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Molecular Modeling and Simulation of the Mycobacterial Cell Envelope: From Individual Components to Cell Envelope Assemblies

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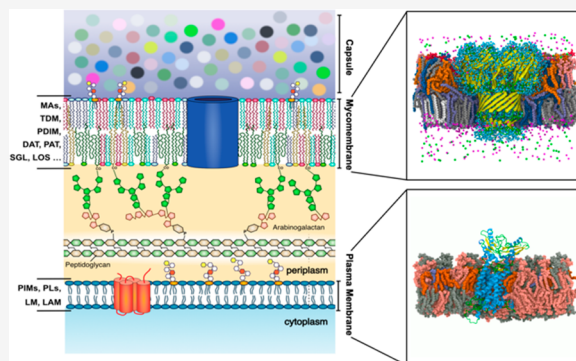
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ABSTRACT: Unlike typical Gram-positive bacteria, the cell envelope of mycobacteria is unique and composed of a mycobacterial outer membrane, also known as the mycomembrane, a peptidoglycan layer, and a mycobacterial inner membrane, which is analogous to that of Gram-negative bacteria. Despite its importance, however, our understanding of this complex cell envelope is rudimentary at best. Thus, molecular modeling and simulation of such an envelope can benefit the scientific community by proposing new hypotheses about the biophysical properties of its different layers. In this Perspective, we present recent advances in molecular modeling and simulation of the mycobacterial cell envelope from individual components to cell envelope assemblies. We also show how modeling other types of cell envelopes, such as that of *Escherichia coli*, may help modeling part of the mycobacterial envelopes.

We hope that the studies presented here are just the beginning of the road and more and more new modeling and simulation studies help us to understand crucial questions related to mycobacteria such as antibiotic resistance or bacterial survival in the host.



INTRODUCTION

Mycobacteria are deadly pathogens, causing severe infections in humans such as leprosy (*Mycobacterium leprae* and *M. lepromatosis*) and tuberculosis (*M. tuberculosis*).¹ *M. bovis* can cause disease in cattle, leading to pneumonia, weight loss, and eventual death. The pathogen is transmitted to humans through the consumption of contaminated or unpasteurized dairy products.² For example, *M. bovis* is transmitted from cattle to other wildlife, such as wild boars, badgers, and deer, increasing the threat of human infection in areas surrounding disease outbreaks in cattle.³ In 2021, there were 133,802 cases of leprosy⁴ and 10.6 million new infections of *M. tuberculosis* (*Mtb*), leading to 1.6 million deaths worldwide.⁵ Although tuberculosis has been controlled in developed countries, incidence is still high in sub-Saharan Africa and southwest Asia.⁶ Thus, there is a pressing need for innovative research to better understand mechanisms of mycobacterial persistence to help develop alternative intervention strategies. One critical issue in antimycobacterial drug discovery is that our understanding of the complex mycobacterial cell envelope is rudimentary at best. This cell envelope consists of an array of lipids and carbohydrates contributing to both hydrophobic and polar regions of various thicknesses and densities, making it extremely challenging to predict how molecules cross this barrier and enter the cell.

Molecular modeling and simulation of realistic biological membranes have been a subject of intense research for decades, starting in the late 1980s with simple, short-time-scale

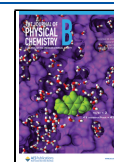
simulations to complex and realistic simulations in the past decade.⁷ Simulation programs such as AMBER,⁸ CHARMM,⁹ NAMD,¹⁰ OpenMM,¹¹ and GROMACS¹² (to name a few) have allowed launching longer simulations on much larger systems, while tools such as CHARMM-GUI and MMTSB have rendered the system modeling much easier for scientists.^{13,14} Efforts to characterize various membrane types have led to a focus on lipidomics and the effects of lipid types on the physical properties of biological membranes. Over the decades, molecular modeling and simulation have provided a wealth of information on the organization principles underlying cell membranes. With continuous advances, nowadays it becomes possible to model complex lipid and/or protein compositions from eukaryotic plasma,¹⁵ bacterial,¹⁶ or even plant membranes,^{17,18} making comparison of the biophysical properties of these different membranes amenable.¹⁸ Yet, there remain classes of lipids (especially from bacteria) that are overlooked. In this Perspective, we present recent progress in the molecular modeling and simulation of one of such classes, mycobacterial lipids, and how this can inform us about the

