

Understanding Lipid Bilayer Disruption by Antimicrobial
Monoglycerides Using Molecular Dynamics Simulations

PhD Thesis

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Abstract

Monoglycerides are glycerol-fatty acid esters of which several molecules, in particular those of medium-chain length, have well documented antimicrobial potency, making them a promising research area for potential future disinfectants and antimicrobials. The antimicrobial mechanisms of monoglycerides have been extensively researched using experimental approaches, identifying that a key aspect to their potency is the ability to insert into and disrupt the biological membranes found in bacterial cells, inhibiting membrane-associated processes and cause changes to membrane properties and structure. Due to the small scale size of individual MG molecules, it is difficult to assess some aspects of this mechanism using current experimental methods, creating a need to utilise computational techniques to simulate monoglyceride behaviour within biological membranes in order to further characterise their disruption mechanisms and address current gaps in knowledge.

The research employed in this thesis aims to fill this knowledge gap by utilising all-atom Molecular Dynamics simulations to explore monoglyceride interactions with lipid bilayers at an atomistic level of detail. In the first phase of research presented in this thesis a series of simulations was created using a simplified lipid bilayer system and a variety of MGs set up in ideal conditions for examining experimental hypotheses regarding monoglyceride behaviour, providing novel evidence that bilayer disruption is disproportionately higher at high monoglyceride concentrations, that at equal monoglyceride concentrations within a bilayer the monoglyceride C8-MG causes more significant disruption than monoglycerides C10-MG and C12-MG, and that asymmetric monoglyceride distributions across leaflets cause significantly more disruption. The second phase of research repeated this methodology with more

complex bilayer models closer replicating bacterial membranes, confirming that bilayer composition affects membrane susceptibility to monoglycerides, though trends from the simplified system remained consistent. Potential bilayer stress relief mechanisms from asymmetric monoglyceride concentrations in the form of monoglyceride flip-flop and leaflet budding were also identified. Finally, free energy barriers to monoglyceride movement through lipid bilayer systems were calculated using umbrella sampling, identifying negligible energy barriers for monoglyceride entry from the water box into cell membranes. Expected timescales for monoglycerides to remain in lipid bilayers were calculated, revealing C12-MG to be the more stable in a leaflet than C8-MG and C10-MG. Energy barriers to monoglyceride movement were also shown to differ based on bilayer composition, and prior disruption of a leaflet by monoglycerides does not lower the energy barriers required for monoglyceride entry, but may lower the free energy barriers for MGs to move within the bilayer system.

The findings presented within this thesis can be used to evaluate existing experimental hypotheses regarding bilayer disruption, as well as highlight the importance and utility of molecular dynamics methodologies as a tool to complement experimental research. The groundwork laid in this thesis provides clear avenues to build upon utilising molecular dynamics simulations in order to further investigate the antimicrobial applications of monoglycerides and produce a strong overall picture of how they can be used for real-world applications in agriculture and healthcare.

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List of Abbreviations

AMR - Antimicrobial resistance

GNIM, G-IM - Gram negative bacterial inner membrane

LNP - Lipid nanoparticle

WHAM - Wheighted Histogram Analysis Method

S_{CD} - Deuterium order parameter

AIPE - 1-(12S-methylmyristoyl)-2-(13-methylmyristoyl)-sn-glycero-3-phosphoethanolamine

AIPG - 1-(12S-methylmyristoyl)-2-(13-methylmyristoyl)-sn-glycero-3-phosphoglycerol

APL - Area per lipid

C10:0 - Capric acid

C12:0 - Lauric acid

C18:0 - Stearic acid

C8:0 - Caprylic acid

CMC - Critical micellar concentration

CMC - Critical micellar concentration

DAG - Diglyceride

DFT - Density Functional Theory

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DPPE - 1,2-dipalmitoyl-rac-glycero-3-phosphoethanolamine

DPPG - 1,2-dipalmitoyl-rac-glycero-3-phosphoglycerol

FA - Fatty Acid

FF - Force Field

GMC, C10-MG, Monocaprin - Glycerol monocaprinate

GMCY, C8-MG, Monocaprylin - Glycerol monocaprylate

GML, C12-MG, Monolaurin - Glycerol monolaurate

GMS, C18-MG, Monostearin - Glycerol monostearate

GNOM, G-OM - Gram negative bacterial outer membrane

GPPM, G+PM - Gram Positive bacterial plasma membrane

IL - Inner leaflet

LJ - Lennard-Jones Potential

LL - Lower leaflet

LNP - Lipid nanoparticle

LPS - Lipopolysaccharide

MAIPE - 1-myristoyl-2-anteisopentadecanoyl-sn-glycero-3-phosphoethanolamine

MAIPG - 1-myristoyl-2-anteisopentadecanoyl-sn-glycero-3-phosphoglycerol

MBC - Minimum bactericidal concentration

MD - Molecular Dynamics

MG, MAG - Monoglyceride

MIC - Minimum inhibitory concentration

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NAMD - Nanoscale Molecular Dynamics

NP - Nanoparticle

NPT - Isothermal-isobaric ensemble

NVT - Canonical ensemble

OL - Outer leaflet

OYPE - 1-oleoyl-2-(9Z-hexadecenoyl)-sn-glycero-3-phosphoethanolamine

PAICL2 - Tetra-(hexadecanoyl/anteiso-pentadecanoyl)-cardiolipin

PAIE - 1-palmitoyl-(12S-methylmyristoyl)-sn-glycero-3-phosphoethanolamine

PAIG - 1-palmitoyl-(12S-methylmyristoyl)-sn-glycero-3-phosphoglycerol

PBC - Periodic Boundary Conditions

PBC - Periodic Boundary Conditions

PC - Phosphatidylcholine

PE - Phosphatidylethanolamine

PG - Phosphatidylglycerol

PME - Particle Mesh Ewald

PMF - Potential of mean force

PMPE - 1-palmitoyl-2-cis-9,10-methylene hexadecanoyl-phosphatidylethanolamine

PMPG - 1-palmitoyl-2-(9R,11S-methylene-hexadecanoyl)-glycero-sn-3-phosphoglycerol

POPC - 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine

POPE - 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine

PVCL2 - 1,3-bis(sn-3'-phosphatidyl)-sn-glycero-2'-phosphatidyl cardiolipin

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PYPG - 1-palmitoyl-2-(9Z-hexadecenoyl)-sn-glycero-3-phosphoglycerol

QMPE - 1-pentadecanoyl-2-cis-9,9R,11S-methylene-hexadecanoyl-sn-glycero-3-phosphoethanolamine

SMD - Steered molecular dynamics

TAG - Triglyceride

UL - Upper leaflet

US - Umbrella sampling

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Chapter 1

Introduction

1.1 Background

1.1.1 The Rise of Antimicrobial Resistance

Antimicrobial compounds are molecules with the capacity to inhibit the growth of one or more microorganisms by targeting specific aspects of a microbe. Microorganisms can be classified as bacteria, archaea, protozoa, fungi, or viruses by their physiology.

