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Multiscale Models of Antibiotic Probiotics

Yiannis N. Kaznessis

Department of Chemical Engineering and Materials Science University of Minnesota,
Minneapolis, MN 55455, USA yiannis@umn.edu

Abstract

The discovery of antibiotics is one of the most important advances in the history of humankind. For eighty years human life expectancy and standards of living improved greatly thanks to antibiotics. But bacteria have been fighting back, developing resistance to our most potent molecules. New, alternative strategies must be explored as antibiotic therapies become obsolete because of bacterial resistance.

Mathematical models and simulations guide the development of complex technologies, such as aircrafts, bridges, communication systems and transportation systems. Herein, models are discussed that guide the development of new antibiotic technologies. These models span multiple molecular and cellular scales, and facilitate the development of a technology that addresses a significant societal challenge. We argue that simulations can be a creative source of knowledge.

Keywords

Multiscale Models; Antimicrobial Peptides; Synthetic Biology

Introduction

The discovery of antibiotics is one of the most important ones in the history of humankind. For eighty years the life expectancy and the standards of living of humans greatly improved largely thanks to antibiotics. But the age of antibiotics may be coming to an end [1, 2]. New and alternative strategies must be explored as antibiotic therapies become obsolete because of bacterial resistance.

Herein we focus on the significant need for technologies to control colonization and transmission of *Enterococcus faecalis* and *Enterococcus faecium* (collectively named EF). Over the last three decades enterococcal strains have evolved to resist almost all antibiotics, including vancomycin, long considered an antibiotic of last resort for many infections [3, 4]. EF are difficult to treat and are the leading cause of surgical site infection in the US, the second leading cause of nosocomial infection, the second most common pathogen for

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nosocomial bacteremia and bloodstream infections, and the third leading cause of urinary tract infections [5].

A technology that can effectively prevent or treat enterococcal EF colonization may reduce the public health threat posed by these microorganisms. In our group, we engineer lactic acid bacteria (LAB) to detect enterococci and then, upon detection, release a molecular arsenal of antimicrobial peptides (AMPs) that specifically targets these enterococci (schematic in Figure 1).

Recently, we reported on a potent, alternative antibiotic technology [6]. We engineered *Lactococcus lactis* to detect enterococcus strains, such as *Enterococcus faecalis* and *Enterococcus faecium*, and then produce antimicrobial peptides that specifically target and inhibit EF.

This work is both a starting point for future work on antibiotic technologies and a culmination of efforts in our group and others over the past dozen years. They are a starting point because they offer a significant proof of concept. The main hypothesis we are intent on validating is that engineered LAB can reduce counts of enterococci in the gastrointestinal tract of animals. This strategy is not limited to EF, and can potentially be extended to other microbes, such as *Salmonella* spp., *Escherichia* spp., *Streptococcus* spp., and *Listeria* spp., to name a few.

The recently reported work is also a culmination of efforts, at the heart of which has been the development of modeling tools. We believe it instructive to provide a perspective on how models have improved our understanding and how they have facilitated the design of an important antibiotic technology. Herein, we summarize important milestones of these research efforts, in order to then discuss the general utility of simulations.

Molecular and cellular models of AMP activity

We have been developing multiscale modeling tools that guide explanations and predictions of the antagonistic activity of AMPs against pathogens. This is a problem that involves multiple length and time scales. In particular, we have modeled five distinct scales, from molecular to ecological interactions (schematically shown in Figure 2). In what follows, we discuss modeling formalisms at various scales and we summarize important results:

Scale 1: Atomistic models of peptides and peptide-membrane interactions

AMPs are small molecules that form an effective first line of defense against invading pathogens as part of the innate immune response of numerous species [7, 8]. Their mechanism of action, although in many ways still a mystery, appears to be based primarily on interactions with the bacterial cell membrane, resulting in rapid membrane depolarization and bacterial death.