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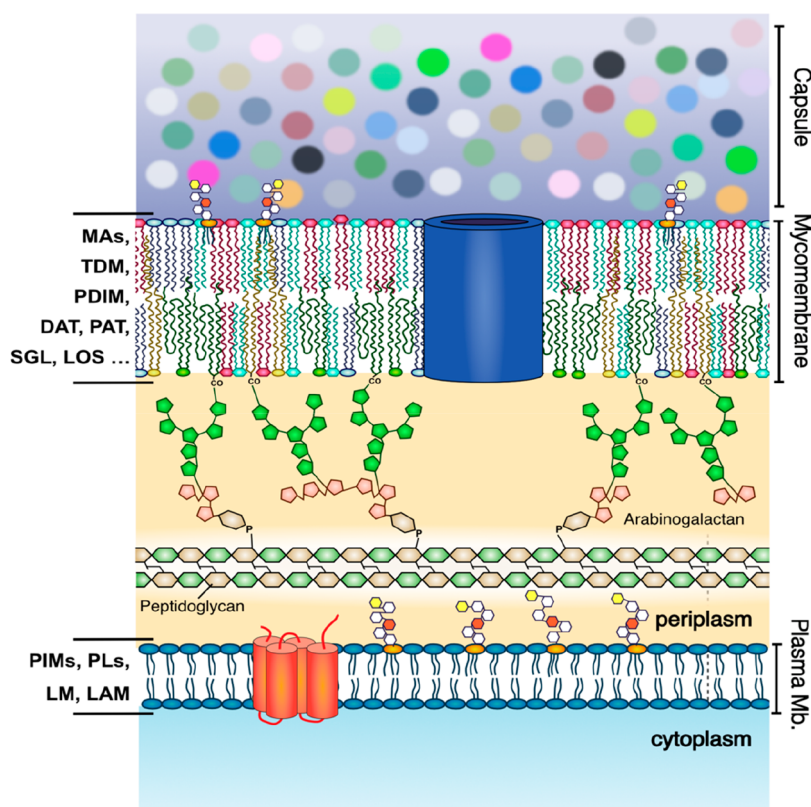


Figure 1. Schematic representation of the three main layers of the mycobacterial cell envelope: the outer membrane (or mycomembrane) covalently bonded to an arabinogalactan layer, the peptidoglycan layer, and the inner membrane (or plasma membrane). The main lipids constituting the mycomembrane are mycolic acids (MAs) and their ester-linked trehalose dimycolate (TDM), phthiocerol dimycoserate (PDIM), diacyl trehaloses (DATs), pentaacyl trehaloses (PATs), sulfated trehalose glycolipids (SGLs), and lipooligosaccharides (LOS). The plasma membrane is composed of different types of typical phospholipids (PLs) (such as cardiolipin (CL), phosphatidylinositol (PI), and phosphatidylethanolamine (PE)), phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannan (LM), and lipoarabinomannan (LAM).

biophysical properties of these molecules from individuals to assemblies.

Unlike typical Gram-positive bacteria having only a plasmic membrane, mycobacteria are unique and their cell envelopes are composed of a mycobacterial outer membrane (MOM), a peptidoglycan (PG) layer, and a mycobacterial inner membrane (MIM). This double bilayer structure of mycobacteria is separated by a PG layer, resembling that of Gram-negative bacteria but with the MOM covalently linked to the PG layer (Figure 1).¹ In this Perspective, we present our view to model this cell wall by discussing what is known, what is done, and what needs to be done for each layer of the cell envelope and how to bridge them together.

■ MODELING A REALISTIC MYCOBACTERIAL ENVELOPE: WHY HAS IT NOT BEEN DONE BEFORE?