Types of microorganism differ in their lifecycle and cellular mechanisms, and so the efficacy of an antimicrobial compound can vary significantly across different target microorganisms. The range of microorganisms an antimicrobial compound can inhibit is referred to as its spectrum of activity, with broad-spectrum antimicrobials being effective against a broad spectrum of species within a group of microorganisms, while narrow-spectrum antimicrobials target a select few [2]. Antimicrobial compounds can be further grouped into classes by similarities in mechanisms of action.

Antimicrobial resistance (AMR) is a phenomenon where a microorganism is not inhibited by an antimicrobial compound it would normally be susceptible to. Microorganisms can have innate resistance to a compound due to exhibited characteristics, the absence of key molecules targeted by a drug, or molecules produced by the organism from their genetic code which inactivate a drug [3]. Microorganisms can also acquire antibiotic resistance mechanisms through multiple routes, including random mutation, direct transfer from neighbouring cells, or transfer mediated by

viruses such as bacteriophages [4].

AMR is most well known as antibiotic resistance in bacteria. Antibiotic resistance is a natural and expected process, with the first records of antibiotic resistance being observed in penicillin-resistant *Staphylococcus aureus* in 1942, within 15 years of penicillin being first discovered, and within two years of penicillin first being introduced for use in hospital settings [5]. Despite this, antibiotic resistant bacterial strains have been a cause of increasing concern over the decades, as the incidence rate of antibiotic resistance has been rising at an alarming rate. This has primarily been attributed to increased exposure of antibiotics to bacterial species with more widespread use in agriculture and healthcare sectors.

In healthcare, AMR can arise from the overuse of antibiotic drugs to treat infections. Antibiotics can be misprescribed by medical professionals to treat non-bacterial infections, generally due to misidentification of the cause of infection. For example viral or fungal infections require antiviral or antifungal drugs respectively to kill the target pathogen rather than antibiotics [6]. In these situations, the drug's mechanisms of action will not treat the infection, and the use of the antibiotic may lead to the development of resistance in harmless bacteria within the host microbiome, which can then be passed to pathogenic bacteria via horizontal transmission [7]. Despite the common belief that prematurely finishing a course of antibiotics increases the risk of antibiotic resistant bacteria developing, there is conflicting evidence as to how true this is, as a week long course of antibiotics has been found to be as effective as a 10-14 day course for several infections, and prolonged use of antibiotics can act as a selection pressure to drive the development of antibiotic resistance [8, 9]. Poor sanitation practices can also transmit antibiotic resistant bacteria between patients, staff, and visitors, colonising the microbiomes of other individuals and therefore spreading the prevalences of antibiotic resistance genes across a community [10].

Antibiotic use is also prevalent in industrial farming and agriculture. Industrial factory farming is used for the mass production of animal products in a relatively small area of land. Factory farming has been associated with several antibiotic resistant bacteria strains, including in common foodborne pathogens *Escherichia coli*

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and *Salmonella enterica* [11]. Over 80% of antibiotics sold are for use in farming, 70% of which share a mechanism with clinically used antibiotics. [12] Antibiotics are used in farming to treat infections in animals, which often arise due to the poor housing conditions of farmed animals. Additionally, sub-therapeutic dosages of antibiotics added to animals feed have been found to enhance growth of livestock [13]. Antibiotic resistant bacteria that emerge in livestock can then be passed on to humans zoonotically. There have been reports of livestock-associated Methicillin-resistant *Staphylococcus aureus* (MRSA), a multidrug resistant bacterial "superbug" which poses particular challenges to both agriculture and medicine being transferred from animals or their products to humans [14, 15]. Furthermore, both traces of antibiotics used in animal agriculture, and antibiotic resistant bacteria present in the animals themselves, can be spread to humans through consumed animal products such as meat, which has been associated with increased prevalence of antibiotic resistant bacteria in humans [16]. Waste from animal agriculture containing antibiotics can also leach into and contaminate water supplies from use as crop fertiliser, increasing the prevalence of antibiotic resistance in waterborne bacteria [17]. Environmental antibiotic pollution has also been associated with poor water treatment, human waste management, and pharmaceutical waste products, although most environmental antibiotic concentrations are below the thresholds required to select for resistance phenotypes in bacteria [18, 19].

While not all bacteria are inherently pathogenic to humans, AMR among pathogenic bacteria has been a cause of concern globally. Antibiotic resistance is considered a leading cause of morbidity, with one study predicting a rise to 10 million deaths a year globally caused by bacterial infections unresponsive to antibiotic therapy by 2050 [20]. A more recent study identified that based on current trends 1.91 million deaths could be directly attributable to antimicrobial resistant infections by 2050, rising from 1.14 million deaths reported in 2021, and a further 8.22 million deaths could be associated with antimicrobial resistant infections [21]. One of the biggest concerns with the increasing prevalence of antibiotic resistance is the reduced ability to treat infections as more antibiotics decline in efficacy [22]. Antibiotic development is a long and costly process, with little financial incentive for companies to engage with, and

as fewer novel antibiotic drugs are produced while antibiotic resistance continues to rise, there has been an increased research focus on finding new strategies to mitigate antibiotic resistance [23]. Mechanisms of antibiotic resistance, the issues with antibiotic development, and methods of overcoming antibiotic resistance are all discussed in greater detail in the next chapter.

1.1.2 Monoglycerides as Antibiotic Alternatives

One strategy to overcome antibiotic resistance is the discovery of molecules that display antibacterial activity through mechanisms not utilised by traditional antibiotics. One of these types of molecules, the focus of this thesis, is monoglycerides (MG). MGs are non-polar single-tailed lipid molecules which are esters between a fatty acid (FA) and a glycerol molecule. MGs can be saturated or unsaturated and are amphiphilic molecules, with a hydrophobic region represented by the acyl chain region, and a hydrophilic head group represented by a glyceryl region (Figure 1.1). MGs within this thesis are commonly referred to with the nomenclature "CX:Y-MG", or simply "CX-MG" for saturated MGs, which refers to a MG with a FA tail that is X carbons long and contains Y double bonds. MGs exist naturally as intermediate molecules in triglyceride synthesis, and are therefore typically present in low concentration in fats and oils where triglycerides are found [24].

