Microbial Membranes: A Challenge for Bioenergy

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Abstract

The production of bioenergy and bioproducts from lignocellulosic biomass promises to contribute significantly to the establishment of a clean, green economy. However, obstacles remain to making this industry economically viable. Among these is achieving high production rates and titers of target molecules through fermentation: microorganisms used in bioprocesses are inhibited by the toxic effects of biomass pretreatment and biofuel solvents which degrade the overall function of biological membranes. Here, we review historical research on the structure of the plasma membrane before detailing some progress on understanding the effects of amphiphilic solvents relevant to biofuel production on microbial membranes at the nanoscale.

Introduction

Living cells are surrounded by a thin, flexible plasma membrane (PM) that demarcates the cell's interior from its external environment and envelope specialized intracellular compartments including spores, vesicles, and organelles[1-2]. PMs are compositionally and functionally diverse biological structures that are multifunctional in that they control essential cellular processes, including cell signaling, nutrient transport, the transduction of energy in the form of ion gradients and cell motility, and also serve as a barrier to inhibitory compounds. However, it has taken us the good part of a century to understand the basic structural features of the PM that enable its multifunctionality.

A research area in which bacterial cell envelope membranes play an important industrial role is the microbial conversion of plant biomass to advanced biofuels and bioproducts. Replacing fossil fuels with renewable biofuels will reduce greenhouse gas (GHG) emissions, while providing an abundant source of energy. Microbial fermentation to achieve high product titers is a key processing step for the efficient and economical conversion of plant biomass into advanced biofuels and bioproducts. However, improvements are needed to address key limitations in these conversion processes. A significant cost barrier to the production of advanced biofuels beyond ethanol (e.g., n-butanol or isobutanol) arises from a physicochemical effect involving organic solvents: i.e., no microbe has yet been found to produce more than 30g/L of n-butanol or isobutanol in batch fermentations[3]. As a result of these limited titers, biofuel production via fermentation is currently not cost effective [4]

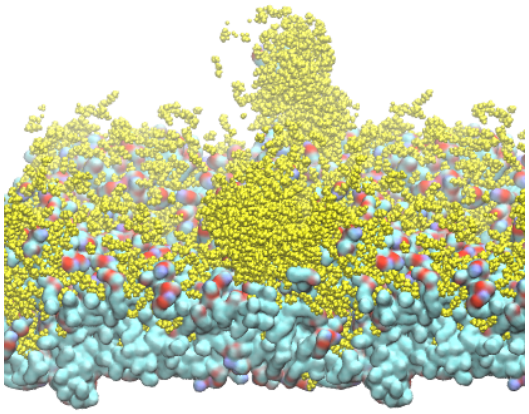

Figure 1. Computer rendering of disrupted *B. subtilis* membrane under 1-butanol stress. 1-butanol shown in yellow, teal represents hydrocarbon tails, red and magenta represent headgroups. The model was extracted from a short (30ns) isobaric-isothermal relaxation molecular dynamics simulation of two *B. subtilis* membrane slabs in an aqueous medium containing 34% (by weight) 1-butanol.

A primary target of microbial solvent toxicity is the cellular membrane, which provides the critical barrier between a microbe and its milieu. Amphiphilic solvents disrupt the molecular packing and organization of lipid membranes (Figure 1), causing altered fluidity and reduced structural integrity[5-6]. Microbes respond to the presence of added or produced solvents by changing the composition of their membranes[7-8]. Eventually, however, a threshold solvent concentration is exceeded, causing membrane rupture and cell death. Moreover, the production of next-generation “advanced” biofuels (e.g., n-butanol and isobutanol) is especially problematic, as they are more toxic than ethanol.

Deriving a predictive understanding of the effects of increased concentrations of solvent molecules on microbial membranes will elucidate the mechanisms that enable microorganisms to adapt to and survive high-solvent conditions, and eventually permit the determination of an optimal membrane composition for any given bioproduct. Based on this knowledge, microbes may then be engineered to favor robust membrane lipid compositions.