We and others developed molecular models to study how AMPs interact with bacterial cell membranes and how they cause cell lysis and bacterial death. Using molecular simulations and free energy calculations, the key interaction steps between peptides and various cell membranes may be explored at atomistic level (schematic in Figure 3). Various classes of

AMPs, including protegrins, ovipirins, indolicins, and bacteriocins have been studied in the recent past [9-11]. In what follows, we focus the discussion on protegrin 1 (PG-1) and its analogues. This is potent, wide-spectrum antimicrobial, first discovered in pig leukocytes [12].

Atomistic molecular dynamics simulations of PG-1 have been conducted in lipid micelles and lipid bilayers as membrane mimics [13-18]. Nowadays bilayers with more than 500 lipid molecules can be modeled, and with realistic bacterial membrane-like compositions.

We explored how PG-1 interacts with lipid bilayer membranes, from a single PG-1 peptide in a zwitterionic lipid bilayer [17], to octameric PG-1 pores in a 3:1 1-palmitoyl, 2-oleoyl phosphatidylethanolamine: 1-palmitoyl, 2-oleoyl phosphatidylglycerol (3:1 POPE:POPG) lipid bilayer [18]. This POPE:POPG composition corresponds approximately to that of the *E. coli* inner membrane, and is often used as a membrane mimic in biophysical studies.

Simulations showed that the structure proposed by NMR experiments [19] is indeed stable, and that the pore opening is large enough to conduct ions. We computed water, sodium, potassium and chloride potentials of mean force through the peptide pore, and conjectured that protegrin pores can eliminate the cell's ability to regulate its transmembrane potential.

Since the free energy is the relevant thermodynamic quantity for constant temperature systems, using molecular simulations we computed the potential of mean force (PMF) for various biomolecular interaction events that underlie the activity of protegrin. We computed PMFs for PG-1 monomer and dimer adsorption to lipid bilayers (3:1 POPE:POPG) [20], PG-1 dimerization in different environments (water, membrane surface, membrane interior) [21], and studied PG-1 monomer insertion into POPE:POPG lipid bilayers.

With atomistic simulations, a clear picture emerged, culminating in a most complete, quantitative narrative of how PG-1 functions. Here, in brief, are the steps of how we now believe PG-1 kills bacteria (depicted in Figure 3):

Step 1)—PG-1 binds lipid bilayer membranes with a free energy of approximately -2.5 kcal/mol. Using atomistic models, we determined the dominant peptide orientations on lipid membranes. We found that electrostatic interactions and counterion release account for the low free energy of peptide-membrane binding [20].

Step 2)—PG-1 inserts inside the core of a lipid bilayer with a free energy of approximately -18 kcal/mol [20]. We determined that hydrophobic interactions and ion-bridges between peptide arginines, solution chloride ions and lipid phosphate groups are largely responsible for the peptide insertion. We also calculated the energy barrier for transport of the peptide inside the membrane and studied the kinetics of insertion.

Step 3)—PG-1 dimerizes preferentially in a NCCN configuration (the two C-termini are next to each other) inside the hydrophobic core of membranes with energies of approximately -8 kcal/mol [21]. We discovered that hydrogen bonds and ionic bridges are responsible for the low free energy of peptide dimerization inside membranes.

Step 4)—Four or five dimers then form a structurally stable PG-1 pore. The peptide hydrophobic cores align with the lipid bilayer hydrophobic core and depending on the type of lipids, toroidal or barrel-stave pores are observed. Carefully conducted atomistic simulations provide a detailed picture of the structure of peptide pores and of lipid arrangements, in agreement with NMR measurements [18].

Step 5)—Ions readily pass through the pore. We determined with molecular dynamics simulations that Cl^- ions move through the pore at a rate of approximately 0.5 ± 0.02 ions per nanosecond, and K^+ and Na^+ at a slower rate of 0.01 ions per nanosecond (there were an insufficiently small number of positive ions moving through the pore during the conducted simulations for accurate statistics in the transport rate to be obtained) [18, 22].

At this point the limitations of atomistic simulations became apparent and taxing. It is not without significant challenges to simulate a system with over 150,000 atoms for more than a few hundred nanoseconds (numerical integration steps are typically 10fs long; for a simulation 100ns long of a system with 100,000 atoms, the result is 3×10^{12} individual integration steps).