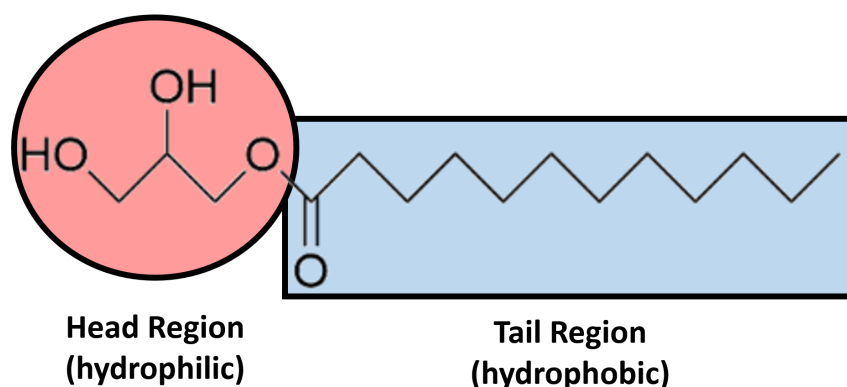


Figure 1.1: Annotated structure of glycerol monolaurate. The red circle represents the hydrophilic head group of the molecule. The blue rectangle represents the hydrophobic acyl tail of the molecule.

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MGs form nanoparticle (NP) structures in aqueous environments, explained in more detail in the following chapter, due to the hydrophobic effect, which is the tendency of non-polar and hydrophobic molecules to cluster together in aqueous environments, maximising water-water interactions. The simplest of these NP structures is the lipid micelle, which is a spontaneously assembled structure that occurs above the molecule's critical micellar concentration (CMC) threshold, a concentration threshold where monomeric MGs will self-assemble into this structure. The CMC threshold differs between MGs, as micelles composed of shorter tailed MGs are less stable [25]. Some of these nanoparticle structures, in particular cubic phase nanoparticles (cubosomes) have been widely researched for their potential as drug delivery systems used to increase the accumulation of drugs at a specific target, as cubosomes can be loaded with both hydrophilic and lipophilic drugs due to their bicontinuous structure which contains both water channels and lipid bilayer regions. [26] Additionally, micelles are commonly utilised in experimental studies to assess MG effects on lipid bilayers [27–29].

Several MGs have been reported to have antimicrobial potency. Antimicrobial activity in MGs has been discussed in literature since the 1910s [30]. Antimicrobial potential has been reported against both Gram-positive (G+) and Gram-negative (G-) bacteria, which are two broad categories of bacterial cell envelope morphology (explained in the following chapter) that display significantly different antibiotic resistance profiles, as well as some fungi and enveloped viruses [31–33]. Against bacteria, MGs have broad spectrum activity, but G+ bacteria are generally more susceptible to the molecules than G- species. [34] An explanation of the architectural differences between G+ and G- bacteria that are attributed to differences in susceptibility towards MGs are discussed in the following chapter. Significant antimicrobial potency in MGs has been widely reported in saturated medium chain length MGs (6-12 carbon acyl chains), with glycerol monocaprate (C10-MG) and glycerol monolaurate (C12-MG) having the greatest efficacy within this group [35]. Some unsaturated MGs also have known antimicrobial potential, such as glycerol monolinolenate (C18:3-MG), and glycerol monoarachidonate (C20:4-MG) [36]. MG tail length in particular has been a key variable tested experimentally with respect

to the disruption mechanism. Even among medium-chain length MGs (MCMGs), where antimicrobial activity is most commonly reported there are notable distinctions between MGs in this group. C12-MG is known to be the most potent experimentally, though it is important to note that in the context of lipid bilayer disruption discussion the greatest potency is not necessarily an indicator of that molecule producing the greatest disruption of bilayer properties at equal exposure within the bilayer. In this context, potency refers to C12-MG causing inhibitory or bactericidal activity at the lowest concentration in solution, which may be related to its comparatively lower CMC threshold and greater micelle stability compared to shorter tailed MGs [28].

The mechanism of antibacterial activity utilised by MGs, discussed in detail in the following chapter, is associated with the disruption of the bacterial cell envelope and associated cellular processes. MGs self-insert into the phospholipid bilayer (cell membrane) and disrupt its properties, increasing small molecule permeability and destabilisation of the bilayer structure. At high MG concentration, generally above CMC, leakage of cellular contents through bilayers exposed to MGs has been observed experimentally, as has the lysis of the cell membrane [27, 37]. Importantly, this membrane disruption mechanism is not shared by traditional antibiotic drugs so there is little . Although some membrane-targeting antibiotics such as polymyxins produce a similar outcome with antimicrobial MGs of compromising the bacterial membrane, there remains distinct mechanical differences between these molecules, as MGs are hypothesised to act through biophysical interactions with the lipid bilayer, while polymyxins target lipopolysaccharide (LPS) thus making them ineffective against G+ bacteria while MGs display efficacy against both G+ and G- bacteria [38, 39]. Additionally, the MG mechanism of action appears to have a high barrier to resistance emergence, as has been observed in time passage studies experimentally, while emerging resistance has been observed in FA counterparts [40]. This high barrier to resistance development is hypothesised to be caused in part by the predominance of hydrophobic interactions in MG-based bilayer disruption in comparison to FA counterparts that also utilise electrostatic interactions due to being charged at relevant conditions [27, 39]. Additionally, lipid membrane targeting mechanisms are generally considered to have

a slower rate of resistance development because they target the integrity of the lipid bilayer rather than a singular molecular target that can be modified through genetic mutation [39].

1.1.3 Potential Applications of Monoglycerides as Antibiotic Alternatives

The antibiotic effects produced by MGs have potential use in several cases. Firstly, their ability to kill bacteria displays potential for MGs as an alternative to antibiotic drugs. Due to mechanisms used by MGs, there is little existing resistance to these molecules within bacterial populations, which could give MG-based therapeutics a niche as treatment options for infections unresponsive to traditional antibiotics. The reported broad spectrum nature of the MG mechanism of action would also enable effectiveness against a range of infections. Antimicrobial MGs can also be incorporated into lipid NPs (LNPs) for use in drug delivery systems. While some MGs without known antimicrobial potency produce more stable NP formations, cubosome formulations that include antimicrobial MGs could be utilised as an additional antibacterial treatment when loaded with antibiotic drugs, creating a drug delivery system with innate ability to destabilise the bacterial cell envelope, which could to improve antibiotic efficacy, as combination antibiotic/cubosome treatments *in vitro* have shown greater bacterial killing than antibiotic alone [41, 42]. Synergy has also been observed between antimicrobial MGs, and as such the use blends of MGs may further improve antimicrobial activity [43]. Ultimately, this use would be far in the future, due to the rigorous trials required to bring a drug to market for human use, however the reported high biocompatibility of MGs suggests these formulations may display low toxicity towards mammalian cells [44].