Even though many types of membranes have been modeled and simulated successfully at the atomic level, the modeling and simulation of mycobacterial membranes remain challenging for a variety of reasons. First, mycobacterial membranes include numerous complex lipids with varying chain lengths and branching structures.¹⁹ The organization and overall molecular composition of the different envelope layers are still up for debate. Second, an exhaustive and explicit membrane proteome is still lacking for mycobacteria, so investigating protein–ligand and protein–lipid interactions has been a slow process. Finally, the current capabilities of membrane modeling

approaches make it difficult to build complex membrane systems, and the force fields used in molecular dynamics (MD) simulations need regular updates to be accurate and specific to the variety of molecules being simulated. This is especially important in the context of mycobacterial cell wall organization with an MOM covalently linked to a PG layer followed by an MIM (Figure 1). Modeling the mycobacterial cell envelope is thus a labor-intensive process, from individually parametrizing each type of molecule to correctly placing many molecules in the same system and designing a large variety of membrane models to deal with uncertainties in the membrane composition. While the idea of modeling the entire mycobacterial cell envelope has been discussed for almost 20 years,²⁰ it is only recent that this endeavor is taking shape. Below, we detail recent advances in modeling the different layers of the mycobacterial (and other bacterial) cell wall that may pave the way to creating a unified model of the whole envelope.

■ MYCOBACTERIAL OUTER MEMBRANE

The outermost layer of the mycobacterial cell wall is the MOM, also called the mycomembrane. The MOM was first described in 2008 by two different research groups,^{21,22} who proposed an average MOM thickness of 7.5 nm. These results gave precious information about the overall structure of the MOM, but did not help decipher the molecular details of MOM organization. To do so, several studies modeled

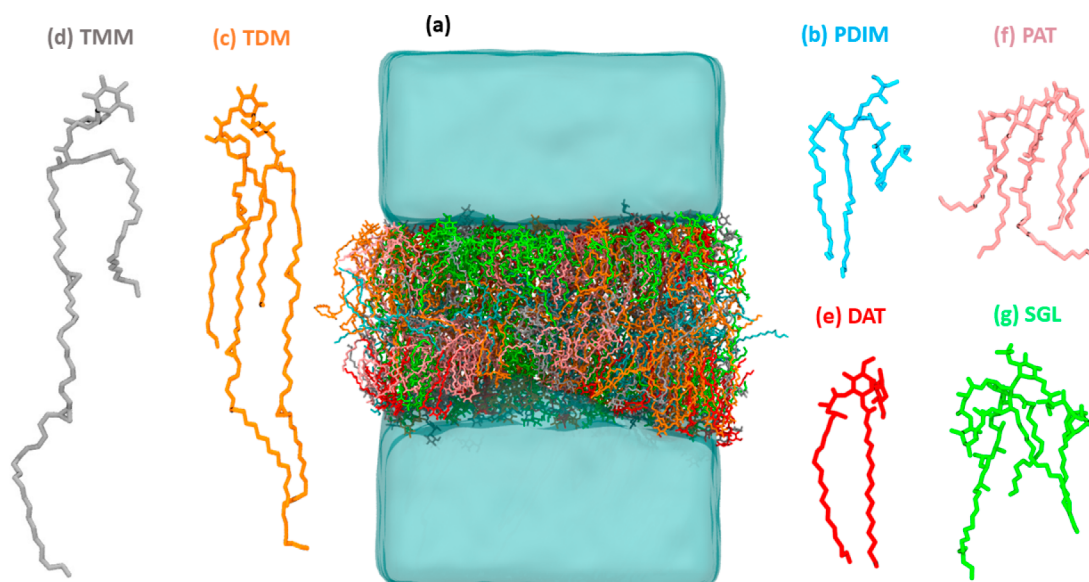


Figure 2. (a) Snapshot of a symmetric all-atom model of the mycomembrane outer leaflet, with equal ratios of (b) phthiocerol dimycocerosate (PDIM), (c) trehalose dimycolate (TDM), (d) trehalose monomycolate (TMM), (e) diacyl trehalose (DAT), (f) pentaacyl trehalose (PAT), and (g) sulfated trehalose glycolipid (SGL).

individual MOM lipid types and characterized their biophysical properties, which is elaborated below.