The question of how bacteria stop growing is then left unanswered, unless models are developed to tackle longer time and larger length scales. With the atomistic simulations as a springboard we can move up to the next level.

Scale 2: Modeling ion transport at biologically relevant scales

We developed mesoscopic, continuous models to study ion transport and depolarization of membranes attacked by AMPs [22, 23]. A Poisson-Nernst-Planck (PNP) model can be constructed fashioned by the molecular models. With the PNP model, the long-time kinetics of cell membrane depolarization can indeed be investigated.

The steady-state Nernst-Planck equation that determines ion concentrations, c_i , of ions with charge q_i , and diffusion coefficient D_i at temperature T , given an electrostatic potential field, is

$$\nabla \cdot \left(D_i \nabla c_i + \frac{D_i q_i e}{k_B T} c_i \nabla \varphi \right) = 0$$

The electrostatic potential field, φ , depends on any fixed charges arising from macromolecular structures (e.g. peptides or lipids) as well as the mobile charge arising from the space-dependent ion concentrations through the Poisson equation of electrostatics:

$$\nabla \cdot (\epsilon \nabla \varphi) = -\rho_f - \sum_{i=1}^N q_i c_i$$

Here, ρ_f represents the fixed charge density and ϵ is the (space-dependent as derived from molecular simulations) dielectric constant. The sum runs over the N mobile ionic species. Coupled equations (1) and (2) represent the PNP system of equations that describe the steady-state distribution of ions around fixed charges [23].

The fixed charge density ρ_f is determined from the positions and charges of the protein and lipid atoms in the pore structure, as determined by atomistic simulations.

The PNP equations are solved, with finite differences employing a successive overrelaxation algorithm and Dirichlet boundary conditions [23]. The electrical current across the pore can subsequently be calculated from the solution for the concentrations (c_i) and the electrostatic potential (ϕ_i), integrating the transport of ions over the membrane [22, 23]. These models use molecular information (structure of peptide aggregates and lipids, charge density, dielectric constant), yet the time scales that become available with this modeling formalism reach into the seconds. We thus provided a clear link between multiple length and time scales (nanometers to millimeters and picoseconds to seconds).

With PNP models the narrative of PG-1 action can be continued as follows:

Step 6)—The transport of K^+ from inside of a cell of 10^{-15} L in size to outside the cell and the transport of Na^+ in the opposite direction results in the collapse of the transmembrane potential of *E. coli* in less than two minutes [22]. Experimentally, we cultured *E. coli* cells and then treated them with PG-1 solutions [22]. Efforts to grow the cells two minutes after treatment were unsuccessful, indicating that transmembrane potential collapse is lethal.

Scale 3: Modeling the time-dependent effects of AMPs on bacterial cells

While membrane permeabilization by AMPs is an important part of the bacterial killing mechanism [24], the highly coupled effects of such permeabilization are not well understood. Furthermore, besides our work with PG-1, no attempts have been made that we are aware of to establish a quantitative link between molecular knowledge of AMP structure and physiological effects measured on whole bacteria.

In order to better understand whole-cell level phenomena, a model was developed to explore cell death kinetics. This model consists of a set of ordinary differential equations, one for each of the ion concentrations and another one for the amount of water within the cell [22]. The model couples the dynamics of transmembrane potential collapse, as derived from the mesoscopic model, with a whole-cell water transport and osmotic swelling model to establish the kinetics of cell death. Here is the summary of results with the modeling formalism, described in detail in ref. [22]:

Step 7)—In an apparently overkill mechanism, as ions transport quickly through pores and the transmembrane potential collapses, water rushes inside the cell increasing the cellular volume by up to 10% of its original volume. The kinetics of this phenomenon unfolds in the next five minutes. Ultimately, the membrane completely loses its structural integrity and erupts, resulting in bacterial cell death.

The seven steps proposed for the activity of PG-1 form a much simplified picture of actual phenomena. We cannot claim that this is the sole important path that explains antimicrobial activity. Indeed, we continue studying this and other alternatives. Nonetheless, this is the first time a sequence of biophysical events that are responsible for biological function is

quantitatively established in the context of antimicrobial peptides. Importantly, this narrative provokes a number of hypothetical questions that we continue addressing.