Antimicrobial MGs can also be utilised in agriculture. Antimicrobial MGs have been considered as an alternative to antibiotics as an animal feed enhancer. The use of specific MGs and MG blends have already been considered for use in swine and poultry farming, where particularly C12-MG have been shown to modulate gut microbiomes for these animals through mechanisms unrelated to their antimicrobial

activity [45]. C12-MG has also been reported to enhance the digestion of dietary fats in animals, improving growth [46]. Antimicrobial MGs have been specifically attributed to preventing the transmission of some enveloped viruses and bacterial necrotic enteritis in pigs [47, 48]. Also within the food sector, MGs have roles in food preservation. Antimicrobial MGs have been assessed for their ability to reduce contamination in a variety of foods. In dairy products, the shelf life of cheese can be increased by 12 days by the addition of a blend of C12-MG and its FA counterpart lauric acid (C12-FA) [49]. Addition of C12-MG to milk was reported to reduced the growth of *Bacillus licheniformis* [50]. C12-MG has also showed promise in inhibiting the spoilage of meat and baked products [51, 52]. MGs have been used in the food industry as emulsifiers to improve product stability [53]. Antimicrobial MGs have also shown potential in disinfectant solutions, both in solutions where they are the primary disinfectant molecule, and when mixed with other disinfectant molecules. C12-MG has been described as having potential as a disinfectant for chicken meat decontamination, and C10-MG has been shown to inhibit the growth of fungi and bacteria associated with dental caries [33, 54]. Emulsions of C10-MG and capric acid (C10-FA) have been reported to maintain antimicrobial efficacy and stability for at least 12 months against *Campylobacter spp.*, indicating potential longevity for such disinfectants [55].

1.2 Thesis Aims

Although antimicrobial MGs show potential for use in a wide array of real-world applications, particularly in the agricultural sector, the full extent of the mechanisms that underpin their antibacterial applications is not fully understood due to inherent limitations of the experimental approaches utilised to characterise these mechanisms. This thesis aims to model the interactions between a range of MGs with or without known antimicrobial potential with various phospholipid bilayer compositions using Molecular Dynamics (MD) simulations in order to improve our understanding of how MGs enter and interact with phospholipid bilayers, and how lipid bilayer properties can be disrupted by their insertion into the structure. In accordance with this aim, the

Chapter 1. Introduction

objectives of this thesis are:

- To provide a review of our current understanding of antimicrobial activity in monoglycerides, and provide the historical context which has led to increased interest in monoglycerides as a potential method of overcoming antibiotic resistance.
- To study how monoglyceride insertion into phospholipid bilayers influence bilayer properties and may lead to bilayer destabilisation using molecular dynamics simulations.
- To understand how differences in monoglyceride structure and bilayer lipid composition may influence the degree of destabilisation that lipid bilayers undergo using molecular dynamics simulations.

The work presented in this thesis is supported by and partially funded by the ABITEC Corporation. ABITEC is a leading global manufacturer of lipids with a vested interest in the potential real-world applications that antimicrobial MGs may provide. While the work produced in this project has a greater focus on the fundamental mechanisms of antimicrobial activity in MGs, it is carried out under the belief that improving understanding of how MG mediated bacterial envelope disruption occurs will enable further optimisation of antimicrobial potential in MG nanoparticles, thereby producing higher quality products which can produce more significant bacteriostatic or bactericidal effects, reducing our reliance on antibiotic-derived products in the future.

1.3 Research Hypotheses

The research presented in this thesis was conducted to address the following hypotheses:

- The extent of monoglyceride-induced membrane disruption is directly correlated with monoglyceride concentration within the bilayer.
- Glycerol monolaurate, which is considered the most potent antimicrobial monoglyceride, causes the most significant disruption to bilayer characteristics.

- Asymmetric stresses generated by monoglyceride self-insertion is a significant driving force in lipid bilayer destabilisation.
- Phospholipid bilayer lipid composition influences susceptibility to monoglyceride-mediated destabilisation.

1.4 Thesis Overview

This thesis is structured as follows:

Chapter 2 describes the biological background of this thesis. An explanation of bacterial envelope structure and how bacteria develop resistance to traditional antibiotics is provided. In addition, strategies for overcoming antibiotic resistance are discussed, alongside an in-depth review of our current understanding of the antimicrobial applications and mechanisms of fatty acids and monoglycerides.

Chapter 3 describes the Molecular Dynamics theory relevant to the computational work carried out in this thesis. This further provides context for the work carried out to produce this thesis.

Chapter 4 provides a detailed methodology for the simulations and analysis techniques utilised to produce the work presented in this thesis.

Chapter 5 analyses the data collected from a series of Molecular Dynamics simulation in which model 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid bilayer systems were simulated with varying concentrations of monoglyceride molecules pre-inserted into the structure. From this, an improved understanding of how monoglycerides disrupt bilayer properties and destabilise its structure is built up.

Chapter 6 further expands on the conclusions of Chapter 5 by simulating monoglyceride interactions with realistic bacterial membrane models representing both Gram-positive and Gram-negative bacteria. This highlights important aspects of how bilayer composition influences bacterial susceptibility to antimicrobial lipids.

Chapter 7 discusses the translocation of monoglycerides across POPC and bacterial membrane models in relation to free energy barriers required for entry and movement across leaflets. This provides evidence for the behavioural differences

Chapter 1. Introduction

between different monoglyceride structures in regards to bilayer disruption.

Chapter 8 summarises the results and findings presented in this thesis, and provides suggestions for future follow-on work.

Chapter 2

Bacteria, Antibiotic Resistance, and Antimicrobial Lipids

The roles played by bacterial communities within an environment must always be considered in related fields and industries. The presence of bacteria in an environment is often not a cause for concern, and can provide significant benefits to some industries which make use of bacteria to produce highly desired products, such as cheese, antibiotics, and biopolymers [56–58]. Despite this, some bacterial species are well known for their pathogenic effects when infecting other organisms, which in some cases can be fatal [59]. This is a significant cause for concern for public health, and consequently, improving the treatment of bacterial infections and preventing the transmission of bacteria are growing areas of research.

Bacterial infections are usually treatable through the administration of antibiotic drugs, however there is an increasing prevalence of resistance to these drugs among clinically relevant species. Consequently, bacterial infections are becoming harder to treat. Antibiotic resistance continues to increase with the improper management of antibiotics, particularly in healthcare and agriculture. This can cause bacterial communities to be exposed to antibiotics at sub-bactericidal levels, increasing the risk of resistance emerging [60, 61]. Antibiotic resistance is considered a leading cause of morbidity, with one study predicting a rise to 10 million deaths a year globally caused by bacterial infections unresponsive to antibiotic therapy by 2050. A more recent study

elaborated on this, suggesting that based on current trends 1.91 million deaths could be directly attributable to antimicrobial resistant infections, rising from 1.14 million deaths reported in 2021, and a further 8.22 million deaths associated with antimicrobial resistant infections [21].