One of the main components in the MOM is mycolic acids (MAs) that are long fatty acids.^{23–25} *Mtb* has three classes of MAs: α -mycolates, methoxymycolates, and ketomycolates.²⁶ The latter two mycolates are composed of two subclasses having either *cis*-cyclopropane rings or *trans*-cyclopropane rings with an adjacent methyl branch. The fact that cyclopropanation of fatty acids may provide both stabilization and fluidity of bacterial membranes against adverse conditions has been shown using all-atom and coarse-grained MD simulations.^{27,28} The MA classes occur naturally with variations in the numbers of carbons ranging from 60 to 90 for *Mtb*,²⁹ and they appear to be predominately located in the inner leaflet of the MOM.^{23,24} Recent physiochemical investigations indicate that MAs adopt different folded conformations from a compact “W” conformation, similar to four parallel chains, to more extended conformations such as semifolded “sZ” and extended “eU” conformations.^{30–32} These different folding patterns were explored some time ago using all-atom MD simulations,^{33,34} while both coarse-grained and all-atom modeling was more recently used to investigate the assembly of MAs in monolayers and bilayers.^{35,36} Trehalose dimycolate (TDM) and trehalose monomycolate (TMM), the so-called “cord factors”, are the best known MA esters in mycobacteria.^{25,37} TDM is formed by a polar headgroup of trehalose ($\text{Glc}\alpha 1 \leftrightarrow 1\alpha \text{Glc}$) and by two mycolates esterified to O6 of the hydroxymethyl group of each glucose molecule. To the best of our knowledge, there are currently no MD simulation studies of membranes constituted by large TMM or TDM molecules, although we are aware of the modeling of a symmetrical bilayer constituted by short TDM molecules from corynebacteria.³⁸ Beyond studying the folding and assembly of MAs and their esters, parts of these molecules were modeled to study their interactions with host-cell receptors,^{39,40} and the interaction of the mycobacterial transporter MmpL3 (mycobacterial membrane protein large 3) with TMM molecules was investigated using all-atom MD simulations.⁴¹

While some MAs in the inner leaflet are covalently attached to the underlying arabinogalactan–PG complex (Figure 1), other lipids can diffuse more freely in the MOM.⁴² One of them is the phthiocerol dimycocerosate (PDIM) that promotes the entry of *Mtb* into macrophage. PDIM is one of the main *Mtb* virulence factors that can transfer and diffuse into host-cell membranes.^{43,44} This lipid is constituted by a long-chain β -diol (the phthiocerol A chain) esterified by two multimethyl branched mycocerosate molecules (Figure 2).²⁶ All-atom and coarse-grained MD simulations along with ³¹P NMR showed that these molecules can adopt a conical shape in phospholipid bilayers mimicking the host-cell plasma membrane, modulating membrane structures, and causing a phase shift from the lamellar to inverted hexagonal phase. This conical shape seems to be linked to the macrophage infection by *Mtb*.⁴⁵ Even though it is not directly related to lipids present in the MOM, the mycobacterial toxin mycolactone is a virulence factor analogous to PDIM that can perturb the host-cell membrane. The toxin’s actions on membranes were studied with all-atom and coarse-grained MD simulations.^{46–48}

Other free lipids present in the MOM contain trehalose moieties such as diacyl trehaloses (DATs), pentaacyl trehaloses (PATs), and sulfated trehalose glycolipids (SGLs).¹⁹ Lipooligosaccharides (LOS) are based on a common trehalose-containing saccharide core, acylated on the trehalose moiety with multimethyl branched fatty acids. Trehalose is further glycosylated with a mono-, or more commonly, an oligosaccharide of 2–6 sugars, which is species-specific.¹⁹ To the best of our knowledge, there is currently no modeling study of these specific lipids, but numerous studies were performed on bacterial glycolipids and can pave the way to model such molecules.⁴⁹ Furthermore, recently developed tools such as CHARMM-GUI⁵⁰ Glycolipid Modeler⁵¹ or Glycosylator⁵² can be expanded to streamline the creation of such complex molecules. Other lipids are present in the MOM in variable quantities, so interested readers can find more details in these review articles.^{19,26,53}

