via mutations (referred as C-rat, N-rat, and T-rat), the N-rat and C-rat structures showed a strong tendency of β-sheet disassociation. The T-rat structure was well maintained in the β-sheet association and the U-shaped topology comparable to the T-WT structure, but interactions between β-sheets were disfavored as compared to the hIAPP T-WT model. Taken together, structural comparison of hIAPP oligomers with rIAPP oligomers suggests that due to high structural stability and
well-preserved U-shape topology, the hIAPP trimer to pentamer could serve as nuclei seeds to grow into fibrils via different growth pathways. For instance, they can longitudinally accommodate new monomers at the edges via the “lock and dock” mechanism\(^{67}\) to form single-strand fibrils, or laterally associate with other oligomers via stable C-terminal or N-terminal β-sheets to form multiple-strand “sandwich-stacking” fibrils, or incorporate more β-sheets via different fold symmetries to form multiple-strand “wrapping-cord” fibrils. Due to the defected U-shape topology of the rIAPP oligomers, it is very unlikely for the rIAPP oligomers to grow into the fibrils via three pathways as described above.

4.3.3. The β-sheet population of hIAPP and rIAPP oligomers

Many studies have shown that the formation of β-sheet structure is a general feature of amyloid nucleus and fibrils\(^{30,34,92}\). Figure 4.5 shows the collective secondary structure of the hIAPP and rIAPP aggregates from monomer to pentamer as a function of time, while Figure 4.6 quantitatively measures the β-sheet population for all hIAPP and rIAPP aggregates using the DSSP algorithm\(^{137}\).
Figure 4.5. Secondary structure of (a) hIAPP and (b) rIAPP aggregates from monomer (the top) to pentamer (the bottom), as calculated by the DSSP algorithm\textsuperscript{137}.
The hIAPP monomer was unfolded into a disordered conformation containing a large population of coil, bend, and turn, and almost negligible β-structure (4.3%) near the turn region, suggesting that hIAPP monomer is not prone to form significant β-structure alone in solution. For the hIAPP dimer, Figure 4.5 shows that the C-terminal β-strands largely lost their initial β-structure and converted into an unstructured conformation, while the N-terminal β-strands (approximately three to five residues) retained a certain degree of β-structure. Although the overall U-shaped topology of the hIAPP dimer was still largely distorted, due to increased inter-peptide interactions, the β-sheet population was increased from 4.3% in monomer to 15.8% in dimer. Barz and Urbanc recently reported that Aβ1-40 and Aβ1-42 dimers contained ~15-25% β-structure, in good agreement with our β-structure propensity of 15.8%. Dupuis and co-workers also reported that hIAPP peptides formed a β-strand-rich dimer. They proposed three different ways for hIAPP dimerization, including side-by-side association of β-hairpin monomers, lateral stack of β-hairpin monomers, and compact assembly of helical-coil monomers that convert into
β-sheet structure at the monomer-monomer interface. Our hIAPP dimer is reasonably good agreement with side-by-side β-hairpin association mode. Surprisingly, as hIAPP peptides continuously assembled into trimer or above, the parallel in-register cross-β-structures were well preserved, and the β-sheet population dramatically increased to 49.2%, 54.3%, and 61.8% for the hIAPP trimer, tetramer, and pentamer, respectively (Figure 4.6).

A secondary structure content analysis of the rIAPP peptides revealed that the β-sheet content was maintained at 12.1% for monomer and 12.3% for dimer, and then monotonically increased to 23.8% for tetramer, 26.6% for pentamer, and 38.2% for pentamer (Figure 4.6), showing an increased trend of β-sheet structure as a function of the rIAPP polymorphic forms similar to hIAPP oligomers. Particularly for the rIAPP trimer to pentamer, the C-terminal residues that have different sequence from the hIAPP suffered from a significant β-sheet reduction, whereas the N-terminal residues retained their initial β-strand conformation. The turn conformation also underwent large fluctuation during the entire simulations (Figure 4.7). Such less β-sheet-rich structure and disturbed U-shaped topology exert large energy penalty for the rIAPP peptides to self-assemble into highly-ordered, in-register β-sheet-rich protofibrils and fibrils, which explain the non-amyloidgenic activity of the rIAPP.
Figure 4.7. Conformation and internal hydration of turn region (residues 16-26) for (a) hIAPP and (b) rIAPP oligomers from dimer (the leftmost) to pentamer (the rightmost). Some structurally important residues are labeled.

4.3.4. **Interpeptide interactions play an important role in maintaining β-strand association**

It is generally accepted that hydrogen bonding interactions between peptides are inherent to β-sheet stability. The number of hydrogen bonds between adjacent i and i+1 peptides was calculated and normalized by the number of adjacent peptide pairs, n-1, where n is the number of peptides, to enable direct comparison for different polymorphic forms of hIAPP and rIAPP peptides (Figure 4.8a). Comparing the β-sheet content in Figure 6 with the number of hydrogen bonds in Figure 8a revealed a clear correlation that...
regardless of sequence difference, the β-sheet content increased as the number of hydrogen bonds.

Figure 4.8. (a) Number of hydrogen bonds and (b) averaged interaction energy between any single peptide and other peptides in a given oligomer. There is a reasonable correlation between β-sheet content (Figure 4.6) and number of hydrogen bonds and interpeptide interactions between peptides.

For the stable hIAPP oligomers (i.e. trimer to pentamer), native hydrogen bonds between adjacent peptides were almost evenly distributed along two β-strand directions throughout the simulations. Once formed, these hydrogen bonds were rarely broken and acted as a zipper to retain the parallel in-register interchain organization. In contrast, the great loss of hydrogen bonds between adjacent peptides was observed for all rIAPP oligomers at the very early stage of simulations of ~10 ns. It can be seen in Figure 4.8a that as compared to each corresponding hIAPP oligomer, the number of hydrogen bonds was largely reduced by 50.0%, 36.6%, 28.1%, and 26.9% for rIAPP dimer, trimer, tetramer, and pentamer, respectively. Proline is known to be a β-sheet breaker. The replacement of Ala25, Ser28, and Ser29 in the hIAPP sequence with three prolines at 25, 28, and 29
positions at the C-terminal β-strand region in the rIAPP sequence, caused a loss of intermolecular hydrogen bonds between adjacent peptides and intramolecular contacts with its counterpart of N-terminal β-strands, leading to a distorted β-sheet structure. Since the formation of hydrogen bonds requires strong distance and angular dependences, hydrogen bonding interactions help to not only retain existing interchain organization, but also reorganize disordered peptide packings into directional and ordered interchain arrangement.

In addition to hydrogen bonds, other inter-peptide interactions such as hydrophobic interactions, π-π stacking, and other sidechain contacts can also contribute to the stabilization of β-sheet structure. To provide a more complete identification of driving forces underlying β-strand association, inter-peptide interactions were calculated to rationale the correlation between overall structural stability and underlying peptide-peptide interactions. Since different oligomers have different numbers of peptides, to consider the size effect of oligomers on inter-peptide interactions and to consistently correlate the inter-peptide interactions with overall structural stability of oligomers, we calculated the average interaction between any single peptide and other peptides in a given oligomer using 

\[ E_{\text{peptide-peptide}} = \frac{\sum_{i=1}^{n} \sum_{j\neq i}^{n} E_{ij}}{n} \]

where \( i \) and \( j \) represent \( i \)th and \( j \)th peptides in a given oligomer, respectively. In Figure 4.8b, the inter-peptide interactions were normalized by the number of peptides (\( n \)) for direct comparison. The inter-peptide interaction energies were -267.8 kcal/mol, -378.6 kcal/mol, -439.7 kcal/mol, and -468.1 kcal/mol for hIAPP dimer, trimer, tetramer, and pentamer, respectively, while these interactions were reduced
to -128.4 kcal/mol, -286.8 kcal/mol, -369.8 kcal/mol, and -382.2 kcal/mol for rIAPP dimer, trimer, tetramer, and pentamer, respectively (Figure 4.8b). Clearly, change of the sequence from hIAPP to rIAPP caused the loss of inter-peptide interactions for each oligomer. Small hIAPP oligomers are more energetically favorable than the rIAPP oligomers, suggesting that the hIAPP peptides are more likely to retain stable in solution for further peptide aggregation. Decomposition of inter-peptide interaction energies into van der Waals (VDW) and electrostatic contributions revealed that regardless of peptide sequence and size, VDW interactions (44.9%–50.8%) were comparable to electrostatic interactions (49.2%–55.1%), suggesting peptide association is in a cooperative mode. Moreover, gain of favorable inter-peptide interactions decreased the most from dimer to trimer by 110.8 kcal/mol for hIAPP peptides and by 158.4 kcal/mol for rIAPP peptides, but the increasing trend was gradually slowed down as the number of peptides. This fact further supports that hIAPP trimer could serve as a nuclei seed for fibril formation and growth. Given an ideal separation distance of 4.7 Å between adjacent peptides and long-range interaction cutoff of 14 Å, it is reasonable to extrapolate that inter-peptide interactions will eventually achieve to a stable plateau for high-ordered oligomers (i.e. ≥ 7-mer). As expected, inter-peptide interactions were well correlated with the β-sheet content, i.e. the peptides have strong intermolecular interactions with their neighboring peptides, producing a well ordered β-sheet structure with more stable and saturated hydrogen bonds between peptides. Thus, structural stability and β-sheet content of hIAPP and rIAPP oligomers are more precisely attributed to a strong network of interactions between peptides. It was speculated that the formation of amyloid oligomers usually can be divided into two steps. The
monomers rapidly collapse into disordered assemblies driven by strong interactions between hydrophobic residues, followed by the slow structural reorganization to characteristic β-structure driven by main-chain hydrogen bonds\textsuperscript{65}.

### 4.3.5. Hydration of hIAPP and rIAPP oligomers

Water plays a significant role in mediating the structure and kinetics of early prefibrillar oligomers and final mature fibrils at different assembly stages\textsuperscript{138}. The solvent accessible surface area (SASA) of hIAPP and rIAPP aggregates, normalized by the number of peptides, was calculated to quantify the hydration for each residue and each oligomer (Figure 4.9 and Table 4.1). Table 4.1 showed that on average, regardless hIAPP and rIAPP aggregates, the SASA values generally decreased as the number of peptides, with more dramatic decreases from monomer to dimer. As the size of peptide aggregates increased, more residues were well protected from solvent due to the formation of a well-ordered β-sheet structure. Similarly, due to high structural stability, stable hIAPP oligomers exhibited relative smaller SASA than corresponding unstable rIAPP oligomers. Large SASA value suggests not only the more exposed residues, but also the larger dewetting barrier upon association with other peptides. In Figure 4.9, the SASA of each residue for both hIAPP and rIAPP aggregates was inhomogeneous, suggesting that residues have various way of exposure to water. For the oligomers with a relative ordered β-sheet structure, outward-pointing residues to bulk water (black bar) generally had larger SASA values than inward-pointing residues (dash bar).
Figure 4.9. Solvent accessible surface area (SASA) of individual residue in hIAPP and rIAPP aggregates from monomer to pentamer. All data were averaged using structures from the last 10 ns of each simulation.

When hIAPP or rIAPP peptides pack in parallel along the fibril axis, they possess internal cavities formed by consecutive U-turns. The initial size of these internal U-turn cavities was ~4 Å, which is slightly large to accommodate water molecules inside. It is thus of interest to explore the structure and dynamics of confined water in the U-turn cavity in
all hIAPP and rIAPP aggregates. Inspection of MD trajectories did not reveal interior hydration within the U-turn cavity in hIAPP trimer to pentamer (Figure 4.7a). In those well-preserved the U-turn cavities, compact inward-pointing side-chain packing prevented water molecules from penetrating into the U-turn region, although the turn region consists of five consecutive hydrophilic residues of Hse18, Ser19, Ser20, Asn21, and Asn22. SASA data also indicated that the interior cavity of each stable U-turn formed by His18-Leu27 residues was almost solvent inaccessible for hIAPP trimer to pentamer. We also extended MD simulations of hIAPP oligomers to 80 ns, no internal hydration was observed for stable hIAPP oligomers from trimer to pentamer due to the highly integrated and compact turn conformation. Due to relative disordered and expanded turn conformation in hIAPP dimer, few water molecules can access to and interact with turn residues. In contrast, the higher internal hydration propensity observed for rIAPP was mainly due to the specific solvation of the disturbed turn region. All rIAPP oligomers displayed disturbed turn conformation (Figure 4.7b), and the expanded interpeptide distances near the turn region allow water molecules to enter into and be trapped into the turn region during the simulations. Interior water would further destabilize intermolecular interactions between rIAPP peptides. It is apparent that a number of water molecules were confined between peptides to form certain hydrogen bonds with peptides.

Figure 4.10 shows the radial distribution function, g(r), of the oxygen atoms of water (O_w) around the β-carbon atoms (C_B) of six inward-pointing turn residues at positions 16, 18, 20, 22, 24, and 26. The height of g(r) represents local water density (bulk water density is equal to 1 g/cm^3). From all the five g(r) profiles in the hIAPP systems, the change in
height of the g(r)s revealed a decreased order of hydration near the turn region of monomer > dimer > trimer > tetramer > pentamer, reflecting the reduction of water molecules in the solvation shells near the turn region.

Figure 4.10. Radial distribution function, g(r), of water molecules around the β-carbon (CB) atoms of six inward-pointing turn residues at positions 16, 18, 20, 22, 24, and 26 for (a) hIAPP and (b) rIAPP polymorphic aggregates.

The number of water molecules dropped >57% in the first solvation shell from the monomer system to trimer/tetramer/pentamer systems. Due to the dry condition inside the turn region (except for the dimer), no obvious hydration shells (i.e. peaks of g(r)) were observed at 3.5-7.5 Å for all systems, consistent with SASA and MD trajectories. On the other hand, g(r) profiles of the rIAPP systems, particularly for trimer to pentamer, displayed almost identical water distribution (Figure 4.10b), in which the first hydration shell appeared at ~5 Å with similar local water density to 40-50% of bulk water density, suggesting that water molecules are able to access to the disturbed turn region. The dramatic difference of water g(r) profiles between rIAPP and hIAPP oligomers further confirms that the well-preserved turn cavity in the hIAPP trimer to pentamer is likely to
prevent water molecules from penetrating into the cavity due to the limited interior space and restricted sidechain movement. It appears that the well ordered and stable oligomers are less solvated than the disordered aggregates, and this evidentially suggests that desolavltion in hIAPP oligomers provides an additional favorable entropic contribution for facilitating peptide aggregation process.

It is interesting to note that unlike the dehydrated U-turn cavity in hIAPP oligomers, internal hydrated U-turn cavity was observed in Aβ oligomers, which adopt similar β-strand-turn-β-strand motifs to hIAPP oligomers. The size of the U-turn cavity in Aβ oligomers (6-7 Å) is larger than that of the U-turn cavity in hIAPP oligomers (4 Å), which may explain penetration of water molecules into the U-turn cavity. Meanwhile, confined water molecules stabilized interior Asp23-Lys28 salt bridges, but were not trapped into a 4.8 Å space between neighboring peptides, which would otherwise induce destabilization. Additionally, Buchete et al. reported that as finite Aβ oligomers grow into infinite Aβ fibrils, the desolvation of the U-turn cavity occurs. Despite of large energy and entropic penalty, desolvation is generally required to facility peptide assembly longitudinally and laterally, which form different dry interfaces between in-register peptides and between multiple protofilaments.

4.4. Conclusions

We have performed all-atom explicit-solvent MD simulations to investigate the structural stability and dynamics of hIAPP and rIAPP aggregates from monomer to pentamer. All hIAPP and rIAPP oligomers are modeled by longitudinally stacking
U-shaped peptide together to form an initial in-register parallel β-sheet. MD results show that hIAPP monomer and dimer are unstable in solution, but increase of peptides even from monomer to dimer can significantly enhance peptide association by increasing favorable inter-peptide interactions (enthalpy) and decreasing intra-peptide conformation change (entropy). This becomes even more pronounced for higher-ordered oligomers. Small hIAPP oligomers such as trimer, tetramer, and pentamer are highly stable with a well-preserved in-register parallel β-sheet at 330 K, suggesting that the minimal seeds for hIAPP aggregation could be quietly as small as a trimer. The stable parallel in-register C-terminal or N-terminal β-sheet enable the lateral association of multiple hIAPP oligomers/protofilaments to form higher-order protofilaments/fibrils. In contrast, rIAPP oligomers from dimer to pentamer suffer from a significant loss of C-terminal β-sheet and turn conformation. As compared to strong inter-peptide interactions stabilizing hIAPP oligomers, the substantial reduction in inter-peptide interactions including hydrogen bonds contributes to destabilization of the rIAPP oligomers. Additionally, unlike solvated and disrupted U-turn conformation in rIAPP oligomers, highly conserved U-turn cavity in the higher-order hIAPP oligomers is dehydrated without accommodating water molecules inside, which help to protect inter-peptide association from penetrating water. Structural comparison of hIAPP oligomers with rIAPP oligomers suggests that the disruption of Uhape turn conformation appears to be a plausible inhibition pathway to prevent hIAPP aggregation. This work provides a molecular basis for better understanding interactions governing the self-assembly of amyloidogenic hIAPP peptides and non-amyloidgenic rIAPP peptides.
5.1. Introduction

Human islet amyloid polypeptide (hIAPP or amylin), a 37-residue hormone peptide, is synthesized and secreted together with insulin by the pancreatic islet β-cells. The normal physiological functions of hIAPP as hormone are to regulate gastric emptying, suppress food intake, and control glucose homeostasis. When hIAPP peptides misfold and self-assemble into β-sheet-rich aggregates upon interacting with cell membrane, they can cause the death of β-cells, which is pathologically linked to insulin secretion in type II diabetes. Increasing evidence suggests that small, dynamic, transient, and heterogeneous hIAPP oligomers are more toxic to β-cells than monomers and fibrils. Several hIAPP oligomeric and fibrillar structures have been determined computationally and experimentally, including linear fibrils by solid-state NMR, annular aggregates by AFM, and linear, annular, and triangular structures predicted by molecular simulations. Although the toxicity of hIAPP aggregates is very likely to be related to membrane disruption, the exact mechanism remains elusive.
hIAPP peptides can interact with membranes to introduce membrane disruption via different mechanisms (i.e. receptor-dependent, ion channel, membrane detergent-like, and membrane thinning/curvature models). hIAPP can interact with a number of membrane receptors such as G-protein-coupled receptor, RAMPs, CT receptor, amylin receptor, and the mechanosensitive calcium channel TRPV4. Misfolded hIAPP oligomers can also bind to membrane receptors. Unlike the traditional detergent-like model for membrane fragmentation that generally requires higher peptide/lipid ratios above a threshold value, IAPP peptides fragment membranes at lower peptide/lipid ratios, while suppressing membrane fragmentation at higher peptide/lipid ratios. This suggests that at lower concentration, hIAPP peptides tend to form intermediate oligomers, which could be bypassed to undergo alternative pathways at higher concentration. hIAPP peptides can directly insert into the cell membrane to form receptor-independent channels, which allow ions and water to cross the membrane and thus to induce abnormal ion homeostasis and oxidative stress. It is also likely that hIAPP peptides form pores by inducing excessive curvature in the membrane. Various ion-permeable channels formed by hIAPP, Aβ, and the K3 peptides, derived from β2-microglobulin, have been visualized and characterized by atomic force microscopy (AFM), electrophysiology, cell calcium imaging, and molecular dynamics (MD) simulations. Aβ channels exhibited Ca$^{2+}$-selective ion-permeable characteristics, while K3 and hIAPP formed relatively nonselective, voltage independent, ion-permeable channels in phospholipid bilayer membranes. Different ion conductivity and selectivity of these amyloid channels could be attributed to the
variability in peptide sequence and composition, sidechain packing and backbone conformation, and channel topology.

Unlike gated transmembrane ion channels such as gramicidin, which have a precise structure and mechanism to control channel conformations between the open and close states for selective transportation of specific ions across the membrane, amyloid ion channels in the cell membrane appear to consist of several loosely contacting mobile subunits, with certain extent of β-sheet structures. Such dynamic and irregular channels formed by amyloid peptides allow heterogeneous ion permeability. More importantly, recent studies have highlighted the striking resemblance in channel structure and activity of amyloid peptides to other ion channels formed by antimicrobial peptide and toxin proteins. NMR reveals that Aβ, K3, and hIAPP fibrils adopt U-shaped, β-strand-turn-β-strand structures, and recent MD simulations further confirm that such U-shaped conformations of Aβ and K3 are able to assemble into channel structures in the lipid bilayers. To further explore whether a U-shaped conformation could be a generic structural motif for amyloid channels, we use a β-strand-turn-β-strand hIAPP monomer derived from hIAPP fibrillar structures by NMR to computationally model a series of hIAPP ion channels with different numbers of peptides (12-mer, 18-mer, 24-mer, and 36-mer), channel sizes (2 - 4 nm inner diameter and 7-9 nm outer diameter), and channel topology (CNpNC channels: N-terminal β-strands facing the solvated pore and C-terminal β-strands interacting with lipids; and NCpCN channels: C-terminal β-strands facing the solvated pore and N-terminal β-strands interacting with lipids) in the zwitterionic lipid bilayer containing 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).
Simulation results show that U-shaped hIAPP peptides form channel-like structures assembled by 3-5 dynamically associated subunits, in line with AFM images and other, Aβ and K3, amyloid channels. The potential of mean force (PMF) indicates that both CNpNC and NCpCN channels of 12-36 peptides induce multiple ion conductivity along the same direction from the lower bilayer leaflet to the upper bilayer leaflet. Comparison of the hIAPP channels with those other β-sheet amyloid channels shows that all modeled channels share structural features including subunit morphology, ion conductivity, and preferential pore sizes.

5.2. Materials and Methods

The monomeric structure of the hIAPP\textsubscript{1-37} peptide was extracted and averaged from 10 solid-state NMR-derived structures from Tycko’s lab\textsuperscript{29}. Each hIAPP\textsubscript{1-37} monomer had a U-bent structure consisting of two β-strands connected by a loop, i.e. β-strand(Lys1-Val17)-loop(His18-Leu27)-β-strand(Ser28-Tyr37). Similar turn conformations centered on Phe23, Gly24, and Ala25 were also obtained in SDS micelles by solution NMR\textsuperscript{164} and in vacuum MD simulations\textsuperscript{165}. The intra-molecular disulfide bond between Cys2 and Cys7 stabilizes the structure at the N-terminus. The N- and C-termini were blocked by NH\textsubscript{3}\textsuperscript{+} and COO\textsuperscript{-} groups, respectively. Note that physiologically expressed IAPP has an amidated C-terminus while the structures of membrane bound hIAPP monomers in detergent micelles show some differences between the amidated and non-amidated forms\textsuperscript{166,167}. How these termini forms affect the oligomer and fiber structure is unknown. To construct the β-sheet channel, a single
hIAPP monomer was taken as a building block, replicated and rotated along the channel axis to build a circular channel-like structure. Channel sizes can be tuned using a varying number of monomers, and initial separation distance between peptides and the axis center. The channel structures were then minimized with a rigid body motion for the peptides to enhance the formation of intermolecular backbone hydrogen bonds between β-strands, followed by the insertion of the channels into the DOPC bilayer using the CHARMM-GUI membrane builder generator. DOPC lipids were randomly selected from a lipid library and assembled around the channel to satisfy a per lipid surface area of ~72.5Å² at 300 K. The resulting systems were solvated by TIP3P water molecules, and KCl, NaCl, and CaCl₂ at the same concentration were added to neutralize the system and to achieve a total concentration of ~210-260 mM.