To better contextualise the work carried out in this thesis, this chapter provides a review of our current understanding in this field, elaborate on the antimicrobial mechanisms of MGs, and explain the key gaps in literature regarding their mechanisms.

2.1 Bacterial Cell Envelope

All cells contains an outer layer which provides protection from the external environment. Structurally this outer layer differs by cell type, for example, plant cells have a rigid envelope composed of a cell wall and a plasma membrane, while the outer layer of mammalian cells is composed only of a plasma membrane [62,63]. This makes individual mammalian cells more susceptible to environmental factors than cells containing a more robust cell envelope such as bacteria. However, mammalian cells generally organise into complex organised arrangements called tissues that primarily perform specific functions (such as muscle tissue movement), but which also provide significant protection to cells through the extracellular matrix that holds cells together. This can significantly hinder the ability of molecules, e.g. MGs, to directly interact with the plasma membrane of mammalian cells [64]. Bacterial cells feature both a cell wall and at least one cell membrane in their envelope, creating an exoskeleton-like structure that allows the cells to exist in environments that would be hostile to eukaryotic cells (cells with a nucleus, e.g. fungal and mammalian), particularly in relation to osmotic pressure, as the bacterial cell envelope has a higher structural integrity [65]. The structure of the bacterial cell envelope can differ significantly between bacterial species, giving rise to identification tests based on these differences. One of the most important of these is a technique called Gram staining, which uses a stain that attaches to peptidoglycan in the bacterial cell wall, classifying a bacterium as G+ or G- dependent on whether the cell envelope can retain the stain. G- bacteria cannot retain the stain as their envelope features an outer lipid membrane which

prevents the stain from reaching the cell wall, whereas G+ bacteria feature the cell wall as the outermost cell envelope component. Although there are some bacteria which are unresponsive to Gram staining, this is a quick identification test which divides bacteria into two broad groups, allowing further differentiation tests to be performed to identify the bacterial species [66]. In the context of understanding lipid bilayer destabilisation in bacteria, the composition of the cell envelope is crucial to both the potential barriers that need to be overcome in order for disruption to be observed and the observed in the bacterial cell membrane, as the lipid composition of the cell membrane can significantly affect its stability [67].

The cell envelope of a bacterium is crucial to both its function and survival [68]. Proteins embedded within the envelope architecture facilitate vital cellular processes to maintain correct cellular function. These processes include nutrient uptake, which can either occur through passive diffusion of small non-polar molecules through the envelope or mediated by transport proteins and channels embedded within the cell membrane, among others. The primary method of energy production utilised by bacteria, the electron transport chain, which produces the essential energy molecule ATP, is also located within the cell envelope [69]. The bacterial cell envelope also has a significant role in bacterial pathogenicity, as this is the region of the cell which interacts with the external environment. Specifically, the cell envelope enables adhesion of bacteria to surfaces, including host tissues, and can prevent the entry of antibacterial molecules to inhibit their function [70, 71]. Additionally, components of the envelope, such as lipopolysaccharides and lipoteichoic acids, are recognised as pathogen-associated molecular patterns by receptors in the host immune system, triggering induction of the innate immune response against the bacterium [72].

2.1.1 Gram-Positive Bacterial Cell Envelope

The G+ bacterial cell envelope is composed of a cell wall and a plasma membrane, where the cell wall is oriented at the interface with the external environment (Figure 2.1) [73]. A small compartment known as the periplasmic space separates the cell wall from the cytoplasmic membrane, which in turn acts as a barrier for the cytoplasm.

The periplasm is an aqueous environment which facilitates the transfer of stress from the plasma membrane to the cell wall. This compartment is very thin in G+ bacteria, and its existence has been debated [74]. The cytoplasm contains most bacterial cellular machinery, vital for crucial cellular processes including protein synthesis and cell metabolism. The cell wall is composed of the polysaccharide peptidoglycan, composed of mainly amino acids and sugars, which cross-links with other peptidoglycan molecules via amine bonds between peptide chains to form a rigid network structure [75]. While peptidoglycan itself is not anionic, the addition of teichoic acids to the structure induces a net negative charge into the cell wall.

Due to the thick exterior peptidoglycan layer, the cationic crystal violet stain used in Gram staining binds strongly to the cell wall, which then complexes with iodine stabiliser and remains bound to the cell wall, causing the purple colour observed under the microscope [66]. The cell wall also contains teichoic acids that are anchored to the peptidoglycan structure and have roles in the cell wall structure. As a standard, the peptidoglycan wall ranges from 20 to 40 nm in thickness, depending on G+ bacterial species [75]. Cell wall permeability can vary, but small molecules can readily diffuse through the structure [76]. The ability for a molecule to pass through the membrane is dependent on several properties, including charge, lipophilicity, and degree of polarity rather than just molecule size. Non-polar gases and polar molecules up to a molecular weight of up to 1000 Dalton have reported to passively diffuse across cell membranes [77].

The composition of the G+ cytoplasmic membrane, which is a lipid bilayer with embedded proteins, can vary significantly between bacterial species but is primarily composed of phospholipids and cardiolipin [73]. The cytoplasmic membrane has a net negative charge, due to both the presence of negatively charged phospholipids and teichoic acids, which are anchored to the cell membrane by a glycolipid tail [65]. Between the cell wall and plasma membrane is the periplasmic space.

Proteins also comprise a portion of the bacterial cell envelope. Proteins exist in different sections of the cell envelope, and can be found as part of the cell wall, the periplasm, and the cell membrane [65]. The cell wall often contains anchoring proteins

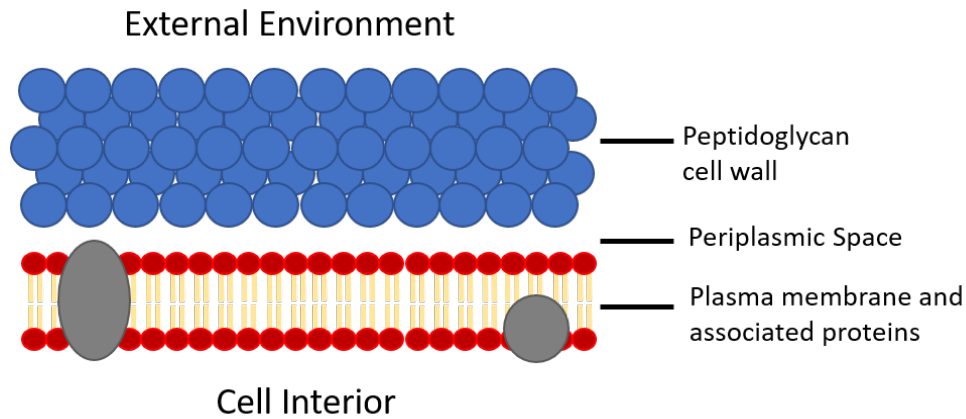


Figure 2.1: Simplified Gram-positive cell envelope architecture.

which allow attachment to host cells, aiding in infection [78]. Proteins present in the cell membrane include enzymes involved in synthetic pathways, particularly those for peptidoglycan, and transmembrane channels to facilitate nutrient transport and efflux of toxic substances [73].