Plasma Membranes and Techniques for Studying Them

We will briefly summarize the historical evolution of PM structure and then discuss some specifics with regards to bioenergy production. Inklings of the existence of the PM started just over a century ago when Charles Ernest Overton, who in 1899 proposed a membrane model known today – not surprisingly – as the "Overton Biomembrane Model"[9]. From his studies, he surmised that the cell boundary was composed of cholesterol, cholesterol esters, and possibly fatty acids and lipids, such as lecithin. Around the same time, Agnes Pockels studied the influence of impurities on the surface

tension of fluids and devised an apparatus of her own invention[10], now commonly known as the Langmuir trough – named after Irving Langmuir – to measure the surface tension of water. Using this new piece of equipment, Gortner and Grendel got interested in determining the amount of lipid in red blood cell (RBC) membranes – RBCs are ideal candidates as they possess no membranes other than the PM[11]. By calculating the surface area of an RBC and determining the area on a Langmuir trough occupied by a monolayer of lipids extracted from RBCs, Gortner and Grendel determined that the amount of lipids extracted from the RBCs was twice their surface area, implying that the PM consisted of a double layer of lipids surrounding each RBC – it should be noted that they came to the correct conclusion even though they did not extract all the lipids from the RBCs and they underestimated the total surface area of an RBC, two errors that canceled each other out.

In 1935, Hugh Davson and James Danielli proposed a membrane model composed of a lipoid core sandwiched by two monolayers of lipids, which in turn were coated by globular proteins[12]. This arrangement of the PM came to be known as the Davson-Danielli model and was later modified by Robertson by removing the Davson-Danielli model lipoid core – i.e., a lipid bilayer was coated by protein[13]. The notion of membrane associated proteins was novel and was used to explain Danielli's surface tension studies of membranes. In the early 1960s, Mueller and Rudin showed that reconstituted membranes were self-healing, exhibited electrical resistance[14], and were fluid in the two-dimensional plane of the membrane – later fluidity was also verified by Frye and Edidin in 1970 using fluorescent tags[15]. Membrane data accumulated over three quarters of a century was eventually assimilated into the so-called fluid mosaic model proposed in 1972 by Singer and Nicolson, where free floating proteins now penetrated the liquid-like lipid bilayer and where lateral heterogeneity was implied[16] – a notion going back to Nathansohn who described the cell surface as resembling a structural mosaic[17].

Prior to *ca*. 1997, membrane-associated proteins were understood to carry out diverse functions, yet the thousands of lipid species in which the proteins resided in or associated with were thought to only play a passive role. However, the so-called lipid raft hypothesis instilled in lipids a regulatory role

whereby they were able to mediate protein diffusion and clustering, thus enabling proteins to perform their preordained roles[18]. To do so, the lipids phase separate into co-existing liquid-disordered and liquid-ordered phases. Membrane proteins then associate with one of the two phases to optimize protein-protein interactions that lead to function. In eukaryotic organisms, lipid rafts are thought to play a role in several membrane associated functions, including endocytosis, signal transduction, and membrane trafficking[19].

The notion that lipids can self-organize into discrete domains with different structural and dynamical properties is central to the membrane raft hypothesis, which postulates the existence of domains enriched in certain types of lipids and cholesterol. While lipid rafts can be studied in precisely controlled model systems, such as synthetic vesicles, until recently, their existence in the PM of living cells was surmised. Nevertheless, the consensus was that the formation of functional lipid domains in cells occurs at the nanoscale, in both temporal and spatial dimensions, making such small and ephemeral structures difficult to observe using traditional imaging techniques[20]. The size, lifetime, and connectivity of domains are all critical parameters that help define the scope of raft functionality, yet these parameters, and how they may be controlled by a cell, are poorly understood.