In the future, the ultimate goal would be to design MOM models constituted of the ensemble of lipids presented above

to see how they can assemble and organize. A very first attempt to model the MOM was recently presented,⁵⁴ but it requires further developments to really understand the biophysical properties of such membranes at the molecular level. To illustrate our view, here, we propose an outer leaflet model of MOM (Figure 2). Our model is a symmetric bilayer consisting of various mycolic acids (TDM and TMM), acyl trehaloses (DAT and PAT), and sulfated trehalose glycolipids (SGL). Building this model will allow us to study how the absence of certain lipids affects the physiochemical properties of the MOM, such as membrane thickness, lipid clustering, and component density along the membrane normal. Later, building a symmetric inner leaflet model of MOM could lead us to build an asymmetric MOM model similar to the previous Gram-negative asymmetric model.^{16,55,56}

■ PEPTIDOGLYCAN LAYER

The PG layer is a mesh-like structure that enhances the rigidity of the cell, allowing it to withstand osmotic pressure while maintaining the cell shape. In *Mtb*, the PG layer sits just below the arabinogalactan layer and between the MOM and MIM bilayers (Figure 1). It is composed of muramic acid residues that contain a mixture of *N*-acetyl and *N*-glycolyl derivatives, whereby the *N*-acetyl functional group can be oxidized to the *N*-glycolyl functional group to form *N*-glycosylated muramic acid. These glycolyl-containing residues may have potential for additional hydrogen bonding interactions between glycans and peptides, strengthening the mesh-like structure. Tetrapeptide side chains consisting of *L*-alaninyl-*D*-isoglutaminyl-*meso*-diaminopimelyl-*D*-alanine are cross-linked with identical short peptides of neighboring glycan chains. The carboxylate groups of MAs in the MOM inner leaflet are linked to the arabinose termini of an arabinogalactan molecule that is connected to the PG layer via a primer region and a phosphodiester linkage to GlcNAc in the PG.³²

While we did not find specific modeling of the PG layer related to mycobacteria, various research groups have modeled the PG of both Gram-positive and Gram-negative bacteria. Chapagain and co-workers built an all-atom PG layer model of *Staphylococcus aureus* with a horizontally aligned mesh. They found that the mesh was porous enough to allow small molecules to diffuse through the layer. Because it is unknown how these meshes align in relation to the plasma membrane, they provided the necessary files for other scientists to extend their model and align new mesh models either vertically or horizontally.⁵⁷ Gumbart et al. built an all-atom PG layer model of *Escherichia coli* (*E. coli*), with a thin PG layer anchored to the outer membrane by lipoproteins. They found that the PG positioning is determined by the length and conformations of these anchors.⁵⁸ Another study by the Gumbart lab found that the PG layer is relatively inelastic in the direction of the glycan strands, but very elastic along the peptide linkages.⁵⁹ Khalid and co-workers investigated how polymyxin B1, a last-resort antibiotic, migrates through an *E. coli* cell wall model and found that the small molecule can lodge itself between glycan strands or attach to the surface of the cell wall, disturbing the PG layer.⁶⁰ In another study by the same lab, interactions between Braun's lipoprotein, outer membrane porin A (OmpA) in both monomer and dimer form, and the PG network were observed in their all-atom simulations. They found that OmpA, when in a dimer formation, readily binds to the PG layer, while Braun's lipoprotein facilitates interactions between the OmpA monomer and the PG layer.⁶¹ In further

studies, Khalid and co-workers found that TolR, a protein anchored in the inner membrane of *E. coli*, along with OmpA in the outer membrane acts together to keep the PG layer from curving.⁶²

Coarse-grained MD simulations of Gram-negative bacterial cell envelopes were also been performed. Ayappa and co-workers built a coarse-grained model of the PG network that agreed with all-atom models in terms of area per disaccharide, equilibrium angle between adjacent peptides, and other properties.⁶³ Jensen and co-workers built coarse-grained models of various transglycosylases, transpeptidases, and endopeptidases surrounding the PG layer and found that cell wall integrity can be maintained with coordination of enzyme activities, bringing new material into the PG layer.⁶⁴

These different works could be used to initiate modeling of the mycobacteria PG layer and then focus on modeling the specificities of the mycobacteria cell wall, such as the arabinogalactan linking this PG layer and the MOM (Figure 1).