2.1.2 Gram-Negative Bacterial Cell Envelope

In contrast to the G+ cell envelope, consisting of a single cell wall and lipid membrane, G- bacteria have an envelope which consists of an outer lipid membrane, cell wall, and inner lipid membrane (Figure 2.2).

The outer membrane (OM) is the outermost layer of the G- bacterial envelope. While the OM is a lipid bilayer, it is not a phospholipid bilayer, as the G- inner membrane (IM) and G+ cytoplasmic membrane (PM) are. The external environment facing leaflet of the OM is composed of lipopolysaccharide (LPS) and glycolipids, while the inner leaflet is composed of phospholipids [65]. Other than its influence on cell morphology, the only known function of the OM is as a barrier [65]. The dense structure of LPS acts as a permeability barrier due to its tight packaging, inhibiting the ability of toxic substances, such as antibiotics, from reaching their target and disrupting bacterial function [79]. The OM also contains associated proteins, which are primarily associated with the phospholipid leaflet. The structure of LPS differs between bacterial species

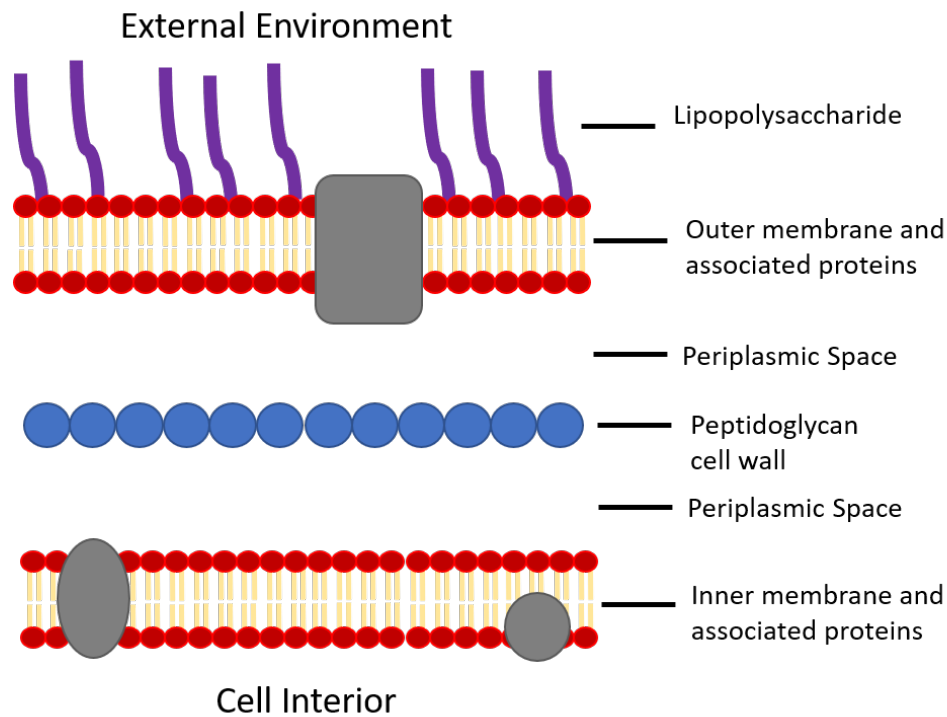


Figure 2.2: Simplified Gram-negative cell envelope architecture.

and its polysaccharide chain, known as the O antigen, is used to classify pathogenicity in some species - namely *E. coli*, as LPS is strongly associated with G- bacterial sepsis due to its potent immunogenicity [80]. The OM has a net negative charge due to the presence of LPS [81].

The G- cell wall is located within the periplasmic space between the outer and inner bacterial membranes. In comparison to the G+ cell wall, the G- cell wall is a thin layer of peptidoglycan, around 2-3 nm and is attached to the OM by Braun's lipoprotein [82].

The periplasm of G- bacteria is much larger than its G+ equivalent, being roughly 15nm thick, and some up to 30 nm in thickness [83, 84]. The periplasm further contributes to the higher antibiotic resistance profile of G- bacteria by creating a compartment to allow the sequestering of toxic molecules before entering the cytoplasm, enabling them to be removed from the cell using efflux channels in the OM. The periplasm is also utilised by the bacterium for several functions, including protein post-translational processing and sequestering of bacterial enzymes that are toxic to cytosolic

components [83].

The IM is a negatively charged phospholipid bilayer [65]. The composition of the bilayer varies between bacterial species, but a large proportion of the structure tends to be composed of phospholipids with phosphatidylglycerol (PG), and phosphatidylethanolamine (PE) headgroups. The G- IM contains a lower proportion of cardiolipin than the G+ plasma membrane [1]. In G- bacteria, the IM is the site of most membrane-bound processes in the cell. It is the site of the electron transport chain, biosynthetic pathways for peptidoglycan and lipids, and facilitates the transfer of proteins produced by bacterial ribosomes in the cytoplasm into the periplasmic space [65]. The combination of the OM and IM in the G- bacterial cell envelope creates a strong permeability barrier, which can significantly inhibit the ability of toxic molecules to permeate through into the cell exterior, which can make G- bacterial infections harder to treat than G+ infections. [79]

2.2 Traditional Antibiotics

Antimicrobial compounds are molecules with the capacity to inhibit the growth of one or more microorganisms by targeting specific aspects of a microbe, which can be classified as bacteria, archaea, protozoa, fungi, or viruses by their physiology.

Types of microorganism differ in their lifecycle and cellular mechanisms, and so the efficacy of an antimicrobial compound can vary significantly across different target microorganisms. The range of microorganisms an antimicrobial compound can inhibit is referred to as its spectrum of activity, with broad-spectrum antimicrobials being effective against a broad spectrum of species within a group of microorganisms, while narrow-spectrum antimicrobials target a select few. [2] Antimicrobial compounds can be further grouped into classes by similarities in mechanisms of action.