In the equilibration stage, each system was gradually relaxed by performing a series of dynamic cycles, in which the harmonic restraints on peptides in the channels were gradually removed to optimize the peptide-lipid and peptide-water interactions. In the production stage, all simulations were performed using the NPAT (constant number of atoms, pressure, surface area, and temperature) ensemble at 300 K. The surface area in the xy plane was kept constant while allowing a volume change in the z direction. The van der Waals (vdW) interactions were calculated using a switch function with a twin-range cutoff at 12 and 14 Å. Long-range electrostatic interactions were calculated using the Particle Mesh Ewald (PME) method. Each hIAPP-lipid system was repeated twice using the same channel configuration, different lipid conformations randomly selected from the lipid library, different initial velocities for all atoms. All MD
simulations were performed using the NAMD software \(^4^4\) with CHARMM27 force field \(^4^5\). MD trajectories were saved by every 2 ps for analysis.

5.3. Results and Discussion

5.3.1. Heterogeneous and dynamic hIAPP channel structures

Figure 5.1. Top-view snapshots of the hIAPP channels in the DOPC bilayer at 0 ns and 60 ns, respectively. Color code: lipid head (red), lipid tail (light blue), polar residues (green), and hydrophobic residues (white).
Figure 5.2. (a) β-sheet population for 18-mer and 24-mer CNpNC channels (black line) and NCpCN channels (red line). (b) Subunit organization for 18-mer and 24-mer CNpNC and NCpCN channels. Subunits are defined by mapping x, y coordinates of Cα atoms of hIAPP peptides onto the x-y plane. The probability to find Cα atoms is represented by an increased color order of red, orange, yellow, green, blue, and purple.

Visual inspection of the MD trajectories showed that all hIAPP channels lost their initial perfect circular shape and gradually transited into several subunits, which loosely contacted with each other to form a pore-like structure. Backbone root-mean-square deviations (RMSDs) of the NCpCN and CNpNC channels reached relatively stable plateaus after 30-ns MD simulations, suggesting that the channels are fully relaxed in the
lipid bilayer (Figure 5.1). The β-sheet population of the individual peptide was used to measure the discontinuous β-sheet network and to determine the number of subunits (Figure 5.2). Due to the small size and low β-sheet population of the 12-mer, there was no obvious clustering effect for both CNpNC and NCpCN 12-mer channels. The 18-mer NCpCN and CNpNC channels had triangular shape with three subunits and the 24-mer channels had rectangular shape with four subunits. The 36-mer channels had relatively smaller curvature, with less discontinuous β-sheet network, but they still tended to break into 4-5 ordered subunits. Due to the highly populated β-sheets in the 36-mer channels, hIAPP peptides tended to form fibril-like structures. High-resolution AFM images also revealed that the pore-like structures were composed of 4-5 subunits in the DOPC bilayer, in agreement with the modeled hIAPP channels. Other amyloid pores in the lipid bilayer obtained from AFM and MD simulations typically consist of 3-7 loosely associated mobile subunits, depending on peptide sequences and their interactions with the surroundings. We suggest that dynamic assembly of several subunits into an amyloid pore is a generic structural feature of amyloid pores.

The averaged pore structures were calculated using the HOLE program. In Figure 5.3, the degree of the pore diameters was indicated by the color in the order of red < green < blue.
Figure 5.3. (a) Averaged pore (solid line) and outer diameters (dashed line) of hIAPP channels and (b) averaged pore structures embedded in the averaged hIAPP channel structures for the 18-mer and 24-mer CNpNC and NCpCN channels. Average pore diameter along bilayer normal is defined by a red-green-blue scale, with small diameter of <14 Å (red), intermediate diameter of 14-16 Å (green), and large diameter of > 22 Å (blue). Color code for protein: hydrophobic residues (white), polar residues (green), positively charged residues (blue), and negatively charged residues (red).
For the CNpNC channels, inner/outer diameters were 1.5/5.1, 1.8/6.2, 2.1/6.9, and 3.5/7.8 nm for 12-mer, 18-mer, 24-mer, and 36-mer, respectively. The NCpCN channels had similar pore and outer diameters to those CNpNC channels, i.e. inner/outer diameters were 0.8/5.0, 1.7/6.3, 2.0/7.1, and 3.6/8.2 nm for 12-mer, 18-mer, 24-mer, and 36-mer, respectively. All channels remained open, but the 12-mer channels had more tortuous pore structure than the intermediate 18-, 24-, and 36-mer channels. Recent AFM images showed that the inner and outer diameters of the hIAPP channels were 1-2 nm and 7-12 nm, respectively, consistent with our 18-mer and 24-mer channels (inner: 1.7-2.1 nm and outer: 6.2-7.1 nm).

As compared to the AFM values, the outer diameters of the 12-mer channels (5.0~5.1 nm) were too small, while the inner diameters of the 36-mer channels (3.5-3.9 nm) were too large. Thus, the 18-mer and 24-mer channels appear to be the more likely structures in the lipid bilayer. In the initial energy-minimized channel structures, both pore-facing and lipid-contacting β-strands contained a large population of β-structure. As the simulations proceeded, all channels gradually relaxed in the lipid bilayer, causing the loss of β-structure to some extent. The averaged β-structure percentages over the last 10-ns simulations, calculated by the DSSP algorithm in VMD, were ~10% for 12-mer, 25%~27% for 18-mer, 37%~43% for 24-mer, and 47%~50% for 36-mer. The intermediate NCpCN channels (18-, 24-, and 36-mer) had slightly higher β-structure population than those of CNpNC channels, because the hydrophobic match between the N-terminal hydrophobic residues near the loop and lipid tails maintained the stability of the β-sheet in the NCpCN channels. In contrast, in the CNpNC channels the positively
charged N-terminal residues of Lys1 and Arg11 induced the larger electrostatic repulsion when they were packed towards the pore. Nevertheless, in general, the pore-facing β-strands were well maintained in all cases, while the lipid-contacting β-strands lost their β-structures to some extent because the larger curvature and separation distance at the channel periphery prevents neighboring peptides from forming intermolecular backbone hydrogen bonds to stabilize β-structure.

5.3.2. PMF for ion binding to hIAPP channels

To characterize channel activity, potential of mean force (PMF) was calculated to measure the relative free energy change required to transfer ions from the bulk water phase to water-lipid interface to channel interior along the z axis, using

\[ \Delta G_{\text{PMF}} = -k_B T \ln \left( \frac{\rho_z}{\rho_{\text{bulk}}} \right) \]

where \( k_B \) is the Boltzman constant, \( \rho_z \) is the ion density at position z along the channel axis, and \( \rho_{\text{bulk}} \) is the ion density in the bulk phase (Figure 5.4). A negative (positive) PMF value indicates favorable (unfavorable) interactions of ions with the surrounding, which suggests the high (low) probability for ions across the bilayers.
Figure 5.4. Potential of mean force (PMF), $\Delta G_{PMF}$, for Cl$^-$ (black), Na$^+$ (red), K$^+$ (green), and Ca$^{2+}$ (blue) as a function of the distance along the channel center axis for the 18- and 24-mer of hIAPP channels. $\Delta G_{PMF}$ is calculated using an equation, $-k_B T \ln(\rho_z/\rho_{bulk})$, where $k_B$ is the Boltzmann constant, $T$ is the simulation temperature, $\rho_z$ is the ion density at the position $z$ along the channel axis, and $\rho_{bulk}$ is the ion density in the bulk region.

For the CNpNC channels, all PMF curves of Cl$^-$ displayed a similar asymmetrical S-shape, with a negative minimum at 0~1 nm and a positive maximum at 1~2 nm. The horizontal S-shape PMF curves clearly indicated that Cl$^-$ preferred to enter the hIAPP channels from the lower bilayer leaflet, because Lys1 (-1.7nm) and Arg11 (-0.7nm) near the lower leaflet formed circular clusters, which provide strong attractive force to drive Cl$^-$ into the interior of the solvated pore. Conversely, the hydrophobic Phe15 and Val17 patches (~1.8 nm) near the upper bilayer leaflet created an energy barrier of 1~2 kJ/mol
for preventing Cl\(^-\) from entering the channels. For the NCpCN channels, the amphiphilic C-terminal \(\beta\)-strands, rather than the positively charged N-terminal residues of Lys1 and Arg11, formed the solvated pore. Consequently, the PMF curves of Cl\(^-\) showed that the minimal peaks appeared at -1 nm in the CNpNC either largely decreased or disappeared, because attractive electrostatic interactions between Cl\(^-\) and Lys1/Arg11 residues in the NCpCN channels were greatly reduced.

Apart from the PMF of the Cl\(^-\) anion, the trends in PMF for Na\(^+\) are qualitatively similar to those observed for K\(^+\) in the CNpNC channels of various diameters, showing strong repulsive energy penalty for these ions to bind to the bilayer and to further enter the channels from either side of the DOPC bilayer. Both Na\(^+\) and K\(^+\) displayed similar unfavorable interactions with the NCpCN channels, but with lower repulsive energy. Unlike K\(^+\) and Na\(^+\) that exhibit a very low binding probability to the bilayers, Ca\(^{2+}\) were highly populated at both bilayer leaflets, as reflected by two minimal peaks at the channel axis of -2\~ -3 nm and 2\~ 3 nm in the PMF curves of Ca\(^{2+}\). This fact indicates that Ca\(^{2+}\) have favorable interactions with the lipids. Visual inspection of MD trajectories also confirmed that Ca\(^{2+}\) tightly bound to both the negatively charged phosphate groups in the DOPC lipid heads and C-terminus of hIAPP peptides. The preferential and dominant binding of Ca\(^{2+}\) to the DOPC bilayer largely reduces the possibility for Na\(^+\) and K\(^+\) to bind to or cross the bilayer. The smallest CNpNC and NCpCN channels (12-mer), which did not collapse, provide ion binding sites at the entry and inner parts of the channels. As the channel size increased to 36-mer, the NCpCN channel showed much lower binding resistance to K\(^+\) and Na\(^+\) than the CNpNC channel, because the repulsion from the
positively charged rings formed by Lys1 and Arg11 were reduced.

5.3.3. Ion permeability of hIAPP channels

![Graphs showing ion permeability](image)

Figure 5.5. Net charge flows of Cl⁻ (black), Na⁺ (red), K⁺ (green), and Ca²⁺ (blue) across the 18-mer and 24-mer channels during the 60-ns MD simulations.

Visual inspection of the MD trajectories showed that all hIAPP channels (12-, 18-, 24-, and 36-mer) were able to induce ion permeability. To quantitatively measure the net ion flux/permeability across the DOPC bilayer, we use a “right-hand rule” to define a positive direction if ions conduct from the lower bilayer leaflet (i.e. Tyr37 for CNpNC model and Ala5 for NCpCN model in N/C-terminus region of channels) to the upper bilayer leaflet (i.e. Ser20 for both CNpNC and NCpCN model in U-turn region of channels), and in a negative direction for ion conductivity. Figure 5.5 show the net fluxes
of ions as a function of time for hIAPP channels with different channel diameters. Simulation results showed that all ions conducted through the hIAPP channels in a positive direction from the lower to the upper bilayer leaflet, although all cations displayed relatively poor directional conductivities. Overall, both CNpNC and NCpCN channels displayed high selectivity of Cl\(^-\) over cations (Ca\(^{2+}\), Na\(^+\), and K\(^+\)). The flux of Cl\(^-\) was significantly higher than the fluxes of cations in all channels, consistent with the PMF profiles.

Transport of ions through a hIAPP channel is generally governed by two steps: the first approaching the entrance of the channel and the second flowing through the solvated pore. The combination of these two steps results in a net flux. The first step provides a critical control for the ion flux. Positively charged Lys1 and Arg11 (located at the N-terminal of hIAPP in the lower DOPC bilayer leaflet) formed anionic binding sites, which induced favorable electrostatic binding for Cl\(^-\). Once the binding sites were fully occupied by Cl\(^-\), increased repulsive force between Cl\(^-\) drove the ions across the channels. In the case of Ca\(^{2+}\), it is observed that Ca\(^{2+}\) barely crosses the bilayer in all channels during the 52 ns of MD trajectories. Since Ca\(^{2+}\) tightly bound to the phosphate groups of the lipid bilayer as shown in PMF profiles, they exhibited very low mobility, mostly occupying these binding sites for the entire simulation. This fact indicates that strong binding between Ca\(^{2+}\) and the lipid bilayer can greatly reduce the ion conductivity across the bilayer. Unlike Ca\(^{2+}\), very few K\(^+\) and Na\(^+\) (<5 ions) were able to conduct through the channels (Figure 5.5). Visual inspection of MD trajectories revealed that K\(^+\) and Na\(^+\) displayed a high mobility within 10 Å of the lipid bilayer, due to lack of binding sites that
have been preoccupied by Ca\textsuperscript{2+}. High mobility and thermal fluctuation caused the weak ion conductivity of K\textsuperscript{+} and Na\textsuperscript{+} through the channels. In particular, those ion conducting events occurred mostly during the first 30-ns of the simulations. Once the systems achieved an equilibrium state, no further K\textsuperscript{+} and Na\textsuperscript{+} conduction through the bilayer was observed. Thermal fluctuation-driven ion conductivity is similar to the case of conducting water molecules through hydrophobic nanotubes.

5.3.4. Interaction of channels with their environment

Figure 5.6. Interaction energy of channel peptides with (a) DOPC lipids and (b) surrounding water molecules. All interactions energies are normalized by the number of peptides.

Interaction of channel peptides with lipids and water molecules in the environment plays an important role in supporting the structure and activity of channels. For each channel, we calculated peptide-lipid interaction and peptide-water interaction, averaged over time and the number of peptides in the channels (Figure 5.6). Overall, all channels
had favorable interactions (negative value) with both lipids and water molecules, suggesting that the environment supports channel conformation. For both NC\_p\_CN and CN\_p\_NC pore topologies, the 12-, 18-, and 24-mer channels had similar interactions with the lipids, as shown by -120--140 kcal/mol for the NC\_p\_CN channels and -100--110 kcal/mol for the CN\_p\_NC channels. But, as the channel size increased to 36-mer, the normalized peptide-lipid interactions were significantly reduced to -45 kcal/mol for the CN\_p\_NC channel and -40 kcal/mol for the NC\_p\_CN channel. As compared to the subunits in the 12- to 24-mer channels, the subunits in the 36-mer channels appeared to be more rigid because they contained a relatively larger number of peptides with a high population of \( \beta \)-structure (47%--50\%). Thus, the rigid subunits had lower flexibility to adapt their conformations to interact with lipids. The less favorable interactions for the 36-mer further support the conclusion that the 36-mer channel structures are unlikely to be lipid-supporting channels, consistent with AFM images. The NC\_p\_CN channels interacted more strongly with the lipids than the CN\_p\_NC channels (except the largest 36-mer channels). Both N- and C-terminal \( \beta \)-strands contributed hydrophobic match near the loop (i.e. Leu12-Val17 in the N-terminal \( \beta \)-strand and Phe23-Leu27 in the C-terminal \( \beta \)-strand) interacting with the lipids. Since the C-terminal residues of 20-29 (SNNFGAILSS) have been shown to be critical for amyloid formation\textsuperscript{173}, packing of the C-terminal \( \beta \)-strands towards the pore preserved the intermolecular backbone hydrogen bonds, which help to stabilize an inner \( \beta \)-sheet and retain peptide association. Meanwhile, the lipid-contacting N-terminal \( \beta \)-strands carrying two positively charged residues, Lys1 and Arg11, in the NC\_p\_CN channels strongly interact with lipid headgroups in the lower
bilayer leaflet, reducing electrostatic repulsion as compared to the pore-facing N-terminal β-strands in the CNpNC channels. Both effects produce more favorable interactions of the NCpCN channels with the lipids. Decomposed energy contributions (i.e., vdW and electrostatic interactions) further revealed that the NCpCN channels were stabilized by comparable 39.9-49.1% vdW and ~51.7%-60.1% electrostatic interactions, whereas the CNpNC channels were mainly stabilized by vdW interactions of 61.2-70.3%. Large differences in energy contribution indicate that different packing between lipids and peptides lead to different vdW and electrostatic contributions in each case.

Visual inspection of MD trajectories showed that all hIAPP channels were well hydrated, including a central water pore and a hydrated cavity inside the U-shape turns. The averaged peptide-water interactions for the CNpNC/NCpNC channels were -798.2/-762.1, -801.0/-710.9, -645.6/-603.3, -473.6/-455.2 kcal/mol for 12-mer, 18-mer, 24-mer, and 36-mer, respectively. The interactions of the CNpNC channels with water molecules were slightly stronger than those of the NCpCN channels. The 36-mer channels had relatively weak peptide-water interactions. The subunits of the 36-mer channels presented ordered β-strand packing with fully saturated hydrogen bonds between peptides, which prevents hydrogen bond formation between the peptides and water molecules. Peptide-water interactions were much stronger than peptide-lipid interactions, suggesting that unbalanced interactions could be attributed to the channel breaking into several subunits.
5.3.5. Structure and dynamics of lipid bilayers

To characterize the perturbation effects of hIAPP channels on the lipid ordering, we calculate the deuterium order parameter $S_{CD}$, using $S_{CD} = 0.5 \left( 3 \cos^2 \theta_{ij} - 1 \right)$ where $\theta_{ij}$ is the angle between the C-H vector and the bilayer normal and the angular bracket represents averaging over lipids and over time. The averaged $S_{CD}$ of two oleoyl acyl chains were compared for various carbon atoms in all simulated channel systems. The $S_{CD}$ values of pure DOPC lipids without hIAPP channels were also calculated for comparison. Higher $S_{CD}$ value indicates more ordered lipid chains. It can be seen that the $S_{CD}$ values of lipids for all channel systems were smaller than those in a pure DOPC bilayer, suggesting that the membrane-embedded hIAPP channels affect the ordering of lipid chains. The shapes of $S_{CD}$ curves were similar for all channel systems, but displayed different extent of lipid ordering, which strongly depends on the local interactions between lipids and channels.

To further quantify whether the amyloid channel induces membrane thinning, we measured the thickness and curvature of the lipid bilayer by projecting phosphorus atoms of the lipid headgroups onto the $xz$ plane (Figure 5.7). The bilayer thickness is measured by the distance between the average positions of the phosphorus atoms in each leaflet, while the bilayer curvature is quantified by the positions of the phosphorus atoms in each leaflet. In all starting configurations, the bilayer thicknesses measured by the phosphate–phosphate distance between two leaflets was 40.2 Å and the bilayer surfaces were flat.
Figure 5.7. The bilayer thickness and curvature of 18-mer and 24-mer hIAPP channels, by projecting phosphorus atoms of the lipid headgroups onto the x-z plane. The probability to find phosphorus atoms is defined by an increased color order of red, orange, yellow, green, blue and purple.

Figure 5.7 shows that for all channel systems, the channel peptides moderately depressed the local bilayer by ~3-5 Å as compared to the starting configurations, forming minor grooves beneath the peptides. The non-peptide contact bilayers remained flat with undisturbed thickness of 40.2 Å at a distance of ~25-42 Å from the center of channels. This fact indicates that bilayer thinning is very localized, and only confined to the peptide–lipid contact region, especially at the two peptide termini. The degree of the
bilayer thinning effect appears not to be correlated with the size and topology of the channels. Instead, peptide insertion depth and buried peptide sequence could play key roles in local bilayer thinning. Similar local bilayer distortion behavior was also reported for other pore-forming peptides \(^{174,175}\). While not addressed here, other components of cell membranes such as cholesterol, membrane proteins, and protein receptors play an important role in regulating peptide conformation, adsorption, and aggregation, and thus in biological function and the dynamic structure of cell membranes, and in hIAPP-lipid interactions. Jha et al. \(^{176}\) and Weise et al. \(^{110}\) have reported that in the raft membrane containing enriched sphingolipids and cholesterol, hIAPP aggregates faster and exhibits a much higher propensity for fibrillation than in the pure lipid bilayer. Wakabayashi et al. \(^{177}\) also clearly showed that hIAPP rapidly accumulated in ganglioside- and cholesterol-rich microscopic domains (i.e. lipid-raft domain), and depletion of gangliosides or cholesterol significantly reduced the amount of amyloid deposits. The results of these studies are also in line with our recent computational work \(^{178}\), showing that increased cholesterol level in lipid bilayer promotes the association of A\(\beta\)42 monomer with the POPC bilayer.

### 5.3.6. Comparison with other amyloid channels

Numerous studies have shown that amyloid peptides can insert into the membrane to form receptor-independent channels as shown by a series of AFM images \(^{144,155,179}\). Amyloid channels formed by different peptides share common structural characteristics despite the sequence variability (Figure 5.8).
Figure 5.8. (a) Averaged pore structures calculated by the HOLE program embedded in the averaged barrel conformations during the simulations for the conformer 1 (turn at Ser26-Ile31) and 2 (turn at Asp23-Gly29) Aβ1–42 barrels. In the angle view (left panel) of the pore structure, whole barrel structures are shown with the ribbon representation. The right panel represents the simulated barrel structures with highlighted subunits for the averaged barrels in the surface representation that are shown in the view along the membrane normal. (b) Averaged pore structures embedded in the averaged channel conformations during the simulations for the K3 channel with NCpCN topology. In the surface representation for the channel, the front part of the channel in the angle view (left
panel) has been removed to allow a view of the pore. In the view along the membrane normal (middle panel), subunits are highlighted by yellow dotted circles. In the right panel, simulated channel structure is shown with the ribbon representation in the view along the membrane normal.

They consist of multiple dynamic subunits, which are loosely associated to form heterogeneous channel-like structures. The size and shape of channels in the membrane vary, but the membrane-supported amyloid channels, which typically appear to have an averaged pore diameter of 1–2 nm and outer diameter of 8–12 nm, are wide enough for conducting ions and water to cross the membrane. Interestingly, some antimicrobial peptides such as protegrin-1 (PG-1) can also form channel-like structures in the cell membrane, with loosely associated subunits. Thus, such ion-permeable channels may represent a general class of toxic channels, which differ from the natively folded, gated ion-selective channels regulated by two, open and closed states.

Aβ CNpNC channels formed by different fragments of Aβ9–42, Aβ11–42, and Aβ17–42 display strong Ca$^{2+}$ binding, in addition to other cations, because the negatively charged Glu22 sidechains near the upper lipid leaflet provide the relative low free energy barriers for Ca$^{2+}$ to bind to and conduct through the channel. K3 (a β2-microglobulin fragment) forms the NCpCN channel-like structures in a DOPC bilayer as observed by modeling and AFM. Electrophysiology studies show that K3 channels permeate multiple ions across the bilayer, with weak cation selectivity, because of an excess of negatively charged groups (Asp34, Glu36, and Asp28) in or near the channel. Our modeled hIAPP
channels with different sizes (12- to 36-mer) exhibit highly directional permeability of Cl⁻ ions and induce strong binding to Ca²⁺ ions. For all modeled Aβ, K3, and hIAPP channels, the U-shaped channel-forming peptides adopt parallel β-sheet arrangement with a “turn-to-tail” orientation, in which the U-turn regions of the peptides located at the upper bilayer leaflet, while the N-/C-terminus locate at the lower bilayer leaflet. However, this may not be the case for other amyloid channels. It is likely that if amyloid peptides insert into the membrane via the U-turn residues first, they can adopt “tail-to-turn” orientation to form a channel with the N-/C-terminus at the upper bilayer leaflet and the turn at the lower bilayer leaflet. Coexistence of “turn-to-tail” and “tail-to-turn” channels in the membrane and their relative populations determine the overall ion conductivity and selectivity. α-helical hIAPP channel structures have also been proposed 180-182, although it is unclear if membrane disruption involves a channel-like mechanism or non-specific disruption of the membrane 181, 183.