■ MYCOBACTERIAL INNER MEMBRANE

The MIM or plasma membrane is the deepest layer of the mycobacterial cell envelope (Figure 1). In comparison to MOM, it is a more canonical bilayer structure found in bacterial or eukaryotic plasma membrane.⁶⁵ It is composed of typical phospholipids such as cardiolipin (CL), phosphatidylinositol (PI), and phosphatidylethanolamine (PE).⁶⁶ Aside from these conventional glycerophospholipids, we note the presence of phosphatidyl-*myo*-inositol mannosides (PIMs) accounting for over half of the dry weight of the MIM lipids. PIMs are present in the membrane with different degrees of acylation and mannosylation.^{67,68} Especially, AcPIM₂ (monoacyl phosphatidylinositol dimannosides) and Ac₂PIM₂ (diacyl phosphatidylinositol dimannosides) may be predominately located in the inner leaflet of the MIM, and AcPIM₆ (monoacyl phosphatidylinositol hexamannosides) and Ac₂PIM₆ (diacyl phosphatidylinositol hexamannosides) are likely distributed in the outer leaflet of the MIM.⁶⁶ It is suggested that a degree of acylation in PIMs may impact the fluidization of the membrane and play a role in the membrane stress response.⁶⁸ Lipomannan (LM) and lipoarabinomannan (LAM) are lipoglycans, and they are synthesized by adding a polysaccharide backbone composed of *D*-mannan and *D*-arabinan to the PIM core.^{69,70} More specifically, LM is a polymannosylated PIM that is built from a conserved (α 1 \rightarrow α 6)-Man backbone in which some units are substituted, generally at O-2 by single α -Man. LAM corresponds to the LM with an attached *D*-arabinan domain. The nonreducing termini of the arabinosyl side chains are capped by dimannose. With their PIM anchors inserted in the outer leaflet, LM and LAM can reach the PG layer (Figure 1). Modeling the MIM in its whole complexity can help us to understand its physicochemical properties and how molecules, such as antibiotics, may diffuse in this environment.

Recent works have started to model the MIM. Adhyapak et al. recently proposed and simulated a first simplified all-atom model containing Ac₂PIM₂, CL, PE, PI, diacylglycerol, and phosphatidylglycerol (PG).⁵⁴ Li et al.⁴¹ proposed a symmetric all-atom model containing PI, PE, and CL as well as PIM₂ and PIM₆, while Brown et al. constructed both asymmetric and symmetric MIM models using coarse-grained MD simulations (Figure 3).⁵