Antibiotic drugs are antimicrobial molecules which specifically target bacteria. Antibiotic drugs have a history spanning nearly 100 years, beginning with the discovery of penicillin by Fleming in 1928, and have been widely used in medicine since the 1940s [85]. Since the discovery of penicillin, numerous classes of antibiotic drugs have

been discovered, targeting different bacterial mechanisms across the whole range of bacterial species. In medicine, both human and veterinary, there are a wide variety of mechanisms which can be used to treat bacterial infections without producing toxic side-effects, as bacterial cells are prokaryotic (lack a nucleus and membrane bound organelles). Therefore, bacteria show less physiological similarity to mammalian cells than other eukaryotic cells such as fungi (Figure 2.3) [86]. Antibiotics are non-specific in their targeting, and therefore the overuse of antibiotics can often kill beneficial bacteria in the gut as a side-effect, which can cause alterations to microbiome diversity and cause unwanted side effects, for example changes to metabolism [87]. Opportunistic pathogenic bacteria normally only present at low concentrations in the body due to being outcompeted by other bacteria may also increase in population significantly due to microbiome changes caused by antibiotic usage, causing opportunistic bacterial infections to emerge [88].

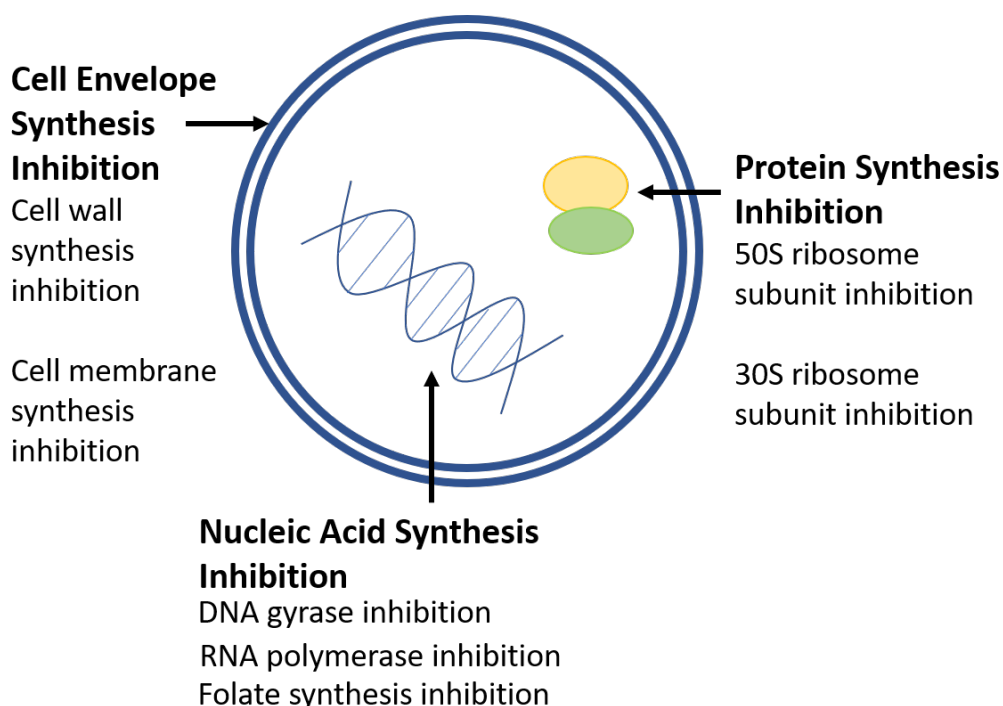


Figure 2.3: Common bacterial pathways targeted by antibiotic drugs.

Broadly, antibiotics can be categorised mechanistically as preventing bacteria from dividing (β -lactams, quinolones) and preventing essential molecules from being

produced (aminoglycosides, tetracyclines, growth factor analogues). The major classes of antibiotic and their respective mechanisms of action are as follows:

- β -lactams (methicillin, amoxicillin) target penicillin-binding proteins (PBPs) found in the bacterial cell wall. The binding of PBPs by penicillin-class drugs halts the cell wall elongation process by inhibiting peptidoglycan synthesis and preventing bacterial cell growth and division [89].
- Growth factor analogues (sulfamethoxazole, sulfacetamide) function by acting as substitutes for typical bacterial growth factors required for bacterial cell growth. Sulphonamides act as an analogue for para-aminobenzoic acid (PABA) which is involved in the synthesis of the growth factor folic acid. The substitution of sulphonamides for PABA prevents the formation of folic acid, therefore inhibiting bacterial growth [90,91].
- Quinolones (ciprofloaxin, levofloaxin) target bacterial DNA replication by targeting DNA gyrase and topoisomerase IV. Both enzymes are utilised in the DNA supercoiling process and function by producing DNA fragmentation. Inhibition of these enzymes prevents the DNA elongation process or causes chromosome fragmentation, triggering DNA repair processes and potential cell death [92].
- Aminoglycosides (streptomycin, gentamicin) target protein translation. Members of the class target the 30S subunit of the bacterial ribosome which functions to bind mRNA in order to initiate protein translation. Without the ability to synthesise new proteins, the bacterial cell cannot carry out protein-dependent cellular processes and undergo apoptosis [93].
- Tetracyclines (doxycycline, tetracycline) have a similar mechanism to aminoglycosides, also interacting with the 30S ribosomal RNA subunit. Tetracyclines instead prevent the binding of tRNA to the ribosome, thereby preventing the elongation of peptide sequences to prevent protein synthesis within the target bacterial cell [94].

Further information regarding the discovery and development of antibiotic drugs can be found in Appendix A.1.

2.3 Antibiotic Resistance in Bacteria

Bacterial phenotypes that tolerate the presence of an antibiotic drug are referred to as a strain which exhibits antibiotic resistance. Antibiotic resistance in bacteria is identified *in vitro* by the use of minimum inhibitory concentration (MIC) testing, which determines the minimum concentration of antibiotic required to inhibit the growth of a specific bacterial species overnight [95]. MIC values are compared against clinical breakpoints established by the European Committee of Antimicrobial Susceptibility Testing in Europe to determine whether a bacterial isolate is susceptible or resistant to an antibiotic at clinically relevant concentrations [96, 97]. A bacterium may be able to resist antibiotics either through the development of specific mechanisms which prevent the activity of an antibiotic drug, which is known as resistance, or through non-specific means which cannot be passed to offspring, which is known as tolerance [98]. Further information on how antibiotic resistance develops and is spread within bacterial populations can be found in Appendix A.2.