Chang and co-workers 184 used MD simulations to study the structure and dynamics of the modeled β-barrel structures formed by Aβ25-35 octamers in the POPG bilayer. They found that these Aβ-barrel channel-like structures adopted a highly populated “in-register mixed parallel-antiparallel” organization, which caused local disturbance of the bilayer and water molecules to pass through. Shafrir et al. 185 reported a computational study showing that six Aβ42 peptides can form stable annular β-barrel pore structures in both bulk water and membrane environments. For the proposed β-barrel structures, hydrophobic and glycine residues of Gly29-Ala42 formed a central hydrophobic pore, while hydrophilic N-terminal residues of Asp1-His14 were wrapped around a central core.
to form a soluble and protective shield. This study did not provide detailed information about the channel activity. Due to a complex energy landscape of polymorphic amyloid oligomers, some experimental structures of Aβ oligomers in solution show a similar subunit structure as the fiber \textsuperscript{186,187}, while the subunit structures in others are different \textsuperscript{188,189}.

To examine whether hIAPP peptides can penetrate the lipid bilayer via the turn region or the N/C-terminal region with further self-assembly into “turn-to-tail” and “tail-to-turn” channels, we applied steered MD and umbrella sampling to explore the possibility of single hIAPP penetration into the bilayer via different orientations. The penetrating process of amyloid oligomers across the membrane is complicated due to various adsorption orientations of amyloid oligomers on surfaces \textsuperscript{190}. Thus, to facilitate peptide penetration across the bilayer and to obtain consensus PMF profiles, three different external forces of 125, 130, and 135 kJ/mol nm$^2$ were applied to either turn region (Asn21) or tail region (Lys1 and Tyr37), depending on the initial peptide orientation, to pull the peptide across the bilayer. In a single PMF profile, the total separation distance between the center of mass of peptides and the center of the bilayer was divided into 30 windows of 0.2 nm each. In each window we used a different initial configuration, with the peptide left free to rotate around their restrained center of mass at a certain distance from the center of the bilayer. Each point in the PMF profiles requires 100 nanoseconds for equilibration. To obtain the unbiased PMFs, we applied the weighted histogram analysis method (WHAM) \textsuperscript{20} using the g\_wham \textsuperscript{191} module in the GROMACS.
Figure 5.9. Potential of mean force (PMF) for the transfer of single hIAPP peptide from bulk water to water-bilayer interface to the center of a bilayer by pulling (a) turn region and (b) tail region into the DOPC bilayer.

Figure 5.9 presents the PMFs for the transfer of single hIAPP peptide from bulk water to water-bilayer interface to the center of a bilayer. Depending on the insertion orientation, the PMF profiles displayed completely different insertion behaviors. By pulling the tail of hIAPP into the bilayer, the hIAPP peptide preferred to stay in the interior of the bilayer with the lowest energy minima of -18 kcal/mol at z=2.2 nm, as compared to bulk water phase at z>5 nm. The peptide also had comparable probability to fully insert into the lipid bilayer compared to staying in the bulk water phase. This type of PMF usually indicates that the peptides seem to form a barrel-stave pores, consistent with our hIAPP channel models. On the other hand, when inserting hIAPP into the bilayer via the turn region, although the peptide still preferred to be partially inserted into the bilayer, an additional barrier of ~11 kcal/mol needed to be overcome so that the peptide can fully cross the bilayer to form a transmembrane pore. Comparison of the two
PMF profiles suggests that a certain preferred orientation of peptide insertion is required in order to form a transmembrane pore; thus hIAPP is more likely to penetrate the bilayer via the tail than via the turn region. On-going studies on the co-existence of “turn-to-tail” and “tail-to-turn” channels in the membrane will provide a more complete picture for ion conductivity and selectivity of hIAPP channels. In parallel to channel-induced membrane disruption by amyloid peptides [56], strong evidence also exists for non-channel like membrane disruption. A number of studies [57-60] show that amyloid peptides can fragment the cell membrane, resulting in non-specific ion leakage.

5.4. Conclusions

In this work, we investigate the structure and activity of hIAPP channels in the DOPC lipid bilayer using computational modeling and MD simulations. We construct two types of hIAPP channels, each with different size (12-, 18-, 24-, and 36-mer): CNpNC channels with charged N-terminal β-strands facing a solvated pore and NCpCN model with C-terminal β-strands facing a solvated pore. During the MD simulations, all channels fragment into three to five oligomeric units that are loosely associated, preserving the channel-like structures in the bilayer. Of particular note, 18-mer and 24-mer channels present similar numbers of oligomeric units and overall dimensions to the channels observed by AFM. The bilayer does not support too small 12-mer channels or too large 36-mer channels, consistent with the Aβ channels (17, 27). Due to the formation of anionic binding sites provided by the positively charged N-terminal residues of Lys1 and Arg11 near the lower DOPC bilayer leaflet, they create an electric potential to drive Cl⁻ through the solvated pore in the same direction, from the lower leaflet to the
upper bilayer leaflet. Cl\(^-\) display much stronger binding activity to the hIAPP channel and higher flux across the bilayer than cations. The PMF profile shows that, unlike Na\(^+\) and K\(^+\) that are barely adsorbed on the lipid bilayer, Ca\(^{2+}\) bind tightly to phosphate groups in the lipid heads, resulting in poor ion conductivity. Na\(^+\) and K\(^+\) exhibit relatively weak ion fluxes due to thermal fluctuation. More importantly, the hIAPP ion channels highlight the striking resemblance of the structure and ion permeability to other amyloid channels, e.g. loosely associated subunits, universal U-shape β-strand-turn-β-strand structure, receptor-independent ion conductivity, and similar dimensions with pore diameters of 1~2 nm and outer diameters of 8~12 nm, which offer unique insights into the membrane disruption mechanism associated with amyloid toxicity. In addition, the local disorder of the membrane upon peptide insertion, together with the tilting of the lipid head, can cause bilayer thinning near the peptide contact region. All hIAPP channels modeled in this work align the hIAPP in parallel to the lipid bilayer via a “turn-to-tail” orientation. However, the hIAPP could also insert into the membrane via “tail-to-turn” orientation to form a channel which is expected to induce an opposite ion conductivity. Co-existence of both channels with different channel topologies could lead to different channel activities. We will address this issue in our on-going studies of different combination of two distinct hIAPP channels in the lipid bilayer, which is expected to provide a more complete picture for the activity of hIAPP channels associated with amyloid toxicity. We further emphasize that amyloid conformations are broadly polymorphic in water, and on the membrane. Not surprisingly, this polymorphic landscape is also reflected in channel morphologies in the membrane. We may expect a broad range of variants, including
channels forming from on-pathway and off-pathway intermediate oligomerization states. It is conceivable that intermediates can also insert into the membrane and at high concentration assemble into toxic channels.
CHAPTER VI
NON-SELECTIVE ION CHANNEL ACTIVITY OF POLYMORPHIC HUMAN ISLET AMYLOID POLYPEPTIDE (AMYLIN) CHANNELS

6.1. Introduction

Human islet amyloid polypeptide (hIAPP or amylin) is a 37-residue hormone peptide that is synthesized, stored, and secreted with insulin from the β-cells of pancreatic islets. Increasing evidence suggests that the interactions of hIAPP aggregates, particularly small and soluble oligomers, with cell membranes are at least partially responsible for the dysfunction and death of β-cells in type II diabetes. However, the exact mechanisms of hIAPP-oligomer-induced toxicity remain unclear, for example, how do hIAPP oligomers interact with cell membranes to induce cell toxicity and which types of oligomer conformations are involved in type II diabetes? Characterization of the conformations of hIAPP oligomers and their interactions with cell membranes at the atomic level still presents a daunting challenge.

Human IAPP and other amyloidogenic peptides have been hypothesized to induce membrane disruption by several mechanisms (i.e. receptor-dependent, ion channel,
activation of endogenous ion channels, membrane detergent-like, and membrane thinning/curvature models\textsuperscript{103}. Among these models, the ion channel hypothesis has long argued that toxicity is mediated by unregulated channel formation by amyloidogenic proteins, leading to ion dyshomeostasis that triggers amyloid-induced neuronal apoptosis\textsuperscript{199}. Unlike receptor-dependent mechanisms that depend on specific amyloid sequences, a number of structurally similar ion channels have been characterized by different amyloid peptides including hIAPP\textsuperscript{153}, A\textbeta\textsuperscript{144, 154-156}, and K3\textsuperscript{157, 158} using atomic force microscopy (AFM), electrophysiology, cell calcium imaging, and other techniques, indicating a common amyloid toxicity mechanism likely exists that is independent of the particular sequence. While channels formed from different amyloid peptides show many similarities and important differences. For example, A\textbeta channels exhibited Ca\textsuperscript{2+}-selective ion-permeable characteristics\textsuperscript{159-162}, while K3\textsuperscript{157} and hIAPP\textsuperscript{144, 153, 163} formed relatively nonselective, voltage independent, ion-permeable channels in lipid membranes. Common experimental tools (AFM, CD, FTIR, etc) provide low resolution information about channel structures and their relation to ion permeability and selectivity, while high resolution characterization (NMR and x-ray) of these heterogeneous and dynamic channels in cell membranes has proved to be extremely difficult. More importantly, recent studies have shown that amyloid channels bear striking resemblance in channel structure and activity to other ion channels formed by antimicrobial peptide and toxin proteins\textsuperscript{75}, further suggesting that a general principle may govern channel-induced membrane insertion/disruption by different peptides.

Recent molecular modeling and molecular dynamics (MD) studies of single
amloid channels in cell membranes by our and others have revealed that although all Aβ
154, 155, 170, 200, K3  157, 158, and hIAPP 12  channel models in lipid bilayers are highly
polymorphic in regards to channel structures and topologies, they also share some
common structural characteristics: (1) the channels have irregular shapes consisting of
several loosely contacting mobile subunits with a certain extent of β-sheet structures; (2)
the channels have similar inner diameters of 1-2 nm and outer diameters of 8-12 nm,
which has been verified by high resolution AFM 144; (3) the channels are assembled by
U-shaped-conformational peptides derived from their parent fibrillar structures; (4) the
U-shaped peptides adopt the same “turn-to-tail” orientation to form the channels, in
which the U-turn region orients towards the upper bilayer leaflet, while the
N-/C-terminus orients towards the lower bilayer leaflet. Computational studies provide
atomic details for illustrating the different ion conductivity and selectivity of particular
amloid channels, which could be attributed to the variability in the peptide sequence and
composition, side-chain packing and backbone conformation, and channel topology.
However, considering the rugged energy landscape of amyloid peptides upon interacting
with cell membrane, a “one-size-fit-all” of a common amyloid channel formed by
amyloid peptides with the same insertion orientation in the membrane may not be
sufficient and accurate enough to illustrate the mechanisms of amyloid channel-induced
toxicity and structural parameters affecting amyloid channels. It is very likely that
amyloid peptides could insert into the membrane with different orientations and
conformations to form heterogeneous channels in membranes.
We have recently simulated two types of single hIAPP channels in the DOPC bilayer: a CN\(_{p}\)NC channel with charged N-terminal β-strands facing a solvated pore and a NC\(_{p}\)CN model with C-terminal β-strands facing a solvated pore, with each type of channels with a varying number of hIAPP peptides (12-, 18-, 24- and 36-mer) all aligned to be parallel to the lipid bilayer via a “turn-to-tail” orientation\(^{12}\). MD simulation results demonstrated that (i) the 18-mer and 24-mer channels of CN\(_{p}\)NC and NC\(_{p}\)CN presented similar numbers of oligomeric units and similar overall dimensions to the channels observed by AFM\(^{144}\); (ii) all hIAPP single channels induced directional permeability of multiple ions across the bilayers from the lower to the upper leaflet; (iii) more importantly, potential-mean-force (PMF) profiles showed a similar energy barrier for a single hIAPP peptide to penetrate into the bilayer via either the tail or the turn orientation.

Overall, these results suggests the coexistence of “turn-to-tail” and “tail-to-turn” channels in the membrane and the relative populations of these channels may play an important role in determining overall ion conductivity and selectivity, which were not achieved in the early studies. Herein, we for the first time modeled and simulated a number of hIAPP “double channels”, two separate hIAPP channels in a single bilayer, in a DOPC bilayer. The double channels are composed of 18 mers by considering a complete combination of channel conformations (CN\(_{p}\)NC and NC\(_{p}\)CN) and orientations (parallel and antiparallel) of the form ↑\(_{N}\)↑\(_{N}\), ↑\(_{C}\)↑\(_{C}\), ↑\(_{N}\)↓\(_{N}\), ↑\(_{C}\)↓\(_{C}\), ↑\(_{N}\)↓\(_{C}\), ↑\(_{C}\)↓\(_{N}\), where ↑ stands for a “turn-to-tail” orientation and ↓ for a “tail-to-turn” orientation and N stands for a CN\(_{p}\)NC conformation and C for NC\(_{p}\)CN. We used the image facility of CHARMM to mimic double channels of ↑\(_{N}\)↑\(_{N}\), ↑\(_{C}\)↑\(_{C}\) using a single channel of either ↑\(_{N}\) or ↑\(_{C}\). We first demonstrated the
feasibility of atomistic modeling of double hIAPP ion channels and provided atomic explanation of non-ionic selectivity of hIAPP channels, due to the co-existence of “turn-to-tail” and “tail-to-turn” channels in the membrane. This work provides a more complete picture of membrane insertion and disruption by amyloid peptides.

6.2. Experimental Section

6.2.1. hIAPP Channel models

Initial hIAPP$_{1-37}$ monomer was extracted and averaged from 10 solid-state NMR-derived structures from Tycko’s lab. The monomeric hIAPP$_{1-37}$ had a U-bend structure with two antiparallel β-strands connected by a loop, i.e. β-strand (Lys1-Val17)-loop(His18-Leu27)-β-strand(Ser28-Tyr37). An intra-molecular disulfide bond was present between Cys2 and Cys7 at the N-termini. The N- and C-termini were blocked by NH$_3^+$ and COO$^-$ groups, respectively. Our previous MD simulations of hIAPP channels in the DOPC bilayer have demonstrated that 18-mer and 24-mer channels presented similar numbers of oligomeric units and overall dimensions to the channels observed by AFM. The bilayer did not support too small 12-mer channels or too large 36-mer hIAPP single channels. In this work, we proposed to use the single 18-mer hIAPP channel extracted from previous 60-ns MD simulations to construct double 18-mer channels by considering different combinations of (CNpNC and NCpCN) and orientations (parallel and antiparallel). The initial separation distance between two individual channels in the same lipid bilayer was set to at least 15 Å to ensure no interactions between them. The channel structures were then minimized with harmonic
position constraints for structural relaxation and optimization with the surrounding water molecules and lipids.

6.2.2. MD simulations

The resulting energy-minimized channel systems were subject to equilibrium and production runs by MD simulations. In the equilibrium stage, each channel system was gradually relaxed by performing a series of dynamic cycles, in which the harmonic restraints on peptides in the channels were gradually removed to optimize the peptide-lipid and peptide-water interactions. In the production stage, all simulations were performed using the NPAT (constant number of atoms, pressure, surface area, and temperature) ensemble at 300 K. The surface area in the xy plane was kept constant while allowing a volume change in the z direction. The van der Waals (vdW) interactions were calculated using a switch function with a twin-range cutoff at 12 and 14 Å. Long-range electrostatic interactions were calculated using the Particle Mesh Ewald (PME) method. The equations of motion were integrated using the velocity Verlet integrator with a step of 2 fs. Each hIAPP-lipid system was repeated twice using the same channel configuration, different lipid conformations randomly selected from the lipid library, different initial velocities for all atoms. All MD simulations were performed using the NAMD software with CHARMM27 force field. All MD simulations ran for 80 ns and MD trajectories were saved by every 2 ps for analysis. A summary of all simulation systems was listed in Table 6.1. 
Table 6.1. Simulation details of hIAPP double channels in the DOPC bilayer.

<table>
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<tr>
<th>Systems</th>
<th>Atom Number</th>
<th>Lipid Number (upper, lower)</th>
<th>Ion Number</th>
<th>Ion Strength (mM)</th>
<th>Time (ns) and runs</th>
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</table>

6.2.3. Population analysis

To quantitatively obtain the relative populations of the variant hIAPP channels, we extracted the channel structures from the last 20-ns MD simulations. A total of 3000 conformations (500 conformations for each double hIAPP channel) were used to construct the free energy landscapes of the channel conformers and to evaluate the conformer probabilities using in-house Monte Carlo (MC) simulations. Specifically, two conformers of i and j were randomly selected, and then compared using $e^{-(E_j - E_i)/kT}$, where $E_i$ and $E_j$ are the conformational energies of the respective conformers of i and j, $K$ is the Boltzmann constant, and $T$ is the absolute temperature of 300 K used here. If the Boltzmann factor value is larger than a random number, the move from conformer i to conformer j is allowed. After 1 million steps, the conformers visited for each channel system were counted. Finally, the relative probability of a channel system (n) was evaluated as: $P_n = N_n/N_{total}$, where $P_n$ is the population of the channel n, $N_n$ is the total number of conformations visited for the channel n, and $N_{total}$ is the total number of steps.
Considering the complex structural polymorphism of hIAPP ion channels, the populations of various channels are only indicative.

6.2.4. Materials

hIAPP and rIAPP (97% purity) was purchased from Anaspec (Fremont, CA). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine sodium salt (POPS) were purchased from Avanti Polar lipids Inc. (Alabaster, AL). 6-carboxyfluorescein was purchased from Sigma-Aldrich (St.Louis, MO). Lucigenin was purchased from Invitrogen (Eugene, OR).

6.2.5. Model membrane preparation

Large unilamellar vesicles (LUV) of POPC/POPS 7/3, were prepared from a chloroform solution of lipids in the desired ratio. The solution was gently dried under nitrogen flow and then placed under a high vacuum overnight to further evaporate any residual solvent. The dry lipid film was hydrated with a buffer solution containing 1 mM Lucigenin (10 mM phosphate buffer, 100 mM KNO$_3$, pH 7.4) to a final concentration of 10 mg/ml. The resulting solutions were extruded 23 times through a 100 nm polycarbonate Nucleopore membrane filter (Whatman) mounted on a mini-extruder in order to obtain LUVs with an average diameter of 100 nm. Removal of any non-encapsulated carboxyfluorescein or lucigenin was performed by running the extruded LUV solution through a Sephadex G50 gel exclusion column (Sigma-Aldrich) and collecting the first band detectable under UV light which contained the separated
6.2.6. Lucigenin assay

The presence of membrane disruption was detected by measuring decreasing in the fluorescence at 520 nm upon binding of Cl\(^-\) to encapsulated lucigenin or through the concentration dependent increase in carboxyfluorescein fluorescence from its release from LUVs upon membrane disruption. Samples were prepared by first diluting the dye-filled vesicles solution with buffer solution (10 mM phosphate buffer solution, 100 mM NaCl, pH 7.4) to a final concentration of 250 µM. Then, hIAPP was added to a final concentration of 5 µM. Experiments were carried out in Corning 96 well non-binding surface plates. Time traces were recorded using Biotek Synergy 2 plate reader using a 2 nm bandpass filters at 440 nm excitation and 520 nm emission at 25°C, shaking samples for 10 seconds before each read. The fraction of membrane disrupted was calculated by:

\[
\text{Fraction of membrane disrupted} = 1 - \left[\frac{(I - I_{100})}{(I_0 - I_{100})}\right]
\]

where I is the emission intensity of the sample, I\(_0\) is the emission intensity obtained in the absence of peptide (baseline control) and I\(_{100}\) is the emission intensity obtained after adding 1 µl of Triton X-100, a detergent which acted as a positive control to give 100% leakage.

6.3. Results and Discussion

Figure 6.1 shows the top-view of channel topologies by projecting the Cα atoms of hIAPP peptides on the bilayer surface averaged from the last 10-ns simulations. It can be seen that all hIAPP double channels lost their initial perfect circular shapes and
gradually formed 3-5 subunits, which loosely contacted with each other to retain the
pore-like structures. The channels kept open during the simulations with average
inner/outer diameters of 1.8/8.0 Å, which allow water and some ions to be readily diffuse
through the channels. The two channels were separated by at least 35 Å, indicating that
the two channels did not interact with each other to induce conformational changes. Such
hIAPP double channels exhibited similar sizes as other single amyloid channels
consisting of several loosely contacting mobile subunits with a certain degree of β-sheet
structure \(^{144, 163, 169, 203, 204}\) and exhibited similar irregularly-shaped morphologies. Visual
inspection of MD trajectories also showed that once the hIAPP channels were formed in
the DOPC bilayer, they remained open pore-like structures without disaggregation of
individual channels or exchange of hIAPP between the two channels during the
simulations.

To confirm the existence of open pores during the early phase of aggregation, we
measured the influx of negatively charged molecules into POPC/POPS (7/3) Large
Unilamellar Vesicles (LUVs) after incubation with hIAPP by using a lucigenin
fluorescence assays along with a comparison to previous results \(^{205}\) with the larger
negatively charged dye carboxyfluorescein as described in the Supporting Information.
Briefly, we prepared POPC/POPS LUV samples filled with either the Cl\(^-\) sensitive dye
Lucigenin or the concentration sensitive dye carboxyfluorescein.
Figure 6.1. Schematic presentation and MD snapshots of the six most likely double channels of hIAPP. Cα atoms of the channels are projected onto the x-y plane to illustrate overall channel topologies. The probability to find Cα atoms is calculated and colored in an increased order of red, orange, yellow, green, blue, and purple to represent subunit organization of the channels. Color codes in the schematic presentation are N-terminal strands (red) and C-terminal strands (blue).
Pore formation by hIAPP allows Cl\(^-\) to enter the previously inaccessible interior of the vesicle, where it can bind the dye and change its fluorescence. A significantly smaller increase is seen for carboxyfluorescein. The results are in agreement with the several aspects of the simulations. First, the fluorescence of lucigenin increases immediately after the addition of hIAPP. The fast response indicates Cl\(^-\) ions can penetrate into the LUVs almost immediately after the addition of hIAPP, well before amyloid formation begins after several hours.\(^{106}\) Immediate membrane disruption is consistent with the early phase of the two-step model of membrane disruption\(^ {206,207}\), in which membrane defects form before the membrane is totally disrupted by fiber growth on the membrane surface\(^ {106,208,209}\). Although we cannot confirm the existence of discrete pores as opposed to other types of non-specific membrane disruption, the results are consistent with pore formation preceding fiber formation, as has been shown to occur with A\(_{\beta}\)\(^ {1-40}\)\(^ {199}\). Second, the higher rate of permeability for the smaller Cl\(^-\) ion (stokes radius = 0.2 nm)\(^ {210}\) compared to the larger carboxyfluorescein molecule (stokes radius = 0.7 nm)\(^ {211}\) suggests the pore may be relatively in narrow in some places, consistent with previous simulations.\(^ {12}\)

To quantitatively examine whether the membrane environment supports channel structures and activity, the interaction of channel peptides with lipids and water molecules were respectively calculated by averaging over time and the number of peptides in the channels. Overall, all double channels had favorable interactions (negative value) with both lipids and water molecules, suggesting that the environment supports channel conformation. Specifically, the total interaction energies between peptides and
lipids for all channel systems were similar to each other (-130 to -122 kcal/mol), but the conformational energy of the CNpNC channels was relatively lower than that of the NCpCN channels, because the CNpNC channels had more favorable hydrophobic interactions between the hydrophobic C-terminal and the hydrophobic lipid tails. Different channel conformations (CNpNC vs. NCpCN) and orientations (parallel vs. antiparallel) also induced different contributions from the decomposed electrostatic and VDW energy. For the double channels with the same channel conformation, ↑N↑N and ↑N↓N channels had comparable VDW and electrostatic interaction energies (~50%), while ↑C↑C and ↑C↓C channels had more favorable electrostatic interaction energies (~62%) because the hydrophilic N-terminal residues (including positively charged Lys1 and Arg11) were packed against the zwitterionic heads of the lipids to enhance electrostatic interactions. In the heterogeneous double channels of ↑N↓C and ↑N↑C was in between ↑N↑N/↑N↓N systems and ↑C↑C/↑C↓C systems.