Modern techniques for membrane characterization

Discovered in 1932, neutrons are non-destructive probes that can interrogate length-scales from angstroms to hundreds of nanometers, and timescales from pico- to nanoseconds. Also, and in contrast to photons and electrons, neutrons can easily distinguish between hydrogen's two stable isotopes, namely protium (H) and deuterium (D)[21]. This is ideal for hydrogen rich molecules such as the lipids comprising the PM. This unique ability of neutrons enables a powerful technique, known as neutron contrast variation, that is widely used to study biological and biologically relevant membranes – it should be noted that although the different classes of biomolecules (e.g., lipids, proteins, nucleic acids, etc.) have different neutron scattering length densities, there is essentially no contrast between

members of the same class — i.e., it is impossible to distinguish, for example, between two lipid species. However, by substituting D for H, either in the solvent or in chemical moieties of the biomolecule, contrast variation can be used to highlight or mask specific biomolecules or parts of a membrane, without altering their chemistries.

The study of lateral phase separated membranes in connection to biology has received considerable attention. Although micrometer-sized lipid domains are routinely observed, those
Much smaller than 100 nm are only "visible" by techniques such as, small-angle neutron scattering (SANS), a probe free technique, that has been used to measure the size of nanoscopic lipid domains in unilamellar vesicles (ULVs) with unprecedented accuracy. Although SANS was previously used to detect lipid domains in binary lipid systems, it has only been recently applied to ternary lipid/sterol mixtures. Pencer et al.[22], for example, used phase separated ULVs to detect lipid domains smaller than 10 nm in radius in ~ 30 nm diameter. More recently, Heberle et al.[23] carried out SANS experiments on a four-component model system mimicking the mammalian PM outer leaflet and found a direct correlation between domain size and the thickness of the coexisting liquid-ordered and liquid-disordered bilayers, implying that line tension plays a central role in controlling domain size.

Significant information on detailed membrane structure has also been provided by molecular dynamics (MD) simulation, which integrates Newton's equation of motion to calculate the movements of each atom in a molecular system. MD has elucidated many aspects regarding the structure and dynamics of biomolecular systems, and this is arguably especially true for biomembranes, which are intrinsically heterogeneous and disordered supramolecular systems[24-30].
In one example, MD simulations of cholesterol, ergosterol and lanosterol in a DPPC membrane shed light on the way these sterols modulate the physical properties of membranes[31] All three sterols order the DPPC acyl tails and condense the membrane relative to the DPPC liquid phase membrane, but they do so to significantly different degrees. For example, the smooth face of ergosterol, together with tail unsaturation, not only allows it to interact more closely with the lipids, but also enables closer packing of the lipids with each other, reducing their area per lipid. Moreover, ergosterol induces a higher

proportion of *trans* lipid conformers that result in a thicker membrane with higher lipid order parameters. Compared to other sterols, ergosterol resides closer to the bilayer/water interface. In contrast, the relatively rough face of lanosterol leads to less interaction of the steroid ring system with lipid acyl chains, resulting in less well-packed lipid acyl chains. Cholesterol is intermediate between ergosterol and lanosterol. These findings may explain why ergosterol is the most efficient of the three sterols at promoting the liquid-ordered phase and lipid domain formation, and may also partly explain why cholesterol is evolutionarily preferred over lanosterol in higher-vertebrate PMs.

In addition to determining the static structures of biomembranes, neutron scattering techniques, such as neutron spin echo (NSE) can be used to accurately determine their mechanical properties. For example, using the same four-component model system studied by Heberle et al.,[32], Nickels et al.[33] combined NSE with an alternate contrast variation scheme and all-atom MD simulations to determine the bending moduli of the liquid-ordered and liquid-disordered phases populating ULVs. They concluded that besides line tension, differences in bending moduli of the two phases need to be considered when explaining the formation of lipid domains, both in model and fully functional biological membranes. In this way, Nickels et al.[33] showed that the local physical properties of phase separated lipid bilayers vary with the local composition. It is a logical extrapolation then that bilayer properties such as bending modulus, compressive modulus, viscosity, or lateral diffusion coefficient will also vary as the local composition and domain size change in response to environmental stimuli.[34] This effect is proposed as a potential buffering effect on bilayer physical properties, with the altered local compositions (transfer of high melting lipids from the ordered to the disordered phase) compensating for disordering effects such as increased temperature or the presence of co-solvents.