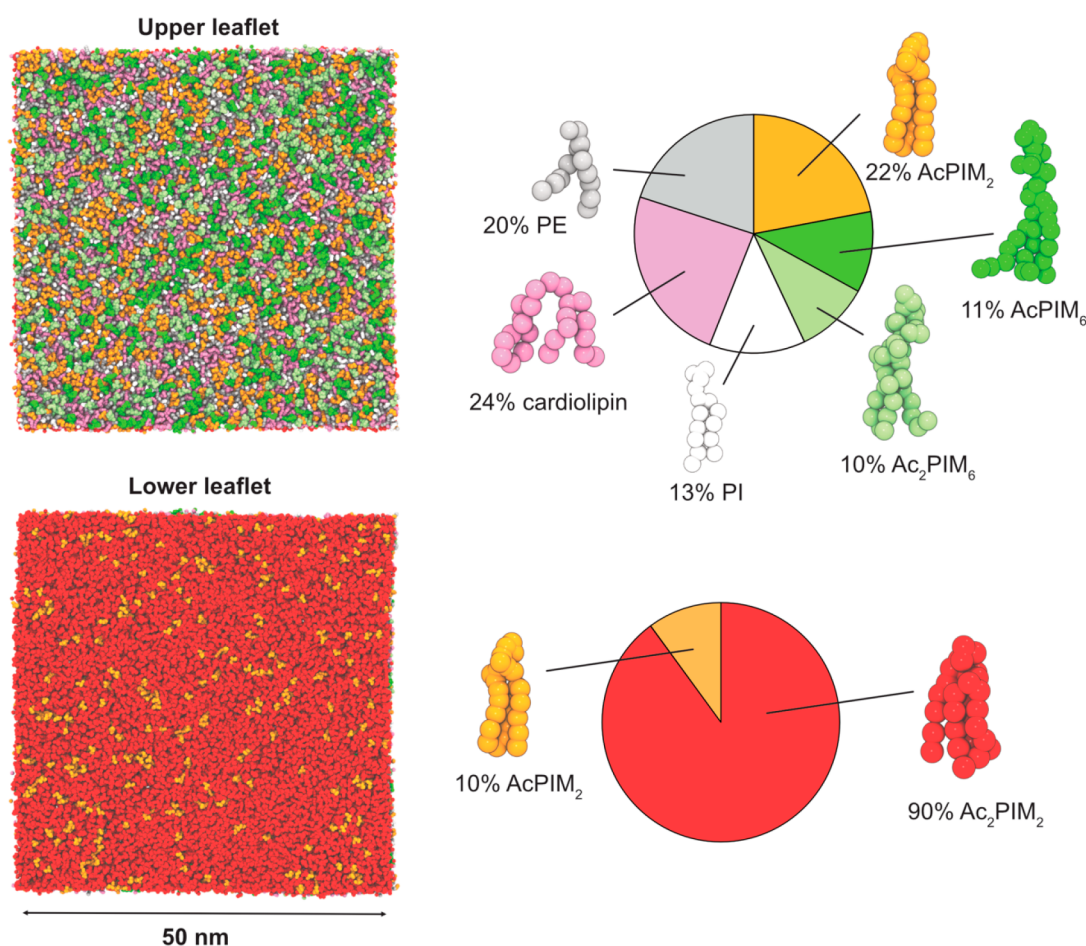


Figure 3. Coarse-grained model of the mycobacterial inner membrane with the different lipid types constituting each membrane leaflet (model from Brown et al.).⁵

■ REALISTIC MYCOBACTERIAL CELL ENVELOPES: MULTIPLE LAYERS AND PROTEINS

Beyond modeling mycobacterial lipids and carbohydrates individually or constructing lipid membranes and PG layers, it is also crucial to model important molecules that can interact with different layers of the cell envelope. Because more and more structures of membrane proteins are being elucidated, here we mention our opinions that could be relevant for this endeavor.

The MmpL family predominantly functions as endogenous lipid transporters, with many involved in the biogenesis of the multilayered cell envelope. Specifically, MmpL3 exports intracellularly synthesized TMM into the periplasm.⁷¹ TMM transport is essential for the formation of the MOM, and its driving force is thought to come from the proton-motive force (PMF) established across the inner membrane, although the precise mechanism by which the PMF drives lipid export remains enigmatic.⁷² Furthermore, several mutations in the protein core can confer resistance to antibiotics.^{73,74} To better understand its function and decipher the role of mutations, it is important to combine structural studies and MD simulations. Different structures of MmpL3 were recently determined.^{74–78} Recent all-atom MD simulations studied how TMM can diffuse from the membrane to the protein and accommodate a binding pocket in the protein.^{41,76} Further simulation studies can be carried out with more realistic MIM models to better understand MmpL3–MIM and MmpL3–TMM interactions.

Another protein of interest is ATP synthase, which synthesizes adenosine triphosphate (ATP) from adenosine diphosphate and phosphate. ATP synthase from mycobacteria possesses specific features in comparison with those in other organisms.^{79,80} ATP synthase's structure from *M. smegmatis* was resolved in the *apo* state, as well as in complex with the antibiotic Bedaquiline, the second generation diarylquinoline TBAJ-876, or a squaramide lead compound SQ31f.^{79,81,82} For Bedaquiline and TBAJ-876, seven different binding sites were identified: five around the c-ring and two at the interface between the c-ring and the a-subunit, the so-called leading and lagging sites, respectively. These sites were also identified using coarse-grained MD simulations.⁵ With the development of the MARTINI3 force field to model small molecules, coarse-grained MD simulations become a promising approach to study diffusion of small molecules toward their target membrane protein and quantify their interactions.^{83,84} Another antituberculosis drug studied by MD simulations is the SQ109 molecule (1,2-ethylene diamine) that targets MmpL3 in *Mtb*. This molecule interferes with the assembly of MAs into the cell wall core of *Mtb*.⁸⁵ Different groups used all-atom MD simulations to understand the interactions of SQ109 and other analogues with MmpL3.^{86,87} We expect that various computational methods will be applied for drug discovery targeting ATP synthase and MmpL3 at the all-atom and coarse-grained levels.