For peptide-water interactions, visual inspection of MD trajectories showed that all hIAPP double channels were well hydrated, including a water pore and a hydrated cavity inside the U-turns. Peptide-water interactions were much stronger than peptide-lipid interactions, suggesting that unbalanced interactions could be attributed to the channel breaking into several subunits. Further, the deuterium order parameter $S_{CD}$ was used to quantify the perturbation effects of hIAPP channels on the lipid orderings. $S_{CD}$ values of lipids for all channel systems were similar to each other, but slightly smaller than those in a pure DOPC bilayer, suggesting that the membrane-embedded hIAPP channels induce only minor local deformation and ordering of neighboring lipids.
The minor local deformation of the membrane seen in the simulations is similar to experimental results \(^{183, 212}\) obtained on an hIAPP variant (hIAPP\(_{1-19}\)) believed to form pores \(^{213}\), and is in contrast to the larger membrane disordering occurring during nonspecific membrane disruption by hIAPP \(^{214, 215}\).

Figure 6.2. Potential of mean force (PMF), \(\Delta G_{\text{PMF}}\), for Cl\(^-\) (black), Na\(^+\) (red), K\(^+\) (green), and Ca\(^{2+}\) (blue) as a function of the distance along the center axis of the hIAPP channels. \(\Delta G_{\text{PMF}}\) is calculated using \(-k_B T \ln(\rho_z/\rho_{\text{bulk}})\), where \(k_B\) is the Boltzmann constant, \(T\) is the simulation temperature, \(\rho_z\) is the ion density at the position \(z\) along the channel axis, and \(\rho_{\text{bulk}}\) is the ion density in the bulk region. PMF represents the relative binding probability of each ion with the channels.
Taken together, the simulation results indicate that different combinations of CNpNC and/or NCpCN double channels in a parallel or antiparallel packing can coexist in the lipid environment and either conformation or orientation is strongly preferred.

To characterize the ion binding selectivity of the channels, the PMF of each channel was calculated along the \( z \) axis of the channel interior to represent the relative free energy profile for each ion across the bilayer via pore permeation, using

\[
\Delta G_{PMF} = -k_B T \ln \left( \frac{\rho_z}{\rho_{bulk}} \right)
\]

where \( k_B \) is the Boltzman constant, \( \rho_z \) is the ion density at position \( z \) along the channel axis, and \( \rho_{bulk} \) is the ion density in the bulk phase (Figure 6.2). The channel systems contained three cations of \( \text{Ca}^{2+}, \text{Na}^+, \text{K}^+ \) at the same concentration (~35 mM) and an anion of \( \text{Cl}^- \). A negative (positive) PMF value indicates favorable (unfavorable) interactions of ions with the surrounding environment, which suggests a high (low) probability for ion binding to the bilayers. In all channels, the PMF curves of \( \text{Cl}^- \) displayed a similar asymmetrical and continuous S-shape along the channels. The horizontal S-shape PMF curves clearly presented an energy barrier of 1-2 kcal/mol for \( \text{Cl}^- \) ion transport through the channels in a preferential direction from the turn (Ser19 to Asn22) to the tail region (Lys1 and Tyr37), almost independent of channel orientations and conformations. For the CNpNC channels, Lys1 (-1.7 nm) and Arg11 (-0.7 nm) near the N-terminal tail formed a circular cluster which provides a strong attractive force to drive \( \text{Cl}^- \) into the interior of the solvated channels, while on the opposite side of the channels hydrophobic Phe15 and Val17 clusters (~1.8 nm) near the turn region created an energy barrier of 1~2 kJ/mol for preventing \( \text{Cl}^- \) from entry into the channels from this direction. Both effects would cause Cl- to preferentially enter the
CNpNC channels from the tail region. This trend remained in the NCpCN channels but was less pronounced. In the NCpCN channels, the amphiphilic C-terminal β-strands, rather than the positively charged N-terminal β-strands, formed the solvated pore, resulting in a decrease of attractive electrostatic interactions between Cl⁻ and Lys1/Arg11 residues. Accordingly, the minima around ±1 nm observed in the PMF curves of CNpNC channels are either greatly decreased or entirely absent in the PMF curves of the NCpCN channels (Figure 6.2). So, the coexistence of the CNpNC and NCpNC channels in a parallel or antiparallel orientation would enhance (reduce) the direction permeability of Cl⁻ ions. The three cations had distinctly different PMF profiles. The energy barriers for Ca²⁺ binding and transport were lower than those of K⁺ and Na⁺. Ca²⁺ ions were dominantly bound to both ends of the channels, as evidenced by two negative minima at ~±2.7 nm. This fact indicates that Ca²⁺ had favorable interactions with the zwitterionic phosphate heads of the lipids, as observed experimentally. Unlike Ca²⁺, which exhibits a high binding probability to bilayers, all channels possessed strong repulsive forces against the entry and binding of Na⁺ and K⁺ to the channels. Moreover, the preferential and dominant binding of Ca²⁺ to the DOPC bilayer also reduces the possibility for Na⁺ and K⁺ to bind to and cross the bilayer due to a Guoy-Chapman effect.

To investigate the net ion conductivity of hIAPP double channels, Figure 6.3 shows the number of ions across each of double channels as a function of time, where a positive value indicates ion transport from the tail to the turn, and a negative value indicates ion transport from the turn to the tail. Because of the low energy barrier for Cl-
transportation, all channels were highly selective and conductive for Cl\(^-\) over other cations.

Figure 6.3. Net ion conductivity of Cl\(^-\) (black), Na\(^+\) (red), K\(^+\) (green), and Ca\(^{2+}\) (blue) across the hIAPP double channels. A positive value indicates ion transport from the tail to the turn, and a negative value indicates ion transport from the turn to the tail.
Cl- conductivity curves, combined with visual inspection of MD trajectories, showed that the entering Cl- ions were hopping concertedely inside the channels in a pulse-like motion. Such hop-jump motion of Cl- ions in a confined space can be described by the continuous-time random walk model\textsuperscript{217}. More importantly, regardless of channel conformations (NCpCN or CNpNC), Cl- conductivity displayed directional permeability in a preferential direction, i.e. Cl- tended to conduct through the channels from the tail side to the turn side, as shown by positive conductivity values in the ↑ channel orientation and negative values in a ↓ orientation, consistent with the PMF results. Specifically, for ↑\textsubscript{NC}↓\textsubscript{N} or ↑\textsubscript{C}↓\textsubscript{C} systems, Cl\textsuperscript{-} exhibited opposite ion conductance in the two conformationally the same, but orientational opposite, channels although all Cl- ions transported from tail side to turn side. The same amount of Cl-ions permeated through the bilayer in each direction for the ↑\textsubscript{NC}/↓\textsubscript{NC} double channel (+12/-12), while only a slight excess of Cl- permeated to the turn side (+10/-13) for the ↑\textsubscript{C}/↓\textsubscript{C} channels, leading to almost “zero” selectivity and conductivity of Cl- for both types of homogenous double channels. For heterogeneous double channels of ↑\textsubscript{NC}↑\textsubscript{C} and ↑\textsubscript{NC}↓\textsubscript{C}, directional permeability of Cl- was also observed. In the ↑\textsubscript{NC}↑\textsubscript{C} system, Cl- exhibited positive conductance for both single channels, although the net conductance was small. In the ↑\textsubscript{NC}↓\textsubscript{C} system, opposite Cl- conductance through ↑\textsubscript{N} and ↓\textsubscript{C} channels was observed. Considering the relatively higher energy barrier for cation permeation observed in the PMF data, most of the double channels exhibited weak or negligible cation conductivities. Nevertheless, there still exists small possibility for the cations to across the channels via thermodynamic diffusion. This is particularly true for Ca\textsuperscript{2+}, since a large amount of Ca\textsuperscript{2+} ions were bound
to the zwitterionic lipid head region, thus increasing the channel conductance probability for this cation. Taken together, all double channels with an antiparallel packing (↓N↑C, ↑N↓N, ↑C↓C) directly exhibited non-selective ion conductivity, while the other parallel-packing double channels (↑N↑N, ↑C↑C, ↑N↑C) showed directional but weak ion conductivity, in agreement with our previous simulations of single hIAPP channels.

Considering the complex structural polymorphism of hIAPP ion channels, our proposed double-channel models and single-channel models are likely to represent only a very small percentage among the channel ensemble, however they cover the most likely membrane-supported channel organizations. We estimated the overall population for (1) six different double channels and (2) two individual channels with different orientations and conformations using an in-house Monte Carlo simulation to calculate conformational energies of the corresponding channels (3000 conformations), as reported in our previous work. For the single-channels, the ↑N model (71.5%) was much more populated than the ↑C model (28.5%) (Figure 6.4). ↑N had a more favorable hydrophobic match between hydrophobic C-terminal residues and interior lipids, while ↑C had the mismatch of cationic N-terminal residues with interior lipids. When individual channels assemble into double channels, the population distribution becomes much wider. Based on the populations and the relative stabilities, these double channel models can be classified into three categories: ↑N↓N occupied the highest population of 30.2%; ↑N↑N (13.1%), ↑N↑C(17.6%), ↑N↓C (19.4%), and ↑C↓C (15.8%) had comparable populations; ↑C↑C only represented 3.8% of the ensemble, but it does existed in varied polymorphic channels. Interestingly, the three double channels with opposite channel orientations (↑N↓C, ↑N↓N,
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Figure 6.4. Population of polymorphic hIAPP channels. The populations had been estimated via in-house Monte-Carlo simulations.

### 6.4 Conclusions

To conclude, we for the first time investigate the structure and activity of double hIAPP channels in the DOPC bilayer using MD simulations and experiments. Six different double hIAPP channels are modeled by considering different channel conformations (NCpCN and CNpNC) and channel orientations. The computational results support that hIAPP peptides can penetrate the lipid bilayer via the turn region or the N/C-terminal region with further self-assembly into “turn-to-tail” and “tail-to-turn” channels. The resultant channels fragment into three to five oligomeric units that are...
loosely associated, but remain open, channel-like structures in the bilayer, similar to other amyloid channels by Aβ and K3. The hIAPP channels exhibit relatively high ion binding selectivity and ion conductivity of Cl⁻ over cations. Although all cations exhibit relatively weak ion fluxes, poor ion conductivity of Na⁺ and K⁺ is due to the bare adsorption on the lipid bilayer while that of Ca²⁺ is due to strong binding affinity to phosphate groups in the lipid heads. A broad range of conformational states of hIAPP channels with comparable populations of these double channels result in non-ionic-selective channel conductance, consistent with experimental data. We expect that this work provides a more complete picture for the activity of amyloid channels associated with amyloid toxicity.
CHAPTER VII
ENGINEERING ANTIMICROBIAL PEPTIDES WITH IMPROVED
ANTIMICROBIAL AND HEMOLYTIC ACTIVITIES

7.1. Introduction

Widespread use of antibiotics and infiltration of antibiotics in the food chain have promoted the enormous and growing threat of bacterial pathogens, which have developed multidrug resistance (MDR) to almost all available antibiotics due to natural evolution, consequently causing many human infectious diseases such as urinary tract infection, tuberculosis, gastroenteritis, pneumonia, and wound infections\textsuperscript{218-220}. Traditional antimicrobial drugs mainly consist of antibiotics and antimicrobial peptides (AMPs). A large number of antibiotics has been contemplated and widely used as FDA-approved drugs such as Penicillins, Polymyxins, Quinolones, Tetracylines, and Sufonamides\textsuperscript{221, 222}. But, conventional antibiotics often suffer from the limited activity to kill both Gram-positive and Gram-negative pathogens and undesirable side effects. On the other hand, naturally occurring AMPs produced by mammals and plants offer many advantages as a next generation of “nature antibiotics”\textsuperscript{223-227}, including a broader spectrum activity to kill both Gram-positive and Gram-negative bacteria, fungi, and parasites (even
clinically common methicillin-resistant S. aureus and vancomycin-resistant Enterococcus), a lower toxicity to host eukaryotic cells, and a synergistic and benign effect with conventional antibiotics\textsuperscript{218, 228}. Meanwhile, many bacteria have also developed resistance to almost all available antibiotics and AMPs to some degrees via natural evolution\textsuperscript{220, 229, 230}. Undoubtedly, there is an urgent need for the development of new effective AMPs to fight against the accelerating MDR bacteria resistance by natural evolution.

Generally, AMPs display high diversity in size (8-50 residues), sequences (overall positive charge and high population of hydrophobic residues), and structures (α-helical, β-stranded, β-hairpin, and extended structures)\textsuperscript{6}. AMPs mainly achieve their functions to kill invading bacteria, fungi, or viruses via a non-receptor mediated mechanism of cell membrane disruption\textsuperscript{220, 231, 232}. Interactions between AMPs and lipid bilayers have been extensively studied by numerous methods including NMR\textsuperscript{233-237}, sum frequency generation (SFG) vibrational spectroscopy\textsuperscript{238, 239}, circular dichroism\textsuperscript{240-242}, membrane partition ability evaluation\textsuperscript{243-247}, and molecular dynamics\textsuperscript{248-252}. However, it still remains unclear fundamentally how AMPs interact with cell membrane to induce membrane disruption. Depending on intrinsic physicochemical properties of AMPs such as size, sequence, structure, and charge distribution and environmental properties such as AMP concentrations, bacteria types, and membrane compositions, AMPs act differently at the membrane to induce different membrane disruptions. AMPs can insert into the cell membranes to form “barrel-stave” or “toroidal” transmembrane pores. The barrel-stave and toroidal structures are fundamentally different: in the barrel-stave pore, AMPs align
vertically and parallel with respect to each other to form a circular pore, while in the toroidal pore, AMPs induce a local curvature of the lipid bilayer to form a highly curved pore. Apart from the transmembrane pore models, AMPs act as “detergents” to distract lipids from the membrane (“detergent model”) or intensively adsorb onto and completely/partially insert into the membrane to induce changes in membrane permeability and integrity (“carpet” model). Combination of these models have also been proposed\textsuperscript{17}. All of these models above could cause similar membrane disruption, leakage of cytoplasmic contents, and concomitant bacterial cell death\textsuperscript{232}.

Due to peptidic nature, most of natural AMPs suffer from poor bioavailability and poor proteolytic stability, which limits their therapeutic applications\textsuperscript{225}. It is often necessary to (i) explore a vast population of diverse chemical and biochemical sequences from other protein/peptide families to increase sequence diversity and (ii) to introduce un-natural, D-amino acids, or β-amino acids via point mutations and appropriate chemical modifications to improve proteolytic stability\textsuperscript{220,253}. The modified AMPs tend to enhance their interactions with cell membranes by facilitating themselves to attach on or insert into the membranes. Apart from bioavailability and stability issues of AMPs, cell selectivity is another major concern for rational design of effective AMPs. Strong membrane binding and insertion ability of AMPs to bacteria could also give rise to human red blood cells, resulting in lysis of human red blood cells via similar membrane-disruption mechanisms. Considering that the structural features common to many AMPs are the presence of cationic and hydrophobic residues\textsuperscript{254-258}, it is reasonable to speculate that cell selectivity of AMPs could be improved by tuning the composition,
position, and ratio of cationic and hydrophobic residues, i.e. balancing the cationic charge and hydrophobicity of AMPs is requisite to obtain nontoxic antimicrobial peptides, which are expected to kill bacteria efficiently while minimizing harm to human cells. Generally speaking, cationic residues of AMPs enhance the specificity of their binding to bacterial cell membranes relative to human cell membranes, because the former contain a greater amount of anionic lipids on the outer leaflet.

Due to the promising activity of AMPs as a substitute of traditional antibiotics, extensive studies have been conducted to screen and design potent AMP candidates using different high-throughput screening methods. These methods include virtual screening, phage-display library, solid-phase synthesis on arrays, and rapid luminescence-based assay for bacterial killing, which lead to the establishment of several AMP databases including Antimicrobial Peptides Database (APD) and Collection of Anti-Microbial Peptides (CAMP). However, most of peptides in the databases have the disadvantage of inconsistent activity data of either MIC or IC₅₀, and such discrepancy is very likely caused by different experimental conditions, which make more difficult to accurately establish the sequence-activity relationship of AMPs. It is thus desirable to use more reliable dataset as model systems to better understand the mechanism and of AMPs.

A bovine dodecapeptide variant (Bac2A, RLARIVVIRVAR-NH₂) is one of the smallest natural occurring antimicrobial peptides. Bac2A has modest activity against Gram-negative bacteria and higher activity against Gram-positive bacteria. Hilpert et al. performed a complete single-point substitution library of Bac2A (12×19=228) for determining the position importance of specific amino acids in Bac2A and the
antimicrobial activity of mutated sequences using a high-throughput cellulose synthesis method. Among 228 substitutions, 46 peptides (~20%) showed improved activity and 52 peptides (~22%) showed equivalent activity to the parent Bac2A peptide, as demonstrated by low IC$_{50}$ and MIC values. This complete substitution library of 12-amino-acid peptides based on Bac2A provides an excellent model system for computationally probing the sequence-structure-activity of AMPs and for rationally designing new AMPs with improved antibiotic activity.

The Bac2A-based peptide library is homogeneous, as all peptides were assayed in the same conditions and thus is of great value to test the sequence-activity relationship in this study. It is an extremely challenging task to study the interactions or insertion of the peptides into a membrane with sufficient temporal and spatial resolution experimentally. Conventional molecular dynamics (MD) simulations are also not expected to observe the spontaneous translocation of the peptides across the membrane due to limited time and length scales, unless advanced simulation techniques and nonphysical restraints are applied to accelerate the peptide penetration process. Here, we developed an integrated platform combining potential mean force molecular dynamics (PMF-MD) simulations and experimental tests to probe an interaction model of AMPs with a mixed anionic POPE/POPG bilayer with a ratio of POPE:POPG=3:1 and a neutral POPC bilayer. The mixed POPE/POPG bilayer mimics the bacterial cytoplasmic membrane, while the neutral POPC bilayer mimics the human red blood cell (hRBC) membrane. The partition of AMPs into different lipid bilayers produced different PMF profiles, which help to determine insertion free energy barriers and to predict both
antibacterial and hemolytic activities of AMPs. PMF-MD results indicated that antimicrobial activity is closely related to the trans-membrane ability, which can be used to evaluate the antimicrobial activity of AMPs. Moreover, some AMPs with high antimicrobial activity also showed low hemolytic activity to human red blood cell membrane. Throughout this straightforward and robust platform, peptides designed and selected by PMF-MD simulations displayed a favorable combination of effective antimicrobial activity and low hemolytic properties \textit{in vitro}. This work provides valuable insights into the mechanism of both activity and selectivity of AMPs underlying peptide-membrane interactions at atomic level. Moreover, the proposed platform, with potential application to high-throughput screening approaches, can also be generally applicable to evaluate the membrane activity of other membrane-associated peptides such as amyloid peptides and cell penetrating peptides.

7.2. Materials and methods

In general, spontaneous translocation of a peptide across the lipid bilayer is difficult to be captured by the conventional all-atom MD simulations due to limited time and length scales. Herein, we applied three advanced computational techniques to overcome this obstacle: First, coarse-grained AMP-membrane systems were constructed to reduce the size and freedom of the modeling systems. Second, the adaptive biasing force (ABF) method was applied to drag and facilitate the AMPs for membrane translocation. Third, the umbrella sampling technique was applied to enhance the sampling of conformations and to obtain the potential mean force upon AMP penetration into membrane. Moreover,
the results from the coarse-grained force field were validated by the united-atom force field, and further bacterial inhibition and hemolytic assays were performed to test computational results.

7.2.1. Coarse-grained AMPs and lipid bilayers

The coarse-grained (CG) simulations in this study were performed with the GROMACS simulation package 4.0.5\textsuperscript{265}. Generally, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are the major lipid components of bacterial membranes with a molar ratio of 3:1\textsuperscript{266}, while phosphatidylcholine (PC) is the major lipid component of normal cell membranes\textsuperscript{267}. To construct the bacterial- or red blood cell-mimic lipid bilayers, we randomly put 156 POPE/52 POPG lipids or 208 POPC lipids into a simulation box containing 1664 water molecules with a 8:1 ratio of water:lipids, and then we performed a series of self-assembly MD simulations allowing to spontaneously form a POPE/POPG or POPC lipid bilayer\textsuperscript{201}. Atomistic structures of the peptides were generated using MEMSAT program \textsuperscript{14,15}, followed by the conversion of atomic structure into CG structure using the CG protocol, parameters, and simulations as proposed by Marrink and co-workers\textsuperscript{268}.

7.2.2. Adaptive biasing force (ABF) method for membrane insertion

To construct the peptide-bilayer system, a single peptide was initially placed at 40 Å above the lipid bilayer with either C-terminal or N-terminal vertically orientating toward the bilayer. The peptide-bilayer system was then solvated in a polarizable water
box. Counter ions of NaCl were added to achieve an electrical neural system with ion strength of ~100 mM. The resulting systems were subject to 10,000 steps of steepest decent minimization with position constraints on heavy atoms of the peptide, followed by additional 10,000 steps of conjugate gradient minimization with position constraint of the peptide as well. To facilitate peptide penetration process across the bilayer, a harmonic potential with a force constant of 1000 kJ/(mol nm$^2$) was applied to the center of mass (COM) of the peptide to pull the peptide across the bilayer along the Z axis. Additionally, to validate the models, two different spring constant of 900 and 1100 kJ mol$^{-1}$ nm$^{-2}$ were used for selected AMPs. Each peptide-lipid system was repeated by two independent ABF simulations with different initial velocities for all atoms.

### 7.2.3. Umbrella sampling for PMF profiles

To determine the free energy profile during the peptide penetration process, PMFs were calculated using the umbrella sampling protocol$^{20}$ as a function of the distance between the COM of the peptides and the lipid bilayer. The initial separation distance between the center of mass of peptides and the center of the bilayer was 4 nm. An asymmetric distribution of sampling windows was used. The window spacing was set to 0.1 nm between Z=0-2 nm and 0.2 nm between Z=2-4 nm, resulting in 31 windows. Such window spacing assignment allows to obtain the more detailed energetic information of the peptide particularly within and near the lipid bilayer. Each window was simulated for 100 ns to achieve adequate sampling of configurations under the biasing potential of 1000 kJ mol$^{-1}$ nm$^{-2}$ applied to restrain the COM of the peptides at a required distance.
from the center of the bilayer. Thus, obtain of a single PMF (free energy) profile requires 31 simulations up to a total of 3.1 μs, covering an entire translocation process. To obtain the unbiased PMFs, the weighted histogram analysis method (WHAM) was used, with 200 bins and a tolerance of $10^{-5}$ kT, for window offsets. PMFs were converged with respect to the number of simulation windows and equilibration time, as established by block analysis (g_wham module in the GROMACS)\textsuperscript{269}, where PMF curves calculated over the two neighboring windows were overlapped by ~50%.