Microbial membranes

Although there are now many studies detailing the physicochemical properties of nanoscopic lipid domains in model membranes, until very recently, they eluded observation *in vivo*. To a great extent, that was due to the inherent complexity of living cells and the fact that high-resolution techniques rely on extrinsic labels to differentiate between groups of biomolecules. Regarding cellular membranes,

their structure has been studied by various techniques, but importantly, by different fluorescence microscopy approaches that have provided details of lateral structure as well as, important information on diffusion and other dynamical processes. However, the success of neutron scattering in the study of nanoscopic lipid domains in model membranes opened the possibility of detecting lipid domains in the PM of a fully functional, living organism. In 2017, Nickels et al.[35], published the first observation of nanoscopic lipid domains in the PM of the Gram-positive bacterium *Bacillus subtilis*, using a combined chemical–biological approach to control neutron contrast. The choice of *B. subtilis* was based on the following: (i) it was extensively studied, with a well-characterized lipid metabolism; (ii) unlike eukaryotic cells, prokaryotes grow well in deuterated media; and importantly, (iii) it has a single membrane whose lipids are constituted by only 7 fatty acids[36] that can also be synthesized in their deuterated forms, thus allowing for the fine tuning of neutron contrast. By exploiting these features, Nickels et al. were able to obtain SANS data that reported on the following: (i) the hydrophobic thickness of *B. subtilis'* PM is 24.3± 0.9 Å; and (ii) that nanoscopic lipid domains populating *its* PM are < 40 nm in size, proving not only the existence of lipid domains in the PMs of living systems, but also in the PMs of prokaryotic cells – it was thought that lipid domains were unique to eukaryotic cells.

While the largest body of information involving lipid-rafts is associated with eukaryotic systems, more recent studies have revealed that parallel membrane-associated process also occur in bacteria[37]. Lopez et al., demonstrated that the Gram-positive bacterium, *Bacillus subtilis*, possesses homologs of flotillin proteins that are functionally similar to those found in eukaryotes[38]. Furthermore, the group showed that the flotillin proteins were enriched in detergent-resistant membrane fractions, which were isolated using methods for detecting raft-associated proteins in eukaryotic cells. The flotillins were also found to co-locate with the histidine kinase, KinC, which is involved in the regulation of biofilm synthesis in *B. subtilis*; optimal function of KinC activity was shown to be dependent on raft formation. Subsequent efforts have shown that flotillin-like homologs are widespread in bacteria and have great importance in the context of antibiotic resistance mechanisms connected to stability of

microbial membrane domains (MMDs) [39]. Indeed, the field of lateral membrane organization within the highly diverse microbial world is just emerging.

As noted above, recently, microbial systems have been developed to examine both the transverse and lateral structure of biological membranes directly in living bacteria. Nickels et al. employed *B. subtilis* as a model Gram-positive organism to create a novel platform where specific isotopic labels could be introduced within the acyl-bilayer of the PM , enabling differential H/D contrast between the hydrophobic center of the membrane and the remaining cellular components, including nucleic acids, proteins, metabolites, and structural carbohydrates[35]. This was achieved by using a background strain devoid of 3-hydroxyacyl-CoA dehydrogenase (FadN), which plays an essential role in the ß-oxidation of fatty acids[40-41]. This prevents the degradation of exogenous fatty acids that are added to the culture medium. The next step involved blocking de-novo fatty acid biosynthesis. Since the type-II fatty acid synthase gene, *fabF*, is essential and cannot be deleted, the fungal-derived small molecular inhibitor, cerulenin, was added to irreversibly block activity and prevent the strain from synthesizing fatty acids. This results in a fatty acid obligate organism which can be propogated via the introduction of the desired exogenous fatty acid. Cultures growing in a minimal glucose medium could synthesize membranes from fatty acids that were supplied exogenously on a carrier protein matrix (bovine serum albumin). This fatty acid feeding strategy was exploited to introduce a defined mixture of fatty acids composed only of anteiso-pentadecanoic acid (*a*15:0) and normal-hexadecanoic acid (*n*16:0), which was sufficient to rescue growth under the imposed conditions. When cells were grown in a minimal medium prepared with 90% (*v/v*) as the solvent, 85% of the skeletal (covalent) hydrogen in the cell was replaced with deuterium. When these cells were emersed in a resuspension buffer composed of 85% D_2O, the cells were essentially “invisible” to neutrons with no detectible difference in scattering spectra relative to controls (buffer blank). When a culture is adapted to take up protiated fatty acids and synthesize membranes, while growing in a high D_2O background, the H-rich fatty acids impart strong neutron contrast within the acyl region of the membrane against the remaining cellular components and solvent. This technique enables SANS methods to be effectively employed to determine accurate physical measurements of the membrane in viable cells placed directly under the