The value of this detailed understanding notwithstanding, a critical barrier in using AMP compounds alone as therapeutics exists: AMPs cannot be administered orally or intravenously for therapeutic purposes, because they may be quickly degraded, and in high initial dosages they may become toxic to host cells. In short, this is the reason why we began exploring LAB as AMP delivery vehicles. Packaging AMPs as DNA in LAB affords the delivery of antibiotic molecules inside the GI tract of hosts.

The challenge at this stage was to develop expression systems for controllable delivery of AMPs. In all cases of antibiotic development, a balancing act must be performed for benefits to outweigh negative effects. We believe that using regulatable DNA promoters may provide a means for better controlling the timing, location and dosing of AMP production. We thus turned to synthetic biology.

In the recently reported experiments of ours, we engineered promoters that respond to sex pheromone peptides secreted by EF [6]. We demonstrated that the well-studied enterococcus pheromone-responding promoter prgX/prgQ of the pCF10 plasmid [25] may be inserted in LAB and confer the ability to detect enterococci in minute environmental quantities.

Again, before experiments we relied on modeling. Multiscale models have improved our understanding of the dynamics of gene regulatory networks and of the competition between bacterial populations. In what follows we summarize the modeling formalisms employed to guide the design and testing of synthetic, inducible promoters in bacteria.

Models of synthetic biological constructs

There are two major scales of models, one implementing the molecular biology dogma on synthetic biological constructs, and the other applying population dynamics of competing microbes.

Scale 4) Gene regulatory networks

Focusing on EF detection by LAB and the subsequent delivery of AMPs, we developed kinetic models of promoter induction by EF pheromones, peptide expression and transport [25]. We discovered that the prgX/prgQ system exhibits fascinating dynamic behavior. It is an intriguingly robust bistable switch, either completely off when small or no amounts of cCF10 pheromone are present, or strongly expressing protein when a specific cCF10 concentration threshold is reached [25]. The improved understanding enabled us to engineer numerous variants of the prgX/prgQ system in LAB that exhibit stronger protein expression than the native EF system [6].

In order to model synthetic biological gene expression systems, we joined the efforts of numerous group to develop algorithms that involve stochastic-discrete models and chemical Langevin equations [26-33], as well as a closure scheme of chemical master equations [34]. We have discussed elsewhere the need for stochastic models of gene regulation and

expression, as well as the simulation of various synthetic constructs, including bio-logical AND gates, oscillators and inducible activators [35].

Here, it suffices to note that models can provide insight into complex dynamics, such as the emergence of known bistability in EF-detecting promoters. Models helped with grasping the impact of stochasticity on the strength of promoters that detect EF pheromones and regulate AMP production [25].

Scale 5)

The fifth level involves the ecology of antagonistic bacteria, e.g. LAB v. EF. At the cell population level, a model of cell-cell communication can be coupled with the negative feedback loop of growth inhibition by AMPs. These models can be parameterized using AMP activity and AMP production data generated by models in scales 1-4. Models in scale 5 can quantify experimental observations of pathogen growth inhibition by modified LAB.

This work is ongoing with numerous group developing similar microbial ecology systems [36, 37]. Here, we note that we have reported on a synthetic ecosystem comprising of bacteria and yeast that communicate with and benefit from each other using small diffusible molecules [38]. In particular, integration of well-characterized molecular regulatory elements into these two microbes allows for communication through quorum sensing. A gene controlling the growth in yeast is induced by bacteria via chemical signals and vice versa.

A stochastic model was developed for the population sizes of *S. cerevisiae* and *E. coli* that captures the relevant intra-cellular and inter-cellular interactions taking place inside the cells and in-between the cells, accounting for the intrinsic and extrinsic noise of the diffusible molecule production and transport. This model described well the dynamics of the synthetic bacteria-yeast ecosystem. Interesting dynamics that are common in natural ecosystems, such as obligatory and facultative mutualism, extinction, commensalism and predator-prey like dynamics were observed [39]. Along these lines, we are developing models of bacterial population antagonism, between LAB and EF and between LAB and salmonella.