### 7.2.4. United-atom models

To further validate the accuracy of the coarse-grained models, atomistic MD simulations of wild type Bac2A sequence (both C-terminal and N-terminal insertion) were performed using GROMACS united-atom (ffgmx) force field\textsuperscript{265}. The atomistic structures of the AMP and lipid bilayer were reconstructed from coarse-grained structures using the structural conversion method as described by Rzepiela et al\textsuperscript{270}, followed by 20,000 steps of the steepest descent method for energy minimization. Then, atomic ABF-MD simulations with constant pressure, temperature, and number of particles (NPT ensemble) were performed. The temperature of 310K was kept constant using the Berendsen thermostat with a relaxation time of 1 ps\textsuperscript{271}. The pressure of the system was semi-isotropically coupled and maintained at 1 bar using the Berendsen algorithm with a time constant of 5 ps and a compressibility of $4.5\times10^{-5}$ bar\textsuperscript{-1}\textsuperscript{271}. The nonbonded potential energy functions were cut off and shifted at 12 Å, with forces smoothly decaying between 9 and 12 Å for van der Waals forces and throughout the whole interaction range.
for the treatment of electrostatic forces. The simulations were performed using a 2 fs integration time step. A 200 ns production MD simulation was performed for each window (total 31 windows), leading to a total ~6.2 μs of united-atom MD simulation. PMF profile was calculated using the same strategy as described in the coarse-grained models.

7.2.5. Bacteria growth inhibition assay

*P. aeruginosa* PAO1 was cultured in separate agar plates overnight at 37 °C in trypticase soy broth (TSB) (BD, Franklin Lakes, NJ). Several colonies of *P. aeruginosa* PAO1 were inoculated in 5 mL TSB medium (10 g/L) at 37 °C under a 280 rpm shaking for 18 h. The received bacteria suspension was then diluted with TSB medium to reach an optical density (OD) of 0.01 at 600 nm (OD600 = 1 ≈ 10⁹ cells/mL culture) as measured by a spectrophotometer (Smartpet 300, Biorad, CA). Peptide stock solution of 1 mg/mL was prepared by dissolving peptides in TSB medium, the sample was mixed by inversion to ensure peptides were completely dissolved in TSB medium. To examine the growth inhibition property of peptides, 2 mL prepared bacteria suspension with OD600 of 0.01 were placed into individual wells of a sterile 24-well plate in triplicates, followed by the addition of calculated volume of peptide solutions to each well for incubation at 37 °C for 24 h. After incubation, the bacteria suspensions were carefully removed from each well and the OD600 of suspensions was measured by a spectrophotometer.
7.2.6. Hemolysis assay

Samples were prepared within 3 hours of performing assay. Peptide stock solutions of 100 µg/mL were prepared by dissolving peptides in PBS (10 mM phosphate buffer, pH 7.4), the samples were mixed by inversion to ensure peptides were completely dissolved in PBS. To prepare Human Red Blood Cell (hRBC) solution, 0.4 mL fresh hRBC used as received (Cat. No: 991-09) from Lee Biosolutions, Inc. were rinsed two times with 7 mL PBS by centrifugation for 10 min at 2500 rpm, and the precipitates were resuspended in 4 mL PBS. To examine the hemolysis property of peptides, 1.6 mL freshly prepared peptide solutions were mixed with 0.4 mL freshly prepared hRBC solution by inversion, and were placed in water bath at 37 °C for 1 hour. 1.6 mL PBS buffer only and 1.6 mL Triton X-100 1% (w/v) was mixed with 0.4 mL hRBC solution as negative and positive control, respectively. After 1 hour incubation, all samples were centrifuged at 2500 rpm for 10 min. 1 mL supernatant was collected and the release of hemoglobin was monitored by measuring the absorbance of the supernatant at 540 nm by a UV-vis instrument at 25 °C with PBS buffer at same pH as the blank. The percent hemolysis was calculated as the following equation.

\[
%\text{hemolysis} = \frac{\text{absorbance}_{\text{sample}} - \text{absorbance}_{\text{negative}}}{\text{absorbance}_{\text{positive}}} \times 100
\]
7.3. Results and discussion

7.3.1. Model validation for the partition of AMPs into lipid bilayer

We have simulated a complete penetration process of AMP across a lipid bilayer and consequently obtained the free energy landscape along a penetration pathway using the MARTINI coarse-grained molecular dynamics (CG-MD)\textsuperscript{272, 273}. MARTINI CG-MD method is one of the most common CG methods for a wide range of simulations of proteins and membranes\textsuperscript{272, 274, 275}. It can reproduce the accurate dynamic behavior of lipid bilayers and can be used to explore interactions between peptides and membranes at much longer time and length scales, which are hardly accessed by all-atom MD simulations. Atomic structures of Bac2A and its mutants were not reported experimentally, but circular dichroism (CD) showed that Bac2A adopted a random coil structure in bulk solution\textsuperscript{276}. We used the MEMSAT program\textsuperscript{14, 15}, which is specifically developed for predicting the 3D structure of membrane proteins, to predict 3D atomic structures of Bac2A-based peptides, in which their secondary structures were predicted to be dominated by random coil structures.

In the Bac2A-based peptide library, we first selected wild type and 19 single-point mutation sequences of Bac2A, which covers a wide range of IC\textsubscript{50} values from 0.03 to 0.75 and mutation sites by 11 different amino acids, for computationally examine the correlation between experimentally determined antimicrobical activities (IC\textsubscript{50}) and computationally obtained peptide-membrane interactions (PMF). Additionally, to rigorously validate our simulation models, we also tested 4 double-substitution mutants and 4 octapeptides derived from the dodecapeptides for comparison. Thus, including wild
type sequence, a total of these 28 sequences were selected by their IC₅₀ values for testing the discrimination accuracy of MD simulations. Among 28 sequences, 17 sequences (10 single substitutions, 4 double substitutions, and 3 octapeptides) had lower IC₅₀ values than wild type (IC₅₀<0.13), 7 sequences (6 single substitutions and 1 octapeptide) had slightly higher IC₅₀ values than wild type (0.13<IC₅₀<0.5), and 3 sequences (3 single substitutions) had much higher IC₅₀ values than wild type (IC₅₀>0.5). As shown in Figure 7.1, for each sequence, we have also considered two distinct insertion pathways of the peptide by pulling either C- or N-terminal into the lipid bilayer separately, yielding two different PMF curves.

Figure 7.1. Schematic of membrane penetration of a given peptide with different C-/N-terminus insertion pathways. The partition of AMPs into different lipid bilayers produced different PMF profiles with three distinct MID, MAX, and MIN values, which help to determine insertion free energy barriers and to predict both antibacterial and hemolytic activities of AMPs.
PMF profiles were calculated by sampling peptide-lipid interactions as a function of
distance between the mass center of the peptide and the center of the lipid bilayer using
the adaptive biasing force (ABF) method. Properties of these simulated AMPs were
summarized in Table 7.1.

To validate our model systems and simulation protocols, we selected three
sequences of WT (IC$_{50}$=0.13), A11C (IC$_{50}$=0.04), and R12C (IC$_{50}$=0.75) to study their
penetration processes across the bilayer. For each sequence, three different external
forces of 900, 1000, and 1100 kJ/(mol nm$^2$) were applied to the center of mass of the
peptide for facilitating its penetration. For each peptide three independent MD
simulations showed similar PMF profiles with very minor differences, indicating that the
effect of external forces on peptide penetration is not significant. Only slight differences
of three PMF profiles occurred at the bilayer interface and the bilayer interior (z<1.0 nm),
which could be due to the local interplay between the external driving forces and the
elastic deformation of the bilayer. Unless stated otherwise, we performed all PMF-MD
simulations at an intermediate external force of 1000kJ/(mol nm$^2$).
Table 7.1. Summary of properties of AMP sequences with known IC$_{50}$.  

<table>
<thead>
<tr>
<th>IC$_{50}$</th>
<th>MAX</th>
<th>MIN</th>
<th>MID</th>
<th>Integration</th>
<th>E$^a$</th>
<th>F$^b$</th>
<th>Length</th>
<th>Scenario</th>
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<tr>
<td>V7K</td>
<td>0.03</td>
<td>8.55</td>
<td>-4.61</td>
<td>3.19</td>
<td>-0.70</td>
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<td>0.24</td>
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<tr>
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<td>-9.28</td>
<td>-11.7</td>
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<tr>
<td>A11C</td>
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<td>-4.64</td>
<td>-0.45</td>
<td>-0.92</td>
<td>5</td>
<td>0.98</td>
<td>12</td>
</tr>
<tr>
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<td>0.17</td>
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<td>-0.53</td>
<td>12</td>
</tr>
<tr>
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<td>1.78</td>
<td>4.17</td>
<td>5</td>
<td>0.28</td>
<td>12</td>
</tr>
<tr>
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<td>5.29</td>
<td>-3.53</td>
<td>-9.63</td>
<td>-13.18</td>
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<td>-0.13</td>
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<td>-0.26</td>
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<td>2.15</td>
<td>5</td>
<td>0.92</td>
<td>12</td>
</tr>
<tr>
<td>V10R$^a$</td>
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<td>5.49</td>
<td>4.57</td>
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<td>0.19</td>
<td>12</td>
</tr>
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<td>1.00</td>
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<tr>
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<td>7.37</td>
<td>5.32</td>
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</tr>
<tr>
<td>R9V</td>
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<td>3.36</td>
<td>4</td>
<td>1.50</td>
<td>12</td>
</tr>
</tbody>
</table>

$^a$ Simulation data from C-terminal penetration data

$^b$ E represented the net charge of the AMP sequences

$c$ H represented the mean values of hydrophobicity, which were represented as Grand Average Hydrophobicity Value (GRAVY), calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. Positive value of the score indicates hydrophobic and negative score indicates hydrophilic peptide$^{277}$. 

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To further test the reliability of the MARTINI force field, we presented and compared the PMF profiles for the transfer of Bac2A peptide across a POPE/POPG(3:1) bilayer using both MARTINI CG force field and united-atom GROMACS (ffgmx) force field. The effect of peptide insertion orientation on the PMF profiles were also examined using both force fields. It can be seen that regardless of different insertion pathways via either N-terminus or C-terminus orientation, PMF profiles obtained by the MARTINI force field were largely in good agreement with the corresponding PMF profiles obtained by the united-atom GROMACS force field, but the MARTINI force field produced more smooth PMF profiles than the GROMACS force field, probably due to the reduced frictional force in the MARTINI-CG model.

On the other hand, the insertion of Bac2A into the bilayer via different orientations indeed yielded different PMF profiles. As Bac2A started to approach to the lipid bilayer via N-terminus, Bac2A showed a favorable affinity to the bilayer surface with an energy well of -3.8 kcal/mol at 2 nm (compared with bulk water phase of z≥4 nm), but penetration of the peptide into the bilayer interior of ~1 nm was hindered by a modest energy barrier (7.1 kcal/mol), followed by a further decreased energy barrier along the penetration pathway towards the center of the bilayer. In contrast, with the C-terminal insertion, Bac2A exhibited similar small affinity (-3.2 kcal/mol) to the bilayer surface, but encountered a significant resistance at the entry and further penetration. Comparison of PMF profiles with different insertion pathways suggests that Bac2A prefers to bind to the water/lipid interface, but the translocation of this peptide into the bilayer interior is much less energetically favorable and encounters a greater barrier. This phenomenon
becomes even more pronounced for peptide insertion via C-terminus, suggesting that N-terminal insertion of Bac2A is more energetically favorable, if such membrane insertion does occur. PMF data are reasonably consistent with experimental observation that Bac2A had modest antimicrobial activity with IC$_{50}$ of 0.13. It should be noted that despite the convergence of the herein calculated PMF profiles, free energy profile is still very difficult to be estimated accurately along a simple reaction coordinate (i.e. distance between the mass center of peptide and the center of the bilayer).

7.3.2. PMFs of antimicrobial dodecapeptides with single substitutions

We first designed 19 single-point mutants to investigate the complete penetration process of the mutants toward the center of a mixed POPE/POPG (zwitterionic/anionic) lipid bilayer as model eukaryotic or prokaryotic cell membranes using the MARTINI CG-MD simulations. The distance-dependent PMF was calculated to determine the trans-membrane capability of the peptides, which will be used to correlate with or complement to experimental IC$_{50}$ values. As shown in Figure 7.2, by varying the mutated sequences of Bac2A and different insertion orientations, different mutants crossed the lipid bilayer differently. To better quantify the transmembrane activity, for a given N- or C-terminal insertion, we classified the mutants into two groups based on their IC$_{50}$: active mutants (IC$_{50}$<0.13, Figure 7.2a1) and inactive mutants (IC$_{50}$>0.13, Figure 7.2a2). In Figure 7.2a1, when 10 active mutants inserted into the lipid bilayer via N-terminus, the mutants displayed a favorable energy well of -7.1~-2.8 kcal/mol at the water/lipid interface (~2 nm) similar to wild type. However, as the mutants further penetrated into
the lipid bilayer \((z=0.5-1.0\ \text{nm})\), 8 out of 10 active mutants (except A11H and A3W) displayed relative small energy barriers of 2.2~8.6 kcal/mol, while A11H and A3W displayed larger energy barriers of 15.2~21.8 kcal/mol. When the mutants were inserted into the lipid bilayer via C-terminus, the mutants also favored to be adsorbed at the bilayer interface. Although all of active mutants had the relatively lower energy barriers than wild type, 6 mutants still suffered from high energy barriers (>12 kcal/mol), which prevented them from membrane insertion, while the remaining of 4 mutants (including A11H and A3W) displayed relative small energy barriers of 3.5~9.9 kcal/mol at \(z=0.5-1.0\ \text{nm}\).

Thus, taken PMFs together, all active mutants, either by N-/C-terminal insertion or both, displayed -7.1~2.8 kcal/mol at the water/lipid interface and 2.2~9.9 kcal/mol at \(z=0.5-1.0\ \text{nm}\). As these barriers were crossed, however, the translocation of active mutants toward the bilayer center seems to be favored (-9.3~3.2 kcal/mol at \(z=0\ \text{nm}\)).
Figure 7.2. PMF profiles for transfer of selected AMPs with identified IC\textsubscript{50} across the POPE/POPG bilayer from bulk water phase, including (a1-a2) single substitution dodecapeptides, (b) multiple substitution dodecapeptides, and (c) multiple substitution octapeptides.
In Figure 7.2a2, when all 9 inactive mutants inserted into the lipid bilayer via either N-terminus or C-terminus, the mutants displayed a favorable energy well of -6.3~–2.3 kcal/mol at the water/lipid interface (~2 nm) similar to wild type and active mutants. However, as the mutants further penetrated into the lipid bilayer (z=0.5-1.0 nm), the inactive mutants encountered the higher energy barriers of 6.7~–15.3 kcal/mol. Moreover, being different from the active mutants, even these barriers were crossed, however, the translocation of the inactive mutants toward the bilayer center seems to be forbidden due to the high energy barriers of 5.5 to 11.2 kcal/mol at z=0. Taken together, comparison of all PMF profiles of the mutants with different insertion pathways reveals several important findings: (1) most mutants display a preferential orientation (N-terminus or C-terminus) for membrane insertion; (2) all mutants tend to adsorb on the lipid surface similar to wild type; (3) only active mutants display strong translocation ability across the membrane. If penetration occurs (in the case of 10 active mutants), 2 mutants prefer to penetrate into the bilayer via C-terminus, 6 mutants via N-terminus, and 2 mutants via either C-terminus or N-terminus, indicating a close relationship between predicted transmembrane ability and IC$_{50}$, and (4) even single substitution can alter the energy landscape for peptide penetration into the lipid membrane, resulting in the change of antimicrobial activity.

7.3.3. PMFs of antimicrobial dodecapeptides with multiple substitutions

Many studies have shown that positively charged residues (Lys and Arg) and aromatic residues (Trp, Phe, and Tyr) play an important role in modulating the membrane penetration process through electrostatic and/or hydrophobic interactions. Herein, we
further selected 4 mutants (sub2= RLRIVVIRVR, sub3= RRWIVVIRVR, sub5=RRWKIVVIRWR, and sub6=RWWKIVVIRWR) with multiple-site mutations by Lys, Arg, and/or Trp for examining how these negatively charged and aromatic residue substitutions affect the transmembrane activity and associated energy landscapes. Experimentally, these four mutants have demonstrated their improved activities (IC$_{50}$=0.04-0.08) as compared with Bac2A (IC$_{50}$=0.13). Consistently, PMF profiles (Figure 7.2b) showed that for both N- and C-terminal insertion, 3 mutants (sub2, sub5, and sub6) displayed the lower energy wells at the lipid interface and the lower energy barriers at 1 nm than wild type, suggesting that these 3 mutants improve their membrane adsorption and membrane insertion capacity compared with wild type. Additionally, when inserting all mutants via C-terminal, energy barriers were greatly reduced by up to 18 kcal/mol. Substitutions of Lys and Arg in multiple sites of Bac2A appear to facilitate electrostatic interaction between the positively charged side chains and the negatively charged phosphate groups of the bilayer, promoting initial anchor and adsorption of peptide on the bilayer surface. Trp substitutions could interact strongly with hydrophobic tails of lipids for further membrane insertion. Additionally, Trp substitutions can also enhance peptide aggregation in solution and on the bilayer surface via π-π interactions, which facilitate conformational change of peptides and increase surface concentration of peptides for membrane disruption. As compared with single substitution, the transmembrane activity of the mutants by multiple substitutions was improved or equivalent to (sub3) the wild type.
7.3.4. PMFs of shorter antimicrobial octapeptides

The shorter AMPs are less expensive and thus more broadly studied. Herein, we truncated Bac2A at 4-11 positions to obtain an octapeptide (i.e. fragment of Bac2A_{4-11}), which was further mutated by Lys, Arg, and/or Trp at different positions to obtain 4 octapeptides (Bac8a=KI\textsubscript{1}W\textsubscript{2}VIR\textsubscript{3}WR, Bac8b=RI\textsubscript{4}W\textsubscript{5}VIR\textsubscript{6}WR, Bac8c=RI\textsubscript{7}W\textsubscript{8}VI\textsubscript{9}WR, and Bac8d=RR\textsubscript{10}W\textsubscript{11}VI\textsubscript{12}WR). Experimentally, as compared to wild type (IC\textsubscript{50}=0.13), three mutants (Bac8a, Bac8b, and Bac8c) had showed their improved activities (IC\textsubscript{50}=0.05-0.06), while one mutant (Bac8d) displayed poor activities (IC\textsubscript{50}=0.39). PMF profiles (Figure 7.2c) showed that three mutants (Bac8a, Bac8b, and Bac8c) displayed relative lower energy well at the lipid interface regardless of N-/C-terminus insertion. Energy barriers of all mutants remained at a similar level to that of wild type for N-terminal insertion, but were greatly reduced for C-terminal insertion. In line with single/double-substitution results, octapeptide and multiple-substitution dodecapeptides had similar energy well (-9.1~-7.1/-14.5~-3.5 kcal/mol) at the water/lipid interface (~2 nm), which was lower than single-substitution dodecapeptides (-7.1~-2.8 kcal/mol). This suggests that the appropriate design of multiple-substitution mutants with different sequence lengths will be more effectively improve their membrane binding/penetrating activities than single-substitution mutants.
7.3.5. Correlation between computational transmembrane ability and experimental antimicrobial activity

All of PMF curves we tested for total 28 sequences displayed similar horizon “S-like” shape (Figure 7.2), with three unique values: a minimum value (MIN) at \( z=\sim 2.0 \) nm (i.e. at the water/bilayer interface), a maximum value (MAX) at \( z=\sim 1.0 \) nm (i.e. at the half depth of a top leaflet), and a middle value (MID) at \( z=0 \) nm (i.e. at the center of the lipid bilayer). The area under the PMF curve was also integrated from \( z=0 \) nm to \( z=4 \) nm to represent a total energy dissipation upon membrane insertion. The MIN value of <0 (compared to the water phase) presents a favorable location for peptides to be adsorbed at the water/bilayer interface, where nonspecific electrostatic interactions between positively charged peptides and anionic lipids could facilitate initial membrane adsorption. MIN values of all AMPs were smaller than 0 and the average MIN value was -6.1 kcal/mol, indicating that all of 28 sequences bearing a net charge of >+1e displayed certain binding affinity to negatively charged POPE:POPG (3:1) membrane. MAX values of all sequences were larger than 0 except sub6, indicating that a common energy barrier exists for peptide penetration. It should be noted that the bilayer interior is a less preferred location for peptide to stay, but should not be fully excluded, particularly when the transmembrane energy barrier (MAX value) is not too high and the MID value is close to or less than 0. This implies that once the energy barrier is crossed, further penetration of the peptide toward the center of the bilayer will follow a downhill pathway with the least-resistance of free energy. MID and integration values showed a wide range
of free energy landscapes from -12.2~11.2 kcal/mol and from -32.1~ 9.9 kcal/mol, respectively.

Based on the MAX, MIN, and MID values in the PMF profiles (Figure 7.2 and Table 7.1), different membrane insertion scenarios and different preferred peptide locations can be identified by mapping out peptide-lipid energy landscapes along peptide insertion pathways: (1) when MIN<0, MAX<12, and MID<0, 9 mutants seem to have two favorable positions in the lipid bilayer: one at the bilayer surface with dominant propensity and the other inside the hydrophobic core of the lipid bilayer with minor propensity; (2) when MIN<0, MAX<12 kcal/mol, and 0<MID<5 kcal/mol, 13 mutants prefer to stay at the water/bilayer interface or partially insert into the transmembrane position, similar to the first scenario; (3) when MIN<0, MAX> 12 kcal/mol or MIN<0, MID> 5 kcal/mol, 6 mutants only show strong preference to stay at the water/bilayer interface. It is very unlikely for peptides to cross high transmembrane energy barrier for membrane insertion; and (4) when MIN>0, mutants should prefer to stay in the water phase, and in our work, no mutants had MIN>0 due to strong electrostatic interactions. We classified 28 sequences into these four membrane-insertion scenarios in Table 7.1.

To quantitatively correlate the MIN, MAX, MID, and integration values with the IC\textsubscript{50} values for all 28 sequences, four stepwise regression models were used to analyze the importance and relevance between four PMF values and IC\textsubscript{50}. Using multiple regression models also help to improve the robustness of the analysis. As shown in Figure 7.3, using IC\textsubscript{50}=0.35 as a threshold value, four regression models enabled to
separate all dataset into a cluster region (IC\textsubscript{50}<0.35) and a scattered region (IC\textsubscript{50}>0.35), as fitted by two linear relationships, respectively.

Figure 7.3. Regression analysis of the correlation between the MAX, MID, MIN, and integration-area values of the PMFs (transmembrane ability) and IC\textsubscript{50} values (antimicrobial activity) for selected AMPs.

The cluster data showed a visible correlation for all four models. The best data fitting was observed for the MAX-IC\textsubscript{50} correlation (R\textsuperscript{2}=0.52), suggesting that the transmembrane energy barrier is critical for antimicrobial activity. The MIN-IC\textsubscript{50} data showed the weakest correlation (R\textsuperscript{2}=0.17), probably because most of peptides tested in
this study displayed membrane binding ability, almost independent of their sequences. R² values were 0.36 for the MID-IC₅₀ data and 0.27 for the Integration-IC₅₀ data, respectively. However, we should point out that the scatter data in the plots indicate that transmembrane ability is likely not the sole determinant of antimicrobial activity. Considering that concentration-dependent membrane disruption mechanisms (i.e. fore-forming models and carpet model), as well as the complex nature of erythrocyte cell membranes containing glycoproteins and glycolipids, are not considered by our PMF-MD simulations. Moreover, in this study, only single peptide was considered for membrane insertion, while multiple copies of the peptides across the lipid bilayers were not simulated to study the concentration effects on membrane deformations via membrane fusion or pore formation. If multiple peptides are involved in membrane penetration, the energy barrier for membrane insertion is likely to be reduced because the peptides are likely aggregate and adsorb at the membrane surface to enhance their interactions with membrane. But, the PMF profile of a single peptide upon membrane penetration could still serve as a good indicator to discriminate membrane insertion ability of the peptides.