beam allowing Nickels et al.[35] to determine the hydrophobic thickness of *B. subtilis'* PM and show that it is populated by lipid nanodomains.

MD simulations (Fig. 1) are tremendously useful in contextualizing the complexity of microbial membranes[42-43]. Atomistic models of the *B. subtilis* lipid bilayer have been developed[44] along with a range of other organisms[42, 45-47]. Introducing this level of compositional complexity is useful in mimicking biology and leads to studies of compositional effects such as the impact of unusual branched chain fatty acids in *B. subtilis* on bilayer structure and fluidity[48] or physical effects like solvent tolerance in *Saccharomyces cerevisiae* model membranes[46]. Model microbial membrane simulations have featured in efforts designed to target improvements in solvent tolerance and production of biorenewable materials[49].

Another experimental technique that can characterize dynamic properties of lipid membrane components, including lipid/water/solvent interactions, is solid state nuclear magnetic resonance (ssNMR)[50]. Line widths and intensities in ^{1}H spectra provide valuable information about molecular dynamics, while chemical shifts in ^{13}C spectra contain structural information. Moreover, the association of solvent molecules with lipid membrane bilayers and their localization in membrane bilayers can be investigated using Nuclear Overhauser Effect Spectroscopy (NOESY) NMR experiments.

Membranes and Bioenergy

The development of bio-enabled conversion processes whereby, second-generation biomass feedstocks (i.e., lignocellulosics) are transformed into high-value biofuels provides a potential pathway towards a low-net carbon future[51]. One could envision an ideal conversion process as a 'one-pot' batch process in which the polymeric matrix of lignin, hemicellulose, and cellulose is simultaneously fractionated, saccharified, and fermented/converted into a practical, easily separated mixture of fuels and high-value chemical precursors. Moreover, while substantial progress has been

made in optimizing each process (fractionation, saccharification, and fermentation), the 'one-pot' system remains elusive[52-59].

To highlight the challenges that remain, it is helpful to consider the somewhat idealized case of simultaneous saccharification and fermentation (SSF) when converting biomass using the most industrially relevant organism namely, *Saccharomyces cerevisiae*[60]. Despite its ubiquity in industry and its high tolerance to stresses such as, pH, efficient SSF is challenging to achieve due to a mismatch in optimal conditions for both processes[61]. A careful examination of the differences in the optimal conditions for both processes suggests that, while temperatures near 50^{o}C are necessary for saccharification, these same temperatures result in increased membrane fluidization, disrupting important microbial processes[60]. Worse still, is that due to changes to membrane fluidity, other stresses may be compounded with fermentation product-related stresses (i.e., the presence of ethanol), further weakening the membrane and killing the fermentation organism[62-63]. It should be noted, however, that while these stresses are a challenge, substantial efforts using non-standard microbes (such as *C. thermocellum)* to withstand these conditions have been quite successful[64], with some microbes able to sustain ethanol titers as high as 71g/L[65] – highest titers reported for 'advanced' biofuels, such as butanol production, are considerably lower at less than 30 g/L[3]While SSF has its own set of challenges, it is helpful to remember that it is not the complete, idealized, one-pot biomass-to-bioproduct conversion pipeline. In addition, it is important to recall that pretreatment stresses, such as organic solvents and biomass degradation products, are also present and impact fermentation efficiency.