It is now possible to determine very large protein complexes such as the type VII secretion system or the mammalian cell entry (MCE) lipid transporter related to mycobacteria.^{88,89} With these structures, it is necessary to take into account not only one membrane but also multiple layers in the cell envelope to propose a complete and accurate model of these super large assemblies. While modeling a simple bilayer is now routinely performed, modeling multilayered systems remains difficult. Yet, several research groups are now able to propose multilayered models for bacterial membranes. The Khalid lab developed several models to study the cell wall of *E. coli* and how the diffusion of the antibiotic polymyxin B1 can be affected by different constituents of this cell wall.^{60,62} The Gumbart lab also studied how proteins can interact with the PG layer and the outer membrane of *E. coli* and recently proposed a model of the whole *E. coli* cell envelope spanned by the super assembly AcrAB-TolC multidrug efflux pump.^{58,90} While these examples constitute tours de force in terms of multilayer modeling, it is now essential to render this type of modeling accessible to the whole MD community. To do so, specific tools need to be developed and deployed. CHARMM-GUI's *Multicomponent Assembler*⁹¹ is being developed to combine multiple types of molecules into a single simulation, while the *polyply* software suite provides a solution to model complex and large systems currently focused on modeling systems involving polymers.⁹² In the future, similar types of tools may be adapted or developed to specifically deal with the construction of multimembrane systems related to the mycobacterial cell envelope.

CONCLUSIONS

With continuous efforts, mycobacterial cell envelope modeling and simulation are now evolving. Ongoing works are paving the way to model all different layers of the mycobacterial cell envelope. It would also be interesting to propose more membrane models related to a specific strain or a bacterial growth phase. To do so, tools to set up systems easily and automatically are essential. Some of the lipids and membrane models are already available through CHARMM-GUI *Membrane Builder*^{51,93–95} and *Martini Maker*,^{96,97} and we plan to continue to expand their repertoires to design mycobacterial cell envelopes in the near future. We hope that the studies presented in this Perspective are just the beginning of the road and more and more new MD simulations help us to understand crucial questions related to mycobacteria such as antibiotic resistance or bacterial survival in the host.

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Notes

The authors declare no competing financial interest.

Biographies

Turner Brown received a Bachelor's degree in Biocomputational Engineering from Lehigh University in 2023. He is currently pursuing a Ph.D. in Bioengineering from Lehigh University. His research interests include membrane dynamics and machine learning for drug discovery.

Matthieu Chavent obtained an Engineer degree in Biotechnology and Bioinformatics from the ENSAIA (2005) and then earned his Ph.D. in Theoretical Chemistry (2009) from the Henri Poincaré University (Nancy, France). He first did postdoctoral research at the Theoretical Biochemistry Lab in Paris, France on visualizing large membrane systems and then became a research fellow at the University of Oxford, UK to work on multiscale simulations of eukaryotic receptors. Currently, Matthieu Chavent is a CNRS researcher at the Institute of Pharmacology and Structural Biology, Toulouse, France. His lab research focuses on the development of methodologies to bridge modeling and experimental results in close collaboration with researchers working in different scientific fields ranging from X-ray crystallography and NMR to fluorescence microscopy. Recently, he is interested by combining modeling and biophysical assays to understand bacterial and eukaryotic membrane organization.

Wonpil Im is a professor of Biological Sciences, Chemistry, Bioengineering, and Computer Science and Engineering at Lehigh University. He received Bachelor's and Master's degrees from Hanyang University in Seoul, Korea. He then earned his Ph.D. in Biochemistry from Cornell University. He did his postdoctoral research at the Scripps Research Institute in La Jolla, California. Research in his lab is focused on the applications of theoretical/computational methods to chemical and physical problems in biology and material sciences. In particular, he is interested in modeling and simulations of biological membranes and associated proteins, glycoconjugates, and protein–ligand (drug) interactions. In addition, his lab has been developing CHARMM-GUI for the molecular modeling and simulation community.

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