Modeling can be a creative source of knowledge

Simulations provide an astonishing wealth of information. They can capture the thermodynamics and kinetics of biomolecular components and interactions, providing fundamental understanding of component function [40, 41].

We have developed models that quantify the molecular interactions between AMPs and cell membranes, the loss of membrane structural integrity, the collapse of the transmembrane potential, cell lysis and death. With these models, a clear timeline of biophysical steps that underlie AMP function can be created. Consequently, peptide sequence and structure may be correlated to antimicrobial activity. Simulations also improved our understanding of the dynamics of non-linear gene regulatory networks, such as the prgX/prgQ system. With these models we have demystified the construction of synthetic biological systems and put them to use in the development of antibiotic technologies.

We note that simulations are carefully crafted, comprehensive processes that involve model development, algorithmic implementation and code running, and then data analysis and inference. Models may be drawn from well-established, universally acceptable theoretical principles. For example, the transport of ions inside an evolving electrostatic field can be modeled using the Poisson-Nernst-Planck equations. Models may also be inspired by hypotheses, physical intuition or speculation. For example, the hypothesis that some antimicrobial peptides penetrate the lipid bilayer membrane of bacterial cell walls may dictate the initial conditions for modeling AMP-membrane interactions. Models can also be fashioned by appeal to experimental lines of evidence. For example, NMR studies have discovered octameric pores of protegrin AMPs to be the structural unit imparting antibiotic function.

Simulations are closely associated with theory, often as numerical solutions to analytically intractable theoretical principles. As a prominent example, consider molecular dynamics. At their heart lies the theory of Newtonian mechanics. Simulations are not however solely extrapolations of theory. Simulation studies can be based on number crunching, but they are much more than that: simulations can help us draw inferences from these numbers.

This is where parallels can also be drawn between simulations and experimentation. With multiple trials possible, changing initial conditions or parameter values, both simulation and experimentation are after significant functional dependencies. They both involve data generation and data analysis through visualization, mining and statistics. Both use these data to draw inferences and to generate hypotheses, often unfathomable with analytical theories.

Building a model is, not unlike experimentation, an arduous, well-scrutinized process that requires effort and expertise, not least because the results are never automatically reliable. The reliability of models is ultimately best sanctioned in light of both theories and experimental measurements. And the process of model development, like the process of setting up an experiment, requires constant questioning and justification of choices, an anticipation of volume and type of data to be generated and physical intuition on what exactly is modeled or measured.

Simulations certainly provide solutions to theories that admit no analytical solution; simulations also provide information scarcely obtained by experimentation. Molecular dynamics, for example, affords atomistic resolution of all the interactions between components modeled. This achievement is implausible either with analytical theory, or with experimentation.

We find simulations to be creative sources of knowledge. They create new descriptions and can be used to draw inferences and draw new hypotheses. Ultimately modeling is a scientific process that gets to the fundamental physicochemical principles of complex phenomena.

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Figure 1.

Engineered lactic acid bacteria detect enterococcal pheromones and produce a battery of enterocins (AMPs naturally produced by EF) that inhibit enterococcus.

Figure 2.

Schematic of time and length scales captured by proposed models: (1) thermodynamics of AMP-membrane interactions and atomistic detail of AMP structure-function relationship; (2) kinetics of ion transport through structurally compromised membranes; (3) time profile of cell death through the collapse of the transmembrane potential and subsequent osmotic swelling; (4) kinetics of AMP expression and production by probiotic lactic acid bacteria; (5) dynamics of the competitive inhibition of EF by LAB.

Figure 3. Schematic of AMP-membrane interactions. Shown are protegrin 1 molecules in red (yellow lines are the two disulfide bonds that stabilize the beta-hairpin structure of PG-1). Antimicrobial peptides bind to bacterial cell membranes (A), insert in the hydrophobic core (B), aggregate (C), form pores (D), and permeabilize the cells.