Recent work considered the question of solvent stresses and found that, similar to thermal stresses found in SSF, membranes can be fluidized via disruption of membrane-water interfaces induced by organic-solvent induced disruption of lipid-lipid interactions [66]. These results suggest that careful membrane engineering that counters organic solvent inclusion may be a pathway toward overcoming solvent stresses; however, further work is needed to support this hypothesis. The fundamental processes of membrane solvation by organic solvents have been investigated by a combined MD and

SANS/SAXS approach [66]. MD and SANS are highly complementary because they overlap in the length-scales they probe and the fact that one can calculate neutron scattering observables from simulations [67]. Importantly, MD simulations provide complementary physical models of membrane systems that rationalize physical properties and enable interpretation of the neutron scattering profiles.

SANS experiments were performed examining the effects of two solvents, n-butanol and tetrahydrofuran (THF) on the structure of model lipid bilayers. These solvent stressors were chosen because THF is an effective biomass pretreatment solvent [68-69], while butanol is a microbial fermentation product. The model microbial membrane was composed of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-1′-rac-glycerol (POPG), at a 70/30 POPE/POPG ratio. SANS was used to study POPE/POPG large unilamellar vesicles (LUVs)at different solvent concentrations. It was reported that both n-butanol and THF thin the membrane, but that n-butanol has a larger membrane thinning effect than THF.

To complement the SANS data, all-atom MD simulations were performed on a bilayer—the lipid composition of which matched the above-mentioned SANS experiments—in aqueous environments containing different amounts of n-butanol or THF. With increasing organic solvent, the membrane thickness again decreases (i.e., the leaflet-leaflet head-group distances decrease), in agreement with the SANS results. The ordering of lipid molecules was also found to change upon exposure to the solvents, with n-butanol having the most pronounced effect. The solvents, especially n-butanol, also induced a clear change in membrane fluidity, evident by an increase in the apparent head-group diffusion constant and a decrease in bending moduli. Calculations of lipid-lipid hydrogen bonding (HB) indicated that both THF and butanol reduce the lipid-lipid HB.

While the SANS data indicated that the solvents partition into the membrane, it was not possible to determine whether the solvent preferentially localizes within the membrane. However, MD calculations of the average transverse distributions of the organic solvent components – relative to the membrane center – clearly showed that both THF and n-butanol were incorporated into the

membrane. Further, the incorporation of n-butanol was slightly more favorable than that of THF. Interestingly, however, THF accumulates near the lipid tails, while n-butanol is localized near the interface between the lipid heads and tails. This preferential solvation has previously been observed in the pretreatment of plant polymers for biofuel production [68, 70-72].

The fact that solvent localization is different between THF and n-butanol, suggests different modes of interactions between the two solvents molecules and the lipids. For example, solvents such as butanol that can form hydrogen bonds with the lipids may thin membranes more than molecules such as THF, which do not contain highly polar chemical moieties.

Conclusion

PT Frangopol was a pioneer in the studies of membrane perturbation by external molecules: in his case, anesthetics such as procaine and lidocaine.[73] In a similar manner, chemicals involved in biofuel generation from lignocellulosic biomass can perturb microbial membranes, and this plays into the economics of green biofuel and bioproduct generation. Here, we have summarized the basic history of the plasma membrane and then gone on to review some modern techniques for characterizing membrane structure and dynamics. Neutron scattering, by virtue of the time and length scales accessible and the possibility of specific labeling, offers opportunities to obtain contrast of heterogeneous membrane structures. Computational science, when combined with membrane experiments such as SANS can reveal the structural, mechanical, and dynamic properties of biological membranes in exquisite detail. The fundamental understanding obtained from the above studies suggests a rational way to optimize microorganisms for biofuel production. Indeed, targeted engineering of the lipid head–fatty acid tail interface may enhance microbial resistance to fermentation and bioprocessing stresses since the disruption of this interface is solvent dependent. Such insights may lead the way in physicochemical bioenergy research.

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