7.3.6. Design of AMPs with double substitutions

Based on the discrimination ability of IC₅₀ as reported by Hilpert et al. and our PMF data, we summarized the favorable amino acid substitutions at various sites in Table 7.2. It can be seen clearly that (1) Arg, Trp, and Cys substitutions are often preferred to the original residues and (2) some positions of 2, 3, 7, and 11 are particularly rich for
substitutions, while the other positions of 4, 8, 9, 10, and 12 have no or less preference for substitutions.

Table 7.2. Favorable single amino acid substitution at different positions of Bac2A (optimized from ref. 264).

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Considering that a complete mutation test requires $20^{12} \approx 4.1 \times 10^{15}$ sequences, which is an infeasible task to be examined by the PMF-MD simulations. Here, based on Table 7.2, we selected two important and representative residues of Arg and Trp to guide our design of new AMPs with double substitutions by Arg, Trp, or combined. We first
designed a total of 40 double-substitution mutants at different favorable sites including 10 R/R, 15 W/W, and 15 R/W. We then examined the transmembrane activity of these designed mutants using the PMF-MD simulations (Figure 7.4). With W/W mutations, regardless of insertion orientation, all W/W mutants displayed similar PMF profiles with pronounced minima (-10.9~4.8 kcal/mol, compared to the water phase) at the bilayer surface (~2 nm) as compared with Bac2A, suggesting that all W/W mutants have strong preference staying at the bilayer surface. 13 out of 15 W/W mutants displayed relative low or moderate energy barriers (3.1 to 8.9 kcal/mol) inside the bilayer of ~1 nm. This implies that there also exists certain possibility for W/W mutants to partially insert into the bilayer, although the center of the bilayer was a less preferred location, but not fully excluded due to relatively small transmembrane barriers. All R/R mutants featured small minima at the interfacial position (~3.8 to -0.9 kcal/mol) similar to Bac2A, but most of them encountered large transmembrane energy barriers (9.1 to 17.2 kcal/mol) for membrane insertion except for 2R11R, 3R11R and 7R11R. Mixed R/W mutants behaved in between R/R and W/W mutants. All 15 R/W mutants showed the lower energy minima in PMF profiles than Bac2A, and this indicates that all R/W mutants had strong preference for the water/bilayer position. 9 out of 15 W/W mutants displayed relative low or moderate energy barriers (5.2 to 11.1 kcal/mol) inside the bilayer of ~1 nm.
Figure 7.4. PMF profiles for transfer of the designed double-substitution mutants (W/W, R/R, R/W, and E/E) of Bac2A across the POPE/POPG bilayer from bulk water phase. Mutants presented by solid lines (dash lines) have a relative strong (weak) trans-membrane ability compared with wild type.
Meanwhile, all R/W mutants disfavored to penetrate into the bilayer via N-terminal, instead of C-terminal compared with Bac2A. Besides the favorable double mutations of WW/RR/RW, we also designed a 9E10E mutation as a negative control. As expected, the presence of two anionic Glu residues resulted in strong repulsive interactions with anionic bilayer, showing much higher values of MIN/AX/MIN values than wild type (Figure 7.4d) and thus indicating a poor membrane penetrating ability.

Besides the W/W, R/R, and R/W double mutations, we also selectively examined the effect of double mutations of other hydrophobic (H), polar (P), and charge (C) residues on the transmembrane activity and associated energy landscapes using the PMF-MD simulations. A total of 10,000 new double-mutation sequences were generated using the mutation table (Table 7.2). Then, we applied the support vector machine (SVM) method to select 17 sequences with high potent antimicrobial activity of ≥ 0.98 and 4 sequences with low potent antimicrobial activity of < 0.6. A total of 21 sequences were classified into three categories: 10 H/H, 6 H/C, and 5 P/X double-substitution mutants, where X represented H, C or P (Figure 7.5). 8 of 10 H/H mutation sequences showed strong membrane penetration potential (Figure 7.5a, solid line), as indicated by the smaller MIN (-8.9 to -3.0 kcal/mol), MAX (-3.7 to 6.0 kcal/mol), and MID (-12.8 to 1.9 kcal/mol) values.
Figure 7.5. PMF profiles for transfer of the designed double-substitution mutants (H/H, H/C, and P/X, where H=hydrophobic, P=polar, and C=charge residue) of Bac2A across the POPE/POPG bilayer from bulk water phase. Mutants presented by solid lines (dash lines) have a relative strong (weak) trans-membrane ability compared with wild type.
The results were consistent with the W/W mutations, suggesting that hydrophobic substitutions can improve the antimicrobial activity of Bac2A. H/C mutation showed similar PMF trends to R/W mutation. 5 out of 6 H/C mutants had the lower energy minima than Bac2A and 3 H/C mutants had the lower or equivalent maxima to Bac2A (Figure 7.5b). With polar substitutions, all 5 P/X sequences showed higher or similar MAX/MID values compared with wild type (Figure 7.5c). It seems that introduction of polar residues hardly improve the membrane penetration ability.

It is generally accepted that hydrophobic aromatic and cationic residues are key residues that contribute to the antimicrobial activity of amphipathic AMPs. In our double-substitution designs, PMF profiles suggest that W/W mutations assist peptides for membrane adsorption and membrane insertion. Increased membrane association ability could be at least partially explained by the enhanced hydrophobic interactions with lipids and entropy effects for water repulsion around the hydrophobic residues. Surprisingly, the quite opposite behavior was observed for most of Arg substitutions at various sites, showing weaker membrane association activity for most of mutants. PMF data did not clearly reveal which position can promote or reduce membrane association activity. Arg substitution at the same position in different sequences often leads to different PMF patterns. Bac2A is an amphipathic peptide carrying a net charge of +4 and 4 Arg residues. Introduction of two additional Arg residues at various positions appears not to greatly promote membrane adsorption via electrostatic attraction between positively charged Arg residues and negatively charged phosphate groups in the POPE/POPG lipids. Instead, imbalance of peptide composition between charge and hydrophobic
residues would reduce hydrophobic interactions and thus prevent the membrane penetration by the R/R mutants. Most of mixed R/W and H/C mutants preferred to be adsorbed on the bilayer surface, similar to Bac2A. Taken together, based on the correlation between IC$_{50}$ and PMF as described above, 40 of the 61 designed mutants were improved or equivalent to Bac2A, indicating that the mechanism of action of the AMPs can be readily modulated by some targeted double substitutions.

7.3.7. Screening of AMPs with low hemolytic activity

Transmembrane ability of the AMPs plays a dual role in antimicrobial and hemolytic activity against both bacterial cells and human blood red cells, respectively. It is critical for improving the selectivity of designed AMPs with improved antibacterial activity against bacterial cells, but reduced hemolytic activity for human red blood cells (hRBCs). The composition of bacterial membrane and hRBC membrane is dramatically different, where the former mainly contain anionic lipids while the latter consists of neutral lipids. Since melittin is well known to induce the lysis of both bacterial and mammalian cells, it is used as a characteristic control to validate the PMF profiles against experimental observation. It can be seen clearly that melittin adopted favorable transmembrane and interfacial locations from 0.5 to 2 nm (i.e. PMF$\leq$0 kcal/mol) in both lipid bilayers, suggesting strong cell selectivity of mammalian cell membrane and thus strong toxicity and hemolytic effect.
Figure 7.6. PMF profiles for transfer of 14 dodecapeptides with IC50 values < 0.13 from the Hilpert’s dataset across both POPE/POPG bilayer (dash lines) and POPC bilayer (solid lines), resulting in (a) 10 mutants with poor cell selectivity and (b) 4 mutants with high cell selectivity. N-terminal (red color line) and C-terminal (blue color line) insertion pathways across both lipid bilayers are also presented for comparison.

To obtain AMPs with high cell selectivity, we selected 14 dodecapeptides with IC50 values < 0.13 from the Hilpert’s dataset (Figure 7.6) and 25 dodecapeptides with potential antibacterial activity from our PMF data (Figure 7.7) for probing their hemolytic activity.
Figure 7.7. PMF profiles for transfer of 25 computationally designed dodecapeptides across both POPE/POPG bilayer (dash lines) and POPC bilayer (solid lines), resulting in (a) 17 mutants with poor cell selectivity and (b) 8 mutants with high cell selectivity. N-terminal (red color line) and C-terminal (blue color line) insertion pathways across both lipid bilayers are also presented for comparison.
We performed CG-MD simulations to compare their PMF profiles across the neutral POPC bilayer (main component of hBRC membrane) with those across the anionic POPE/POPG (3:1) bilayer (main component of bacterial cell membrane). A total of selected 39 Bac2A variants and wild type sequence have already demonstrated their antimicrobial activity computationally and experimentally as described above. In Figure 7.6 and Figure 7.7, regardless of the lipid bilayers, all PMF profiles displayed similar shapes with three distinct MIN, MAX, and MID values, so we used the previous PMF analysis based on MAX, MIN, and MID values to evaluate the transmembrane ability of the peptides across the POPC bilayer, which will be correlated with their hemolytic activities. Specifically, we identify the AMPs with high cell selectivity (i.e. strong transmembrane ability for POPE/POPG bilayer vs. weak transmembrane ability for POPC bilayer) using the following criteria:

(i) Using a MAX value of 12 kcal/mol as a threshold, if the MAX values of POPC is > 12 kcal/mol and the MAX values of POPE/POPG is < 12 kcal/mol, this indicates that the peptide is more difficult to penetrate into POPC (low toxic to hBRC) than POPE/POPG (high toxic to bacterial cell), suggesting a high cell selectivity.

(ii) If both POPC and POPE/POPG display the lower MAX values of <12 kcal/mol & MID_{hRBC}>5kcal/mol (unfavorable penetration process for hRBC-mimic bilayer) & Δ(MID_{hRBC}-MID_{bacterial})>5 kcal/mol (membrane discrimination ability), the peptide has a high cell selectivity.

(iii) Otherwise, the peptide is identified as a poor selectivity in other situations.
With the criteria, among 14 mutants selected from the Hilpert’s dataset, 4 mutants (A3R, A11H, V7Q, and V7K) displayed high cell selectivity. A3R, V7Q, and A11H encountered much higher resistance to penetrate into the POPC bilayer than the POPE/POPG bilayer, as evidenced by large MAX values of >12kcal/mol (Figure 7.6b). V7K displayed large membrane discrimination ability as evidenced by $\text{MID}_{\text{hRBC}} > 5\text{kcal/mol}$ and $\Delta(\text{MID}_{\text{hRBC}} - \text{MID}_{\text{bacterial}}) > 5\text{kcal/mol}$, although V7K is likely to penetrate into both lipid bilayers (Figure 7.6b). The remaining 10 peptides and wild type showed similar PMF profiles as they traveled through both POPE/POPG bilayer and POPC bilayer (Figure 7.6a). This indicates that the peptides have similar transmembrane ability for both lipid bilayers and thus have similar antimicrobial and hemolytic activities (i.e. poor cell selectivity). Following similar line, among 25 computationally designed mutants (Figure 7.7), 5 mutants (3W5W, 5W10W, 1W10W, 2W5R, and 7R11R) displayed good discrimination ability between POPC and POPE/POPG bilayers, while 3 mutants (2R6W, 3R6W, and 2R11R) are likely lack of potential transmembrane ability to cross the POPC bilayer relative to the POPE/POPG (Figure 7.7b). This suggests that these 8 peptides are likely to be potentially harmful to bacterial cells, but not hBRCs because of their different membrane permeability. The other 17 mutants showed similar transmembrane ability to both POPE/POPG and POPC bilayers and thus cannot differentiate both bilayers effectively (Figure 7.7a). Moreover, comparison of the PMF profiles of the same peptide transporting through different lipid bilayers in Figure 7.6 and Figure 7.7 revealed some interesting similarities for peptide transmembrane behaviors across different lipid bilayers. First, no matter how these peptides approach to both
bilayers via either N-terminus or C-terminus, they all feature energy minima at the water/bilayer interface position ($z=2$ nm) and present energy barriers at the bilayer interior ($z=1$ nm) to prevent peptide penetration. This indicates that all peptides possess the membrane adsorption ability, which cannot be easily disturbed by substitutions. Additionally, consistent with previous results, all peptides showed a preferred insertion orientation, and the peptides take the least-resistant pathway via either N- or C-terminus for membrane insertion, depending on peptide sequence/composition, lipid composition, and interplay peptide-lipid interactions.

7.3.8. Experimental tests for computationally designed AMPs

To validate our design principles based on the PMF-IC$_{50}$ relationship, we selected 10 peptides (2 peptides from Hilpert’s dataset including wild type$^{264}$, 5 peptides from designed double mutation sequences with potential high therapeutic index, 1 peptide with low therapeutic index, and 2 peptides with no antibacterial activity as negative control) for testing their antimicrobial and hemolytic activities using bacteria growth inhibition assay and hemolysis assay, as reported in Figure 7.8 and Table 7.3.
Wild type and sub3 were used as positive control, while 9E10E and 5R7R as negative controls. We first tested the antimicrobial activity of the peptides against P. aeruginosa under different peptide concentrations of 5, 10, 20, 50, and 80 μg/ml. As shown in Figure 7.8a, different peptides displayed different concentration-dependent antimicrobial activity, apparently reflecting the substitution effect. 9E10E and 5R7R peptides displayed poor antimicrobial activity, which was independent of peptide concentrations, as evidenced by almost unchanged OD600 of 2.1 that was comparable to untreated bacterial cells. Consistent with the prediction by our double-substitution simulations described above, 9E10E and 5R7R peptides showed relative high free energy resistance upon membrane insertion (Figure 7.4b and 7.4d).
Table 7.3. Property summary of selected dodecapeptides for experimental tests.

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<td>-3.10</td>
<td>1.3</td>
<td>4.4</td>
<td>+5</td>
<td>0.30</td>
</tr>
<tr>
<td>VAR-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3W5W</td>
<td>N/A</td>
<td>-0.92</td>
<td>7.64</td>
<td>8.56</td>
<td>+5</td>
<td>0.24</td>
</tr>
</tbody>
</table>

<sup>a</sup> MID values from bacterial membrane penetration PMFs.

<sup>b</sup> MID values from hRBC membrane penetration PMFs.

<sup>c</sup> Therapeutic index = MID<sub>hRBC</sub>-MID<sub>Bac</sub>

<sup>d</sup> E represented the net charge of the AMP sequences

<sup>e</sup> H represented the mean values of hydrophobicity, which were represented as Grand Average Hydrophobicity Value (GRAVY), calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. Positive value of the score indicates hydrophobic and negative score indicates hydrophilic peptide<sup>277</sup>.
Substitution of neutral hydrophobic residues with two positively charged residues appears to imbalance the cooperative electrostatic and hydrophobic interactions, and compromise membrane activity and antimicrobial activity. Conversely, other 7 peptides (sub3, 7R11R, 3R6W, 2R6W, 2W5R, 2W3W, and 3W5W) displayed the enhanced antimicrobial activity as the concentration of the peptides increased. Among them, 3R6W and 2R6W with combined substitutions of positively charge residue of Lys and aromatic residue of Trp at a particular position 6, were the most active peptides against P. aeruginosa at 50 and 80 μg/ml. The sub3, 7R11R, 2W5R, 2W3W, and 3W5W showed modest antimicrobial activity to inhibit bacterial growth at the highest concentration of 80 μg/ml.

As a measure of biocompatibility, we further reported the hemolytic activity of the 8 peptides (except 9E10E and 5R7R) to hRBCs using a fixed peptide concentration of 50 μg/ml. In Figure 7.8b, all of 8 peptides tested can be classified into three groups based on hemolysis (%): (1) 7R11R, 3R6W, 2W5R, and 3W5W exhibited substantial low hemolytic activity of ≤10%; (2) WT, 2R6W, and 2W3W showed moderate hemolytic activity between 10%-50%; and (3) sub3 had a severe hemolytic toxicity of ~55%. Comparison of antimicrobial and hemolytic results reveals that 3R6W and 2R6W with balanced substitution of charged and hydrophobic residues can be tuned to achieve the best antimicrobial activity while minimizing red blood cell lysis. 2W3W, 3W5W, and 7R11R containing either two aromatic Trp substitutions or two positively charged Arg substitutions show a general moderate activity for both bacterial cells and hRBCs. To better understand the membrane activity of these peptides, PMF data also confirm that
3R6W and 2R6W are more surface resistant to neutral POPC bilayer than to anionic POPE/POPG bilayer. The R/W substitutions suggest the enhanced affinity of the peptides to anionic lipids, but not neutral lipids, resulting in enhanced antimicrobial and reduced hemolytic activity. Additionally, because the MIC values often depend on the assay conditions and bacterial strains, and because many laboratories have developed their own protocols, it would be impractical to compare the MIC values for different compounds reported in the literature.

![Figure 7.9](image.png)

Figure 7.9. Mapping of the predicted cell selectivity (i.e. therapeutic index) of the AMPs onto the two reaction coordinates of hydrophobicity and net charge of the peptides.

To better determine possible design principles, Figure 7.9 shows the mapping of the predicted cell selectivity (i.e. therapeutic index) onto the two reaction coordinates of hydrophobicity and net charge of the 8 peptides from Figure 7.8b. Cell selectivity from high to low values is presented by a red-green-blue color scale. Peptides located in the red region bear both high hydrophobicity and net charges, indicating that simultaneous
increase of hydrophobicity and charge characters together will improve the cell selectivity of designs. However, sole increase of either hydrophobicity or charge achieves limited cell selectivity in the green region. Low hydrophobicity and low charge in the blue region should be avoided. From these results, we should also note that the membrane activity of the AMPs cannot be simply explained by a particular sequence or sequence motif, rather than more complex interplay among intrinsic physicochemical properties of peptides (i.e. size, composition, overall charge, hydrophobicity, secondary structure) and lipids (i.e. composition) and external conditions (peptide concentrations, assay conditions, and bacterial strains). Use of coarse-grained MARTINI model to accurately describe the peptide-membrane interactions still remains a great challenge under intense development for better representing the structure and dynamics of water, peptides, and lipids. Actually, the potential structures and phenomena captured in the CG simulations can serve as a starting point and be refined in fully atomistic models and simulations. In addition, only single-peptide penetration was evaluated rather than synergistic multiple-peptide penetration. Even with these limitations, our PMF data obtained from the translocation of single peptide across different lipid bilayers still can help to distinguish between different possible models of membrane adsorption and insertion.

7.4. Conclusions

In this work, we presented a comprehensive MD investigation of the membrane insertion of AMPs into anionic POPE/POPG and neutral POPC bilayers, which mimic the main components of bacterial and hRBC membranes. Specifically, we used a hybrid
simulation method combining the coarse-grained MARTINI model, adaptive biasing force (ABF) method, and the umbrella sampling technique with MD simulations to probe a complete translocation process of Bac2A and its variants across different lipid bilayers. Potential mean force (PMF) associated with the peptide translocation process was calculated to directly determine the free energy barrier required to transfer the peptides from the bulk water phase to the water-membrane interface to the bilayer interior. A number of computationally designed sequences were identified and predicted with enhanced membrane association ability to bacterial cell membrane while retaining membrane resistance to hRBC membrane, which are well correlated with antimicrobial and hemolytic activities, respectively. Further bacterial inhibition and hRBC assays confirm that the most active mutants (3R6W and 2R6W) can indeed kill bacteria efficiently while minimizing harm to human cells. The balanced substitution of charged residue (Arg) and hydrophobic residue (Trp) in Bac2A can be tuned to achieve the best antimicrobial activity while minimizing red blood cell lysis via the cooperative interactions. Comparison of computational PMF data with experimental cell assay results reveals a statistical correlation between transmembrane activity of peptides across different lipid bilayers and antimicrobial and hemolytic activities with reasonable accuracy. This work provides a platform for screening and design of new effective AMPs with good cell selectivity.
CHAPTER VIII
MOLECULAR DYNAMICS SIMULATIONS OF LOW-ORDERED ALZHEIMER
B-AMYLOID OLIGOMERS FROM DIMER TO HEXAMER ON SELF-ASSEMBLED
MONOLAYERS

8.1. Introduction
Alzheimer’s disease (AD) is the most common neurodegenerative disease and the
most common cause of dementia. The key pathological hallmark of AD is the presence of
extracellular senile plaques formed by amyloid-β (Aβ) peptides and intracellular
neurofibrillary tangles formed by tau proteins in the patient’s brain. Traditionally, insoluble Aβ fibrils found in senile plaques are believed to be responsible for neurological degeneration, but increasing evidence has implicated that small soluble Aβ oligomers formed during the early stage of aggregation are major toxic species, which interact with cellular membranes to disrupt membrane permeability and integrity leading to neuronal cell death. Additional evidence also shows that the clearance of Aβ fibrils or senile plaques in AD patients do not prevent progressive neurodegeneration. Other amyloidogenic peptides/proteins such as prion protein associated with prion disease, α-synuclein with Parkinson’s disease, and amylin with diabetes type II have also
experienced similar peptide misfolding and aggregation process to form amyloids and to induce membrane disruption.

Aβ peptide, a 39-42-residue long, amphipathic molecule with a hydrophilic N-terminal domain (residues 1-28) and a membrane-inserted hydrophobic C-terminal domain (residues 29-40/42), is normally cleaved from the transmembrane amyloid precursor protein (APP) and then released into the extracellular fluid during normal cellular metabolism. Upon cleavage, some of Aβ peptides are rapidly cleared from brain to blood, while the others tend to misfold and self-assemble into different heterogeneous species of lower-ordered oligomers from dimers to hexamers, Aβ-derived diffusible ligands (ADDL), globulomers, protofilaments, and fibrils. Many in vitro studies have shown that the presence of both artificial (mica, graphite, self-assembled monolayer, and nanoparticle) or biological (lipid monolayer/bilayers and cell membrane) surfaces can greatly accelerate both structural transition of Aβ from random coil to β-sheet structure and subsequent formation of heterogeneous Aβ species. However, the atomic-level details about how Aβ peptides interact with these surfaces are still limited, presumably due to the intrinsic aggregation nature of Aβ peptides and the complex topological and chemical nature of a given surface, especially for cell membranes involving membrane proteins, cholesterol, ganglioside, and lipid bilayer. Self-assembled monolayer (SAM) is an ideal model surface with a well-defined atomic topology and surface chemistry at a nanoscale, which is very suitable for the study of protein adsorption behavior at interface. Although the SAM cannot directly mimic the biological properties of cell membrane, the study of interactions of Aβ oligomers with
model SAMs can help to derive underlying driving forces that control structural dynamics, binding affinity, and aggregation of Aβ on the surface, which are very important for deciphering the biological role of Aβ oligomers in Aβ aggregation and toxicity on cell membranes.

Our recent experimental study of Aβ adsorption on different SAMs terminated with CH₃, OH, COOH, and NH₂ groups using circular dichroism (CD), atomic force microscopy (AFM), and surface plasmon resonance (SPR) has revealed that all of these SAMs greatly accelerate the formation of β-sheets and amyloid fibrils, but the kinetics of Aβ aggregation on these SAMs is dramatically different from each other strongly depending on Aβ seeds in solution and SAM surface chemistry. Complement to macroscopic views from experiments, molecular dynamics (MD) simulation provides atomic-resolution details (i.e. structure, dynamics, and driving force) of Aβ assembly on the SAM surfaces, which is currently lacking. Our previous MD simulations of Aβ monomer and pentamer on the SAMs provide the first glimpse on the preferred conformation of Aβ monomer and the preferred orientation of Aβ pentamer on the SAMs. Aβ monomer prefers to adopt α-helical or unstructured conformation, rather than β-hairpin structure, when bound to CH₃-SAM, OH-SAM, COOH-SAM, and NH₂-SAM, while Aβ pentamer is likely to be adsorbed on the CH₃-SAM and OH-SAM via the hydrophobic C-terminal residues.

Building on our previous experimental and simulation results of Aβ adsorption on different SAMs, here we aimed to fill a gap by studying the surface interaction with Aβ oligomers from dimer to hexamer using MD simulations and to obtain a more complete
picture for Aβ adsorption, aggregation, and conformational changes upon adsorption on different SAMs at the early stage of aggregation. Fifteen Aβ-SAM simulation systems were reported to study small Aβ oligomers from dimer to hexamer to interact with CH₃-SAM, OH-SAM, and COOH-SAM, respectively. The structure and dynamics of Aβ oligomers in bulk solution was also studied for comparison. Simulation results showed that unlike the weak adsorption of Aβ oligomers on the OH-SAM, the CH₃-SAM and the COOH-SAM induced the strong adsorption of Aβ oligomers with distinct orientation preference, depending on competitive interactions between Aβ-SAM and SAM-water interactions. Combining with experimental results, all of SAM surfaces exhibited a strong seeding effect for Aβ polymerization. Stable Aβ trimer with enriched β-structure appeared to be the smallest seeding nucleus on the SAMs. This work provides parallel insights into interactions between Aβs and cell membrane at a molecular level.

8.2. Materials and Methods

8.2.1. Molecular models of Aβ₁₇-₄₂ oligomers.

Initial monomer coordinate of Aβ₁₇-₄₂ peptide was taken and averaged from 10 NMR structures (PDB code 2BEG), derived from quenched hydrogen/deuterium-exchange NMR. Residues 1–16 were omitted due to disorder and unavailable crystal structure. The Aβ₁₇-₄₂ monomer consisted of two β-strands, β₁ (residues V₁₇-S₂₆) and β₂ (residues I₃₁-A₄₂), connected by a U-bend turn spanning four residues N₂₇-A₃₀. D₂₃ and K₂₈ formed an intrastrand salt bridge to stabilize this U-bend conformation. Since the experimental crystal structures of Aβ oligomers are
currently not available, we used Aβ monomer with a “β-strand-turn-β-strand” motif as a building block to construct a series of Aβ oligomers from dimer to hexamer by stacking Aβ monomers on top of each other in a parallel and register way with an initial peptide-peptide separation distance of ~4.7 Å, corresponding to the experimental data. The N- and C- termini of Aβ monomer were blocked by COO⁻ and NH₃⁺ groups, respectively, yielding a total net charge of -1e.

8.2.2. SAM surfaces

Three types of SAMs on Au(111), including hydrophobic methyl S(CH₂)₁₁CH₃ (CH₃-SAM), carboxyl-terminated S(CH₂)₁₁COOH (COOH-SAM), and hydrophilic alcohol S(CH₂)₁₁OH (OH-SAM), were constructed in a xy plane. The force field parameters for the SAMs were adopted from polarizable ether parameters in the CHARMM top_all35_ethers.rtf file developed by Vorobyov and co-workers. This force field used in our prior and others’ work can well produce the experimental tilt angle of the alkanethiol chains by ~30° and water contact angles. Each SAM surface has a (\(\sqrt{3} \times \sqrt{3} \))R30° lattice structure with a sulfur-sulfur spacing of 4.995 Å, consistent with electronic diffraction and scanning tunneling microscopy studies of alkanethiol monolayers on Au(111). The size of each SAM surface was constructed to be large enough to accommodate Aβ oligomers with a minimal distance of 10 Å between any edge of SAM and Aβ. Although three SAM surfaces are neutral in electrostatics, the COOH-SAM has relative stronger partial charge distribution in the headgroups than the CH₃- and OH-SAMs.
8.2.3. Aβ-SAM systems

To avoid the biased orientation of Aβ relative to the SAM, any Aβ oligomer were initially placed at ~6.0 Å above the SAMs with both hydrophobic C-terminal and hydrophilic N-terminal β-strands in contacts with the SAMs, i.e. fibril axis of Aβ oligomers was parallel to the surface normal (i.e. z axis). There were approximately two water layers between Aβ oligomers and the SAM surface, allowing to directly simulate a pre-adsorbed state of Aβ and to reduce the diffusional time of Aβ oligomer approaching to the SAM. Each Aβ-SAM system was solvated by a TIP3P water box with a margin of at least 30 Å from top edge of the water box to any Aβ atoms. The solvated systems were then neutralized by adding Cl⁻ and Na⁺ ions to achieve ~190 mM ionic strength, which is close to a physiological value. The resulting systems were energy minimized to remove any bad contacts using the conjugate gradient method for 5000 steps with the peptide backbones constrained and the sulfur atoms fixed, followed by additional 5000 steps with only sulfur atoms fixed.

8.2.4. MD protocol

All MD simulations were performed using the NAMD software package with CHARMM27 force field. The MD simulations were performed in the canonical ensemble (NVT, T=300K). Temperature was controlled by the Langevin thermostat method with a damping coefficient of 1 ps⁻¹. All sulfur atoms were fixed to maintain the \((\sqrt{3} \times \sqrt{3})R30^\circ\) lattice structure of SAMs and all covalent bonds involving hydrogen were constrained using the RATTLE method during simulations. 2 fs timestep was used.
in the velocity Verlet integration. Van der Waals (VDW) interactions were calculated by the switch function with a twin-range cutoff at 12 and 14 Å, while long-range electrostatic interactions were calculated using the force-shifted method with a 14 Å cutoff. Each system was run twice with the same initial coordinates but different initial velocities to verify statistical accuracy. Structures were saved every 2 ps for analysis. To ensure the systems reach equilibrium, the first 30 ns data are discarded from analyses (referred as equilibrium runs), while the last 20 ns data are sampled for equilibrium properties (referred as production runs). Analyses were performed using tools within the CHARMM, VMD\textsuperscript{46}, and in-house FORTRAN, Tcl, Python codes.

8.3. Results and Discussion

8.3.1. Adsorption behavior of Aβ oligomers on SAM surfaces

Analysis of the distance and orientation of Aβ oligomers relative to the SAM surfaces, coupled with MD trajectories, can provide quantitative details of Aβ adsorption/desorption behavior on the SAM surfaces. Figure 8.1 shows final MD snapshots of Aβ oligomers from dimer to hexamer on three SAMs at 50-ns, in comparison to Aβ oligomers in bulk water (i.e. totally 20 systems).
Figure 8.1. Final MD snapshots of Aβ oligomers interacting with various SAMs at 50 ns, in comparison with corresponding oligomers in bulk solution. Water and ions are omitted for clarity. Color codes: N-terminal residues (green), turn region residues (blue), and C-terminal residues (red). Headgroups of CH3-SAM, COOH-SAM, and OH-SAM are colored by blue, red, and pink, respectively.

<table>
<thead>
<tr>
<th>Bulk solution</th>
<th>CH3-SAM</th>
<th>COOH-SAM</th>
<th>OH-SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimer</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
</tr>
<tr>
<td>Trimer</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>Tetramer</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>Pentamer</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
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<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 8.2. Relative position and orientation of Aβ oligomers with respect to the SAMs. (a) Separation distance between the topmost heavy atoms of the SAMs and the bottom peptide of Aβ oligomers and (b) Orientation of Aβ oligomers on the SAMs, where 0° represents no orientation change of Aβ oligomers with respect to the SAMs.

Figure 8.2a shows the separation distance along the z axis between the mass center of the bottom Aβ peptide and the topmost heavy atoms of the SAMs (the topmost heavy atoms of the SAMs are defined as carbon atoms of CH$_3$, oxygen atoms of OH, and two oxygen atoms of COOH), while Figure 8.2b shows the orientation change of Aβ oligomers on the SAMs, as measured by averaged angles between the vectors normal to two β-strand vectors (one β-strand vector is defined by its direction from Phe20 to Val24 at the N-terminus and the other from Ile32 to Val36 at the C-terminus) and z axis. 0°
indicates no orientation change of Aβ oligomers relative to its initial orientation on the SAM. Specifically, the adsorption/desorption behaviors of Aβ oligomers on each of SAMs are described below.

8.3.2. CH$_3$-SAM

Visual inspection of 50-ns MD trajectories showed that all Aβ oligomers were able to tightly bind to the CH$_3$-SAM surface (Figure 8.1), as indicated by small separation distances of ~6.5 Å between the mass center of bottom peptide of Aβ dimer, trimer, or tetramer and the SAM surface (Figure 8.2a) and ~6 Å between the mass center of...
C-terminal β-sheet residues of Aβ pentamer or hexamer and the SAM surface (data not shown). Upon Aβ adsorption, dimer largely lost its initial β-strand-loop-β-strand structure, leading to partially folded random conformation at the N-terminus and a large root-mean-square deviation (rmsd) of 5.5 Å (Figure 8.3b). Unlike Aβ dimer, other Aβ oligomers were able to well preserve the β-strand-loop-β-strand conformation with a large population of β-structure, which could be attributed to the enhanced stabilization of intermolecular hydrogen bonds between adjacent Aβ peptides.

Trimer and tetramer tended to orientate their hydrophobic C-terminal β-sheets toward the SAM surface by 10° and 20°, respectively (Figure 8.2b). The orientation change became even more pronounced for Aβ pentamer and hexamer. After 20 ns, Aβ pentamer and hexamer almost completely changed their initial orientations by ~60° from whole bottom peptide to the C-terminal β-sheet plane facing the SAM, and the new orientations remained stable for the rest of MD simulations (Figure 8.2b). During the reorientation process, C-terminal residues of Ile31, Ile32, Leu34, Met35, and Val36 formed intensive contacts with the CH$_3$-SAM, while N-terminal residues completely lost all contacts with the CH$_3$-SAM. These results suggest that the extent of the reorientation of Aβ oligomers on the CH$_3$-SAM depends on the size of Aβ oligomers, because all initial orientations of Aβ oligomers were set up to be unbiased, i.e. the only bottom Aβ peptide initially contacted with the SAM and no direct contacts between N-/C-terminal β-sheet and the SAM (i.e. both N/C-terminal β-sheet planes are parallel to surface normal).
Table 8.1. Simulation systems of Aβ oligomers on SAMs and in bulk solution. All data are averaged from the last 20-ns MD simulations.

<table>
<thead>
<tr>
<th>System</th>
<th>Adsorption State</th>
<th>RMSD (Å)</th>
<th>Rg (Å)</th>
<th>Number of Atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CH₃-SAM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimer</td>
<td>adsorbed</td>
<td>5.5 ± 0.2</td>
<td>11.8 ± 0.1</td>
<td>29378</td>
</tr>
<tr>
<td>Trimer</td>
<td>adsorbed</td>
<td>4.4 ± 0.3</td>
<td>13.3 ± 0.1</td>
<td>29874</td>
</tr>
<tr>
<td>Tetramer</td>
<td>adsorbed</td>
<td>5.6 ± 0.3</td>
<td>14.6 ± 0.1</td>
<td>30465</td>
</tr>
<tr>
<td>Pentamer</td>
<td>reoriented</td>
<td>5.0 ± 0.2</td>
<td>14.2 ± 0.1</td>
<td>27466</td>
</tr>
<tr>
<td>Hexamer</td>
<td>reoriented</td>
<td>4.7 ± 0.3</td>
<td>16.1 ± 0.1</td>
<td>42985</td>
</tr>
<tr>
<td><strong>COOH-SAM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimer</td>
<td>adsorbed</td>
<td>6.3 ± 0.4</td>
<td>11.6 ± 0.2</td>
<td>29399</td>
</tr>
<tr>
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<td>adsorbed</td>
<td>3.9 ± 0.2</td>
<td>12.8 ± 0.2</td>
<td>29789</td>
</tr>
<tr>
<td>Tetramer</td>
<td>adsorbed</td>
<td>2.6 ± 0.2</td>
<td>14 ± 0.1</td>
<td>30233</td>
</tr>
<tr>
<td>Pentamer</td>
<td>adsorbed</td>
<td>3.3 ± 0.2</td>
<td>14.2 ± 0.1</td>
<td>31715</td>
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<tr>
<td>Hexamer</td>
<td>adsorbed</td>
<td>3.1 ± 0.3</td>
<td>15 ± 0.1</td>
<td>37349</td>
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<td><strong>OH-SAM</strong></td>
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<td></td>
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<tr>
<td>Dimer</td>
<td>desorbed</td>
<td>6.8 ± 0.4</td>
<td>11.2 ± 0.2</td>
<td>29056</td>
</tr>
<tr>
<td>Trimer</td>
<td>adsorbed</td>
<td>5.0 ± 0.3</td>
<td>12.8 ± 0.2</td>
<td>23608</td>
</tr>
<tr>
<td>Tetramer</td>
<td>reoriented</td>
<td>5.2 ± 0.6</td>
<td>13.7 ± 0.2</td>
<td>24526</td>
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<tr>
<td>Pentamer</td>
<td>reoriented</td>
<td>3.4 ± 0.3</td>
<td>14.5 ± 0.1</td>
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<tr>
<td>Hexamer</td>
<td>reoriented</td>
<td>5.1 ± 0.2</td>
<td>15.4 ± 0.1</td>
<td>42421</td>
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<tr>
<td><strong>Bulk solution</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Dimer</td>
<td>-</td>
<td>6.3 ± 0.2</td>
<td>10.9 ± 0.1</td>
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<tr>
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<td>12.8 ± 0.1</td>
<td>18028</td>
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<td>13.5 ± 0.1</td>
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</tr>
<tr>
<td>Hexamer</td>
<td>-</td>
<td>4.7 ± 0.3</td>
<td>15.3 ± 0.1</td>
<td>24566</td>
</tr>
</tbody>
</table>

As the size of Aβ oligomers increases, the increased hydrophobic interactions between hydrophobic C-terminal β-sheet and the CH₃-SAM will outcompete the interactions between hydrophilic N-terminal β-sheet and the CH₃-SAM, driving Aβ
oligomers (particular pentamer and hexamer) to adopt more energetically favorable orientation via the C-terminal β-sheet in contacts with the SAM surface.

COOH-SAM. Similar to the conformational change of Aβ oligomers on the CH₃-SAM, Aβ dimer experienced larger structural deviation than other oligomers on the COOH-SAM (Table 7.1). Interestingly, MD trajectories revealed that all of Aβ oligomers were tightly bound to the COOH-SAM with limited movement, as indicated by small self-diffusion coefficient (D) of 0.228-0.362×10⁻⁶ cm²/s (Table 7.2). The initial separation distance and orientation of Aβ oligomers relative to the COOH-SAM remained almost unchanged over 50-ns MD simulations (Figure 8.2). The contacts between the Aβ oligomers and the COOH-SAM surface barely break once formed. The strong adsorption of Aβ oligomers on the COOH-SAM reflects strong electrostatic interactions between Aβ and COOH-SAM, especially three charged residues of Glu22, Asp23, and Lys28 and two charged terminus interacted strongly with the SAM surface. No preferential Aβ orientation on the COOH-SAM was observed and this can be attributed to the balanced competitive interactions between two N-/C-terminal β-sheets and the COOH-SAM. Despite strong interactions with the COOH-SAM, except for Aβ dimer, primary structural integrity and secondary β-sheet content of other Aβ oligomers were virtually unchanged.

8.3.3. OH-SAM

Unlike strong adsorption of Aβ oligomers on the CH₃-SAM by hydrophobic interactions and on the COOH-SAM by electrostatic interactions, Aβ oligomers displayed
relatively large mobility on the OH-SAM. Aβ oligomers had much larger self-diffusion coefficients on the OH-SAM than on CH₃-SAM and COOH-SAM (Table 7.2).

Table 8.2. Self-diffusion coefficients of interfacial water molecules (D_{water}) a and Aβ oligomers (D_{Aβ}) on the SAMs. All data are averaged using the last 20-ns MD simulations.

<table>
<thead>
<tr>
<th>System</th>
<th>D_{water} (×10^{-5} cm²/s)</th>
<th>D_{Aβ} (×10^{-6} cm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃-SAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimer</td>
<td>1.73</td>
<td>0.321</td>
</tr>
<tr>
<td>Trimer</td>
<td>1.30</td>
<td>0.357</td>
</tr>
<tr>
<td>Tetramer</td>
<td>1.25</td>
<td>0.350</td>
</tr>
<tr>
<td>Pentamer</td>
<td>1.18</td>
<td>0.326</td>
</tr>
<tr>
<td>Hexamer</td>
<td>1.26</td>
<td>0.341</td>
</tr>
<tr>
<td>COOH-SAM</td>
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<tr>
<td>Dimer</td>
<td>0.61</td>
<td>0.362</td>
</tr>
<tr>
<td>Trimer</td>
<td>0.66</td>
<td>0.233</td>
</tr>
<tr>
<td>Tetramer</td>
<td>0.63</td>
<td>0.251</td>
</tr>
<tr>
<td>Pentamer</td>
<td>0.55</td>
<td>0.228</td>
</tr>
<tr>
<td>Hexamer</td>
<td>0.67</td>
<td>0.248</td>
</tr>
<tr>
<td>OH-SAM</td>
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<tr>
<td>Dimer</td>
<td>0.64</td>
<td>0.811</td>
</tr>
<tr>
<td>Trimer</td>
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<td>0.423</td>
</tr>
<tr>
<td>Tetramer</td>
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<td>0.457</td>
</tr>
<tr>
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<td>0.463</td>
</tr>
<tr>
<td>Hexamer</td>
<td>0.77</td>
<td>0.529</td>
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</tbody>
</table>

a Interfacial water molecules are defined by a separation distance of 6 Å within the topmost heavy atoms of the SAM.
In all of the Aβ−OH-SAM simulations, Aβ oligomers were not able to retain their initial position and orientation on the OH-SAM. Specifically, Aβ dimer gradually drifted away from the OH-SAM by lifting its N-/C-terminals, leading to the turn region marginally contacted with the surface. For Aβ oligomers from trimer to hexamer, they tended to reorient by ~10°-35° to allow the C-terminal residues in contacts with the SAM surface for adsorption. This orientation preference is very similar to the reorientation of Aβ pentamer and hexamer on the CH$_3$-SAM, but with a less extent in orientation degree. Comparing the Aβ adsorption behaviors on CH$_3$-SAM and OH-SAM, it seems that high-order Aβ oligomers are more likely to be adsorbed on both SAMs via the hydrophobic C-terminus so as to maximize hydrophobic interactions with hydrophobic alkyl chains. Despite relative large mobility and preferred orientation of Aβ oligomers, all of Aβ oligomers still remained some partial contacts with the OH-SAM within 50-ns MD simulations and a complete desorption of Aβ oligomers from the surface was not observed. It is likely to occur on substantially long time scale, if desorption does occur.

It is worth emphasizing that the weak association of Aβ oligomers with the OH-SAM in simulations does not necessarily imply the non-adsorption or desorption of Aβ oligomers on the OH-SAM. Instead, it may suggest that Aβ oligomers are not favorably adsorbed on the OH-SAM using the only bottom peptide (referred as U-bend orientation), instead Aβ oligomers could temporarily desorb from the surface and then re-adsorb on the surface again using the other preferred binding sites (e.g. C-terminal β-sheet). This speculation is supported by our recent experimental study of Aβ aggregation on different SAMs (Figure 8.10, later discussion) and our previous
simulation studies of Aβ pentamer adsorption on the OH-SAM with three different orientations. We have shown that if Aβ pentamer initially adsorbed on the OH-SAM using the bottom peptide (the same orientation as in this work), it tended to reoriented itself by ~40° to create new adsorption sites for the C-terminal β-sheet to associate with the OH-SAM. If Aβ pentamer initially adsorbed using the N-terminal β-sheet, Aβ moved away from the OH-SAM but retained the turn region to weakly contact with the OH-SAM. Only when Aβ pentamer initially adsorbed using the C-terminal β-sheet, it remained tightly adsorbed on the OH-SAM. These results suggest that the extent of adsorption of Aβ oligomers on the SAM surface strongly depends on Aβ binding sites. It may require long time for Aβ oligomers to search energetically favorable binding sites for surface adsorption by adjusting their orientation and conformation.

8.3.4. Conformational dynamics of Aβ oligomers on SAM surfaces

To characterize overall conformational change of Aβ oligomers, Figure 8.3 shows the time evolution of backbone root mean-square deviation (rmsd) of Aβ oligomers in solution and on different SAMs. It appears that some Aβ oligomers on SAMs experienced less structural deviations than those in bulk solution, probably due to restricted freedoms at the interface.
Figure 8.4. Correlation of β-structure content with inter-peptide interactions for Aβ oligomers. (a) β-structure content of Aβ oligomers, (b) hydrogen bonds between Aβ peptides, and (c) peptide-peptide interactions. Hydrogen bonds and peptide-peptide nonbonded interactions are normalized by the number of Aβ peptides. All data are averaged using the last 20-ns MD simulations.

Although different SAM surfaces induced different extents of conformational changes in Aβ oligomers, unlike disordered Aβ dimers, other Aβ oligomers were found to largely maintain stable parallel β-structure during the entire 50-ns simulations. This observation suggests that small Aβ oligomers, such as trimer or tetramer, can act as stable
seeds for prompting amyloid fibril formation on the SAM surfaces. Many studies have shown that the formation of β-sheet structure is a general feature of Aβ nucleus and fibrils. Regardless in solution or on different SAMs, a secondary structure content analysis (Figure 8.4a) revealed that β-structure population was rapidly increased from 20-41% in dimer to 60-68% in trimer, and then slowly increased to 65-75% in tetramer, 67-76% in pentamer, and 68-79% in hexamer. A similar increasing trend was also observed for intra-peptide and inter-peptide hydrogen bonds as a function of the size of Aβ oligomers (Figure 8.4b). For even small Aβ oligomers (3≤n≤6), these hydrogen bonds were very stable. Once they were formed, they were rarely broken for all cases. Comparing of β-sheet content (Figure 8.4a) with intra-peptide and inter-peptide hydrogen bonds (Figure 8.4b) clearly reveals a positive correlation that as the number of inter-peptide and intra-peptide hydrogen bonds increases, the population of the β-sheet structure is correspondingly increasing as a function of the order of Aβ oligomers, implying that hydrogen bonds play a key role in stabilizing the parallel β-strands.

More precisely, as the number of peptides increases, inter-peptide interactions (Figure 8.4c) that include the contributions from hydrogen bonds, π-π interactions, homo-residue stacking ladders, and other sidechain contacts were enhanced because more peptides were involved to stabilize its neighboring peptides. Considering an ideal separation distance between peptides is ~4.7 Å and the cutoff distances are 10 Å for VDW interactions and 12 Å for electrostatic interactions, the gain of favorable inter-peptide interactions became more pronounced especially as Aβ changes from dimer to trimer.
8.3.5. Interactions of Aβ oligomers with SAM surfaces

To rationalize the driving forces that control Aβ adsorption on the SAMs, Figure 8.5 shows the averaged interaction energies between Aβ oligomers and different SAMs and their partition into electrostatic and van der Waal (VDW) terms from the last 20 ns simulations. Clearly, all Aβ-SAM interactions were energetically favorable, but Aβ oligomers interacted much more strongly with COOH-SAM (-197.9~-258.2 kcal/mol) than with CH$_3$-SAM (-73.0~-115.8 kcal/mol) and OH-SAM (-15.1~-108.6 kcal/mol).
Decomposition of total Aβ-SAM interaction energies into VDW and electrostatic components showed that VDW and electrostatic interactions contributed differently to each Aβ–SAM system, strongly depending on the surface chemistry of SAM surfaces. Specifically, for the hydrophobic CH₃−SAM, major attractive forces driving Aβ oligomers to be adsorbed on the SAM surface were VDW interactions occupying over 90% of energy contribution to Aβ-SAM interactions, while electrostatic interactions were almost negligible. For Aβ oligomers interacting with the hydrophilic OH-SAM, VDW and electrostatic contributions were comparable. For the COOH-SAM with relative large partial charge distribution in the headgroups, electrostatic interactions were dominant forces, but VDW interactions also contributed to ~30-40% of total Aβ-SAM interactions for Aβ adsorption. Despite some fluctuations in Aβ-SAM interactions, the order of Aβ oligomers appears to have a little effect on total Aβ-SAM interactions, resulting in a general decreasing order of Aβ interactions with COOH-SAM > CH₃-SAM > OH-SAM.

To further identify the energy contribution of each individual residue to total Aβ-SAM interactions, Figure 8.6 shows the interaction energies of each residue of Aβ oligomers interacting with SAM surfaces. Although the residue-SAM interactions were highly heterogeneous and strongly depended on the size of Aβ oligomers and the surface chemistry of SAMs, some residues showed apparent preferential interactions with the SAM surfaces.
Figure 8.6. Interaction energy between each individual residue of Aβ oligomers and (a) CH₃-SAM, (b) COOH-SAM, and (c) OH-SAM. All data are evaluated using the last 20-ns MD simulations.

It can be seen that except for Aβ dimer on the OH-SAM, the C-terminal residues of 30-42 generally involved stronger interactions with the CH₃-SAM and the OH-SAM than the N-terminal residues of 17-29. This fact clearly supports observation from MD trajectories that Aβ oligomers tend to reorient themselves so as to make the C-terminal residues in more contacts with these SAM surfaces. Due to this reorientation, some interactions from N-terminal residues were lost. On the other hand, in all simulations of
Aβ oligomers on the COOH-SAM, residue-SAM interactions from N-terminal and C-terminal residues were comparable and no distinguishable interaction patterns were observed for individual residue interacting with the COOH-SAM. But, two positively charged residues of protonated N-terminus and Lys28 are found to have the strongest interactions with the COOH-SAM, suggesting two strong sites for Aβ binding to the COOH-SAM. Additionally, a number of hydrophobic residues near C-terminal region (Ile31, Ile32, Leu34, Met35, Val36, Val50, and Ala42) had relative strong interactions with all SAMs. Such hydrophobic interactions also facilitate Aβ to be stabilized on the SAMs.

Aβ peptide is an amphiphilic peptide, while SAM has varied interfacial properties according to hydrophobic alkyl chains terminated with different functional groups (hydrophilic vs. hydrophobic or neutral vs. charge in this work). To further characterize the nature of surface interactions of Aβ oligomers with SAMs, Figure 8.7 shows the number of hydrogen bonds and VDW contacts between Aβ and SAM. It can be seen that Aβ oligomers formed a large number of stable hydrogen bonds (~24) and VDW contacts (~93) with the COOH-SAM, showing little dependence of Aβ size effect on these contacts. As compared to Aβ-COOH-SAM systems, although both hydrogen bonds and VDW contacts were also formed between Aβ oligomers and OH-SAM, the number of these contacts was largely reduced. The CH₃-SAM only involved a large number of stable VDW contacts (~75), and no hydrogen bonds with Aβ oligomers due to devoid of hydrogen bond donors or acceptors in the CH₃-SAM. Taken together, SAM surfaces can
induce the adsorption of different Aβ species via different surface interactions, providing further support for Aβ polymorphism.

Figure 8.7. (a) Number of hydrogen bonds and (b) number of VDW contacts between Aβ oligomers and SAMs. A hydrogen bond is assigned if the distance between donor D and acceptor A is ≤ 3.2 Å and the angle D–H . . . A is ≥ 120°. A VDW contact is defined if the distance between the center of mass of two sidechains is ≤ 5.5 Å.

8.3.6. Effect of surface hydration on Aβ adsorption on SAM surfaces

Many studies have shown that surface hydration can affect protein adsorption on different biological and artificial surfaces\textsuperscript{309-315}, because a strong hydrated layer provides a physical and energetic barrier for biomolecules to overcome prior to adsorption on the surface. Figure 8.8 shows the averaged interaction energies between the SAM and interfacial waters, where interfacial waters are identified if they are within 6 Å of the topmost heavy atoms of a given SAM surface. The SAM-water interactions are averaged from the last 20 ns and are normalized by the SAM surface area because varied sizes of the SAMs are used for different systems depending on Aβ sizes. Interaction energies of hydrophilic OH-SAM and COOH-SAM with interfacial waters were comparable, but
much lower (i.e. more favorable) than those of hydrophobic CH₃-SAM with interfacial waters. Strong water-SAM interactions are mainly attributed to the formation of a large number of hydrogen bonds at the SAM interface.

Figure 8.8. Averaged interaction energy between SAMs and interfacial water, normalized by the surface area of SAMs. All data are evaluated using the last 20-ns MD simulations.

Simple comparison of water-SAM interactions and Aβ-SAM interactions could provide a quantitative explanation for Aβ adsorption on different SAMs. For instance, the CH₃-SAM had slightly favorable interactions with Aβ oligomers than the OH-SAM (Figure 8.5), but the CH₃-SAM has much weaker interactions with water than OH-SAM (Figure 8.8). Comparison of these interactions with MD trajectories reveals that unlike tightly bound Aβ oligomers on the CH₃-SAM, Aβ oligomers are very mobile on the OH-SAM. This observation suggests that Aβ-CH₃-SAM interactions outcompete weak water-SAM interactions to induce protein adsorption on the CH₃-SAM, but similar Aβ-OH-SAM interactions are largely compromised by strong water-SAM interactions, resulting in relative weak adsorption of Aβ oligomers on the OH-SAM. Following the
similar analysis, the strongest Aβ-COOH-SAM interactions observed in this work are sufficient enough to compensate large desolvation penalty caused by strong water-SAM interactions and thus to drive Aβ adsorbed on COOH-SAM. Clearly, Aβ adsorption on the SAM surfaces is the outcome of competitive interactions between Aβ-SAM and water-SAM interactions.

To explore the structure and distribution of interfacial water near the SAM surfaces, Figure 8.9 (left panel) shows the radial distribution functions (RDFs) of the oxygen atoms of water (Ow) around the top-most heavy atoms of three SAMs (i.e. methyl carbon C12 atoms for CH$_3$-SAM, carboxyl oxygen OT1 and OT2 atoms for COOH-SAM, and hydroxyl oxygen OG atoms for OH-SAM). In all COOH-SAM and OH-SAM simulations, the Ow-OT1/OT2 and Ow-OG RDFs had almost identical first peak positions at ~2.9, but different first peak heights (i.e. the height of g(r) represents the local water density) of 1.7 for Ow-OT1/OT2 and 3.8 for Ow-OG RDFs. The relatively lower first-peak height in Ow-OT1/OT2 RDFs could be due to the loss of interfacial water molecules that are replaced by the adsorbed Aβ atoms. This fact suggests that the first hydration shell around both COOH-SAM and OH-SAM surfaces appears at the nearly same coordinate, but the water density of the first hydration shell is higher on the OH-SAM than on the COOH-SAM. On the other hand, the first peak of the water RDFs in the CH$_3$-SAM simulations was shifted to a relatively large separation distance of 3.9 Å with a reduced height of 0.86.
Figure 8.9. Radial distribution function between oxygen Ow of water molecules and the topmost heavy atoms of (a) methyl carbon C12 in the CH3-SAM, (b) carboxyl oxygen OT1 and OT2 in the COOH-SAM, and (c) hydroxyl oxygen OG in the OH-SAM. Three representative MD snapshots of solvated SAM surfaces interacting with Aβ dimer. Interfacial waters that form hydrogen bonds (blue dash) with SAM are displayed. Aβ dimer is omitted for clarity.
Comparison of the location and density of water RDFs near three SAMs suggests a decreasing order of well-hydrated layers on OH-SAM>COOH-SAM>CH$_3$-SAM. Figure 8.9 (right panel) also shows three MD snapshots of solvated SAM surfaces interacting with Aβ dimer, where Aβ dimer is omitted for clarity. It can be clearly seen that no interfacial water molecules formed hydrogen bonds with the CH$_3$-SAM, while a large number of water molecules were able to form an intensive hydrogen bond network with the COOH-SAM and OH-SAM, consistent with RDF results. The hydration layer on the COOH-SAM was not as intact as the one on the OH-SAM, but with a few small vacancies, indicating the replacement of some interfacial waters by adsorbed Aβ atoms on the COOH-SAM.

8.3.7. Adsorption mechanism of Aβ oligomers on SAM surfaces

Aβ adsorption and aggregation on a surface is a complex and dynamic assembly process, generally involving a slow lag phase for seed formation and a fast growth phase for peptide elongation and lateral association. Both phases require Aβ to undergo conformation change, peptide-peptide association and reorganization, peptide adsorption, desorption, and migration on the surface. It is not likely to simulate such complex process involved multiple assembly pathways using conventional MD simulations with limited timescale. Additionally, in experiments when peptide concentration increases in the presence of the SAM surface, peptides in solution may interact with peptides on the surface or surface-bound peptides may also interact with each other; these interactions will mediate interfacial peptide orientation and conformation, and thus simulations of
single oligomer on the SAM may not adequately represent the real experimentally observed systems.

But as a first approximation, our previous and current MD simulations of Aβ aggregates from monomer to hexamer on different SAMs provide some atomic-level insights into interaction between small Aβ oligomers and SAM surfaces, including (i) different Aβ oligomers can adopt different orientations and conformations upon adsorption on the SAMs, (ii) Aβ adsorption on the SAMs is driven by the competition between Aβ-SAM and SAM-water interactions, (iii) SAMs can induce the adsorption of different Aβ species with different sizes and conformations, and (iv) even small number of peptides such as trimer, which retain parallel structural integrity and 60-80% β-sheet rich structure, can act as a stable nucleus for amyloid formation.

As discussed above, since hydrophobic interactions are mainly responsible for Aβ adsorption on the CH₃-SAM, overlapped surface contact area between Aβ oligomer and CH₃-SAM is crucial for maximizing such hydrophobic interactions. The NMR derived Aβ monomer has a U-bend conformation, with a hydrophobic length of ~40 Å measured by the distance from residue 30 to 42 along the C-terminal β-strand and a width of ~20 Å measured by the distance between Met35 at the C-terminal and Glu22 at the N-terminal, thus the cross section area for this U-bend conformation (referred as U-bend area) is approximately 40×20=800 Å². On this basis, when a number of identical Aβ peptides are stacked on the top of each other to form an oligomer with a peptide-peptide separation distance of ~4.7 Å, a surface area formed by the parallel stacking C-terminal β-strands (referred as C-β-sheet area) can be easily estimated by (n-1)×4.7×40 Å² where n is the
number of peptides. Simulation results showed that small Aβ oligomers (n≤4) adsorbed steadily on the SAM using relative large U-bend area. When n ≥ 5, Aβ pentamer and other high-order oligomers prefer to use the C-β-sheet area (≥752 Å²) to interact with the CH₃-SAM for maximizing hydrophobic interactions. Similarly, high-order Aβ oligomers also showed the preferred interaction and orientation for C-terminal residues to interact with and face toward the OH-SAM. But, this orientation and interaction preference was not observed for Aβ oligomers on the COOH-SAM. Aβ oligomers barely changed their orientation and position on the COOH-SAM due to the comparable interactions with the SAM from N-terminal and C-terminal residues. Moreover, to bring the Aβ peptides to the SAM surface, it is necessary to overcome a dewetting barrier at the SAM interface by freeing surface-bound water molecules to bulk solution. Unlike the CH₃-SAM which induces little desolvation penalty for Aβ adsorption, strong hydration layer near the OH-SAM and COOH-SAM needs to be disrupted by Aβ-SAM interactions to make Aβ adsorbed on the surfaces. Thus, Aβ adsorption on the SAMs depends on the competitive interactions between Aβ-SAM and SAM-water interactions that promote or prevent Aβ aggregation, respectively.

Although small Aβ oligomers can adopt different orientations on different SAMs for maximizing their interactions with surface, from the macroscopic point of view, when Aβ oligomers grow into large species such as high-order oligomers, protofibrils, and fibrils, it is not feasible for such large species to “stand” on the SAM using the U-bend area, followed by further fibril elongation via monomer addition along fibril axis parallel to surface normal. Instead, these Aβ species would “lie down” on the SAM using the
C-terminal β-sheet observed by our simulations or the N-terminal β-sheet for fibril elongation along fibril axis perpendicular to surface normal. Our AFM images\textsuperscript{291} also supported that Aβ fibrils have long lengths ranging from 50-400 nm, but small heights ranging from 2-22 nm on different SAMs. Moreover, surface plasmon resonance (SPR) was used to characterize Aβ adsorption and aggregation on the SAMs at the very early stage of aggregation within 8 hours (i.e. at the oligomeric state)\textsuperscript{291}. SPR results showed that the adsorption amounts of Aβ were \(~7.5\) ng/cm\(^2\) on the CH\(_3\)-SAM, \(~1.45\) ng/cm\(^2\) on the OH-SAM, \(~0.55\) ng/cm\(^2\) on the COOH-SAM, and \(~5.2\) ng/cm\(^2\) on the NH\(_2\)-SAM, respectively. The CH\(_3\)-SAM had much larger Aβ adsorption amount than the OH-SAM, consistent with observation and explanation from MD simulations. But, an apparent discrepancy between tightly adsorbed Aβ oligomers on the COOH-SAM by MD simulations and the least adsorption amount of Aβ on the COOH-SAM by SPR is due to different oligomeric sizes used in simulations and experiments. Within 8-hour incubation, Aβ is very likely to form higher-ordered oligomers in experiments, rather than small oligomers of dimer and up to hexamer. Moreover, the lack of 1-16 residues bearing -4e charges in Aβ and the deprotonation state of COOH groups in the COOH-SAM in our MD simulations could also attribute to different interaction patterns obtained from simulations and experiments. The presence of both negatively charged Aβ\(_{1-16}\) residues and negatively charged and mixed COO-/COOH SAM would induce strong repulsive Aβ-COOH interactions, which can reasonably explain the discrepancy between simulation and experiment. On the other hand, the lack of 1-16 residues in Aβ models
would not change the nature of hydrophobic interactions between Aβ and CH₃-SAM and of comparable electrostatic and hydrophobic interactions between Aβ and OH-SAM.

Figure 8.10. Tapping-mode AFM images of Aβ1-42 fibrils on (a) CH₃-SAM, (b) COOH-SAM, and (c) OH-SAM at the incubation time of day 1, in comparison with (d) Aβ fibrils in bulk solution at day 7.

Finally, AFM images (Figure 8.10) actually showed that regardless of the SAM surfaces, all SAMs can eventually develop a large amount of Aβ fibrils at day 1, exhibiting similar morphologies of Aβ fibrils formed in solution at day 7. This suggests that Aβ fibril formation and fibril growth are a dynamic process, involving distinct and multiple-step aggregation pathways. For example, desorbed Aβ oligomers can grow into large species in solution, and then re-adsorb on the surface. The desorption of such large species from the surface would exert large energy penalty. Another scenario is that
weakly surface-bound Aβ species can interact with each other to form large species to enhance their interactions with the SAMs.

8.4. Conclusions

We employ a series of MD simulations to study the conformational change and adsorption behavior of small Aβ oligomers (from dimer to hexamer) on various SAMs terminated with CH$_3$-, COOH-, and OH- functional groups. In combination with our previous MD simulations of Aβ monomer and pentamer on the SAMs and experimental study of Aβ aggregation on the SAMs, our results collectively suggest that the conformation and adsorption of Aβ peptides on the SAMs are regulated through a complex balance of different interactions between Aβ-SAM, Aβ-Aβ, and SAM-water interactions. Briefly, Aβ oligomers are tightly bound to the CH$_3$-SAM due to strong hydrophobic Aβ-SAM interactions and weak SAM-water interactions. Considering a large dewetting barrier on the COOH-SAM and the OH-SAM, strong (weak) adsorption of Aβ oligomers on COOH-SAM (OH-SAM) is determined by whether the gain of favorable Aβ-SAM enthalpy and desolvation entropy can compensate for the loss of solvation enthalpy. Moreover, due to distinct properties of hydrophilic N-terminal and hydrophobic C-terminal residues, hydrophobic C-terminal residues of higher-order Aβ oligomers appear to have preferential interactions with the SAM surfaces for facilitating Aβ fibril formation and fibril growth. Moreover, stable Aβ trimer with well preserved parallel β-strands may act as the smallest seed for Aβ polymerization. This study provides a more complete and detailed information of Aβ adsorption and aggregation on
the SAM surfaces, which may be useful for understanding interactions between Aβ and cell membrane.
Membrane associated peptides (MAPs) contain tens of thousands of short, simple, and membrane active peptides, which closely related to essential biological processes. The aggregation, folding, adsorption, and membrane penetration behaviors of MAPs define their activity or toxicity. Interactions between MAPs and lipid bilayers have been extensively studied by numerous methods including circular dichroism $^{240-242}$, NMR $^{233-237}$, membrane partition ability evaluation $^{243-247}$, sum frequency generation (SFG) vibrational spectroscopy $^{238, 239}$. However, due to the transient and heterogeneous nature of MAPs when associated with membrane, it is extremely difficult to study the mechanisms of MAPs with traditional experimental methods. Thus, theoretical simulation, which construct the system from a nano-scale level base on classical or quantum theories, provide us powerful tools to study the microscopic and dynamic systems as complementary methods to experiments. The computational tools in this dissertation effectively construct and screen polymorphic structures, rank oligomer populations, monitor the ion leakage through pores, capture the adsorption preference, and
characterize the membrane penetration potential profiles. The integrated computational methods in this dissertation provide us a powerful platform to study the MAPs, and other membrane associated molecules, and finally to find the potential drug candidates or inhibitors.

9.1. hIAPP polymorphic oligomeric structure prediction and evaluation

Misfolding and self-assembly of human islet amyloid polypeptide (hIAPP) into polymorphic amyloid oligomers is pathologically linked to type II diabetes. But, atomic structure and biological role of these hIAPP oligomers are still unclear. In this dissertation, a computational framework is developed to search for a diverse set of hIAPP oligomers with the lowest energy landscape, two “stacking-sandwich model” and “wrapping-cord model” are proposed to describe polymorphic structures of hIAPP oligomers, and all-atom molecular dynamics simulations are used to examine the structure, dynamics, and interactions of the self-assembled hIAPP oligomers. Seven oligomers from the stacking-sandwich model and three oligomers from the wrapping-cord model are determined by their high structural stability with favorable peptide-peptide interactions, although all of them display completely different structures in symmetry and β-sheet packing. These oligomeric structures can also serve as templates to present double- and triple-stranded helical fibrils via peptide elongation, explaining the polymorphism of amyloid oligomers and fibrils.

Sequence-induced differences were further probed in structural stability, conformational dynamics, and driving forces between different hIAPP and rIAPP
polymorphic forms from monomer to pentamer using molecular dynamics (MD) simulations. Simulations show that hIAPP trimer to pentamer exhibit high structural stability with well preserved in-register parallel β-sheet and the U-bend conformation. The hIAPP trimer appears to be a smallest minimal seed in solution. The stabilities of parallel hIAPP oligomers increase with the number of peptides. Conversely, replacement of hIAPP sequence by rIAPP sequence causes a significant loss of favorable inter-peptide interactions in all rIAPP oligomers, destabilizing the C-terminal β-sheet, turn conformation, and overall stability. Such less β-sheet-rich structure and disturbed U-shaped topology exert large energy penalty for the rIAPP peptides to self-assemble into highly-ordered, in-register β-sheet-rich protofibrils and fibrils, which explain the non-amyloidgenic activity of the rIAPP. Moreover, the absence of interior water within the U-turn region in the well-packed higher-order hIAPP oligomers, not in the poorly packed rIAPP oligomers, also stabilizes peptide association.

9.2. hIAPP ion channel-like pore structure and the toxicity mechanisms

Formation of receptor-independent channels by hIAPP in the membrane is regarded as one of the membrane-damaging mechanisms associated with the dysfunction and death of pancreatic islet β-cells in type II diabetes. Here, we investigate dynamic structure, ion conductivity, and membrane interactions of hIAPP channels in the DOPC bilayer using molecular dynamics simulations. In the simulated lipid environments, a series of annular-like hIAPP structures with different sizes and topologies are compatible with the doughnut-like images obtained by AFM, and with those of modeled channels for Aβ, K3
peptide, and antimicrobial peptide PG-1, suggesting that loosely-associated β-structure motifs can be a general feature of toxic, unregulated channels. The hIAPP channels exhibit relatively high ion binding selectivity and ion conductivity of Cl\(^-\) over cations. Although all cations exhibit relatively weak ion fluxes, poor ion conductivity of Na\(^+\) and K\(^+\) is due to the bare adsorption on the lipid bilayer while that of Ca\(^{2+}\) is due to strong binding affinity to phosphate groups in the lipid heads. This study represents a first attempt to delineate some of the main structural features of the hIAPP channels, for a better understanding of the origin of amyloid toxicity and the development of pharmaceutical agents.

9.3. Transmembrane activity evaluation of antimicrobial peptides

The widespread use of antibiotics has promoted the growing threat of bacteria that are resistant to almost all available antibiotics. Antimicrobial peptides (AMPs) represent a promising generation of antibiotics because they have broad-spectrum activity and lower toxicity. However, due to the limited sequence diversity of AMPs, it is challenging to identify and design new potent AMPs by quickly and accurately evaluating their antimicrobial activity. In this dissertation, we develop potential-mean-force (PMF) coarse-grained molecular dynamics simulations to measure the free energy required to transfer the peptides from bulk water phase to water-membrane interface to bilayer interior. The PMF results reveal that different PMF shapes can indeed identify different membrane insertion scenarios by mapping out peptide-lipid energy landscapes during peptide insertion. Antimicrobial activity/toxicity appears to be closely related to the
transmembrane ability across different membranes. The PMF profile can be an instructive index to distinguish and identify antimicrobial activity and hemolytic toxicity of designed and existing AMPs.

9.4. Aggregation and adsorption behavior of amyloid on self-assembled monolayers

Accumulation of small soluble oligomers of amyloid-β (Aβ) in the brain is thought to play an important pathological role in Alzheimer's disease. The interaction of these Aβ oligomers with cell membrane and other artificial surfaces are important for the understanding of Aβ aggregation and toxicity mechanism. In this dissertation, we present a series of exploratory molecular dynamics (MD) simulations to study the early adsorption and conformational change of Aβ oligomers. Aβ oligomers from dimer to hexamer in bulk solution and on different self-assembled monolayers (SAMs) terminated with -CH₃, -OH, and -COOH groups were put into simulations. Within the timescale of MD simulations, the conformation, orientation, and adsorption of Aβ oligomers on the SAMs is determined by complex interplay among the size of Aβ oligomers, the surface chemistry of the SAMs, and the structure and dynamics of interfacial waters. Energetic analysis of Aβ adsorption on the SAMs reveals that Aβ adsorption on the SAMs is a net outcome of different competitions between dominant hydrophobic Aβ-CH₃-SAM interactions and weak CH₃-SAM-water interactions, between dominant electrostatic Aβ-COOH-SAM interactions and strong COOH-SAM-water interactions, and between comparable hydrophobic and electrostatic Aβ-COOH-SAM interactions and strong OH-SAM-water interactions. Atomic force microscopy images also confirm that all
SAMs can induce the adsorption and polymerization of Aβ oligomers. Structural analysis of Aβ oligomers on the SAMs shows a dramatic increase in structural stability and β-sheet content from dimer to trimer, suggesting that Aβ trimer could act as seeds for Aβ polymerization on the SAMs. This work provides atomic-level understanding of Aβ peptides at interface.
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Author: Jun Zhao, Xiang Yu, Guizhao Liang, and Jie Zheng

Publication: Biomacromolecules

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