2.4 Combating Antibiotic Resistance in Bacteria

As reported by the UK Health and Security Authority, antibiotic resistance has continued to increase in prevalence, rising from 62,314 reported in 2019 to 66,730 serious human infections in 2023 [99]. Due to the rising rates of multi-drug resistance (MDR) in bacterial pathogens, there have been growing concerns amongst healthcare and academic professionals that a point will be reached where potential treatment options for bacterial infections will become severely limited as MDR phenotypes become more widespread within bacterial populations. These concerns have been further amplified by some bacterial strains isolated from nosocomial environments displaying significant resistance to last resort antibiotics used in their treatment [100]. To combat this, there are several strategies which have potential for overcoming

current emerging trends in increasing antimicrobial resistance. These strategies include the development of novel antibiotic drugs, enhancing the abilities of existing antibiotic drugs to overcome resistance mechanisms, and the development of therapies which utilise alternative antimicrobial molecules - molecules which can inhibit bacterial growth or kill bacterial cells through mechanisms which are unrelated to existing antibiotic pathways and therefore have low naturally occurring resistance among bacteria. An overview of antibiotic production and strategies to discover new antibiotics can be found in Appendix A.1. Further information on strategies to enhance the efficacy of existing antibiotics, and a general review of alternative antimicrobial molecules can be found in Appendix A.3.

One of the most promising prospective alternative antimicrobial strategies might be to utilise lipids. Lipids are a broad class of both natural and synthetic molecules, some of which display antimicrobial activity. Lipid-based compounds have shown promise as a means of overcoming antimicrobial resistance. Structurally, lipids are typically amphiphilic esters of carboxylic acids and alcohols. The structure of a lipid is typically identified by an alcohol backbone with one or more carboxylic acids bound to it by ester links, though structural variance exists among different subclasses (Figure 2.4). Lipids have three major roles in maintaining cellular function: formation of cell membranes, acting as a source of energy storage, and functioning as both primary and secondary messengers in signalling pathways. Lipids can be divided into several subclasses, many of which are produced for specific functions (Table 2.1).

Table 2.1: Select lipid species and their common functions.

Lipid Species	Functions and Roles	Ref
Triglycerides	Lipoprotein production, dietary fats	[101, 102]
Sphingolipids	Signal transduction in mammalian cells	[103]
Polyketides	Signal transduction, antimicrobial activity	[104]
Sterols	Signal transduction, membrane component, lipoprotein synthesis	[105]
Prenols	Signal transduction, vitamin synthesis	[106]
Saccharolipids	Lipopolysaccharide synthesis, pro-inflammatory agent	[107]
Phospholipids	Signal transduction, membrane component	[108, 109]

While primarily known for other functions, a subset of lipids have known antimicrobial activity, making them a potential source of novel antimicrobial

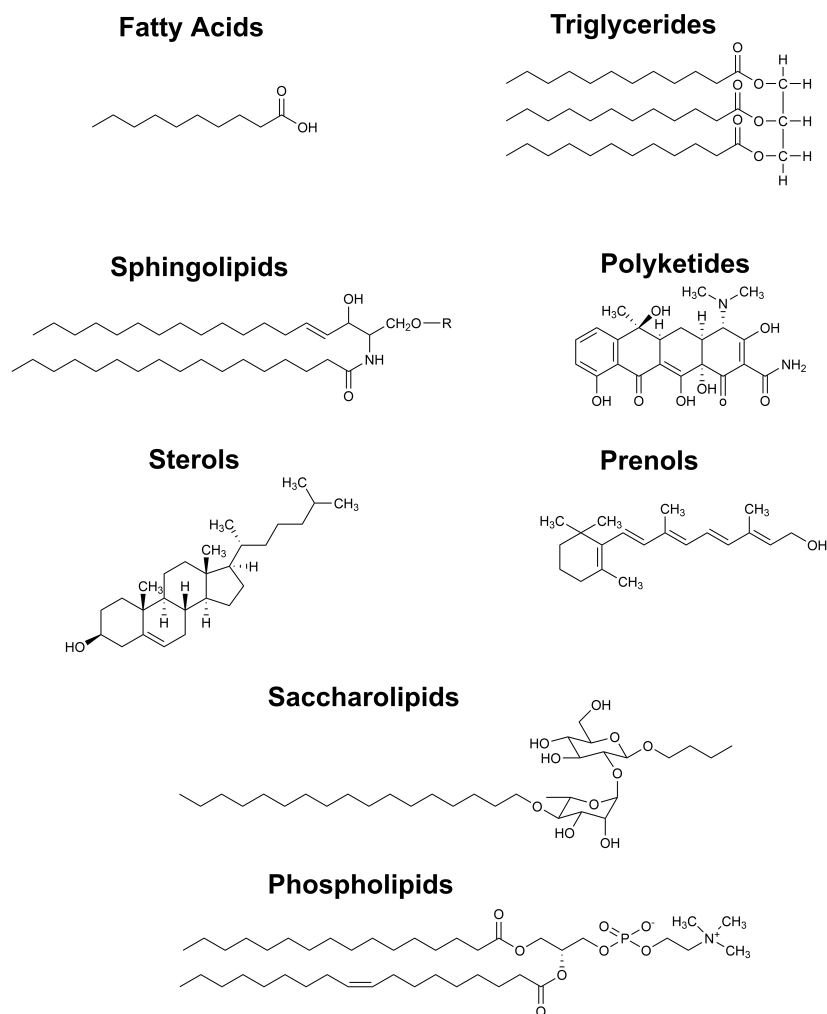


Figure 2.4: Major lipid groups and example molecular structures.

therapies [110, 111]. Antimicrobial activity in lipids is commonly seen in the mammalian immune system, where they are known to be an important component of innate immunity. Sphingoid bases and FAs have displayed a broad spectrum of antimicrobial activity towards G⁺ and G⁻ bacteria [112]. Antimicrobial activity in lipids has also been observed in the plant immune system against both eukaryotic and prokaryotic organisms [113]. Bacterial species may also produce lipids with antimicrobial efficacy themselves, which can be isolated for use as antimicrobial agents. Bacterially produced antimicrobial lipids include rhamnolipids, which have among the most notable antimicrobial activity within the lipid family and have been observed

experimentally to produce significant destruction of lipid membranes when treated with rhamnolipids through solubilisation of the lipid bilayer [114,115].

Among lipids with known antimicrobial activity, some of the most well studied groups are the FAs and MGs [39]. FAs are structurally simplistic molecules, being a hydrocarbon chain containing with a single carboxylic acid head group. MGs are esters of a single FA molecule with a single molecule of glycerol, creating a lipid with a FA tail and glycerol head group (Figure 2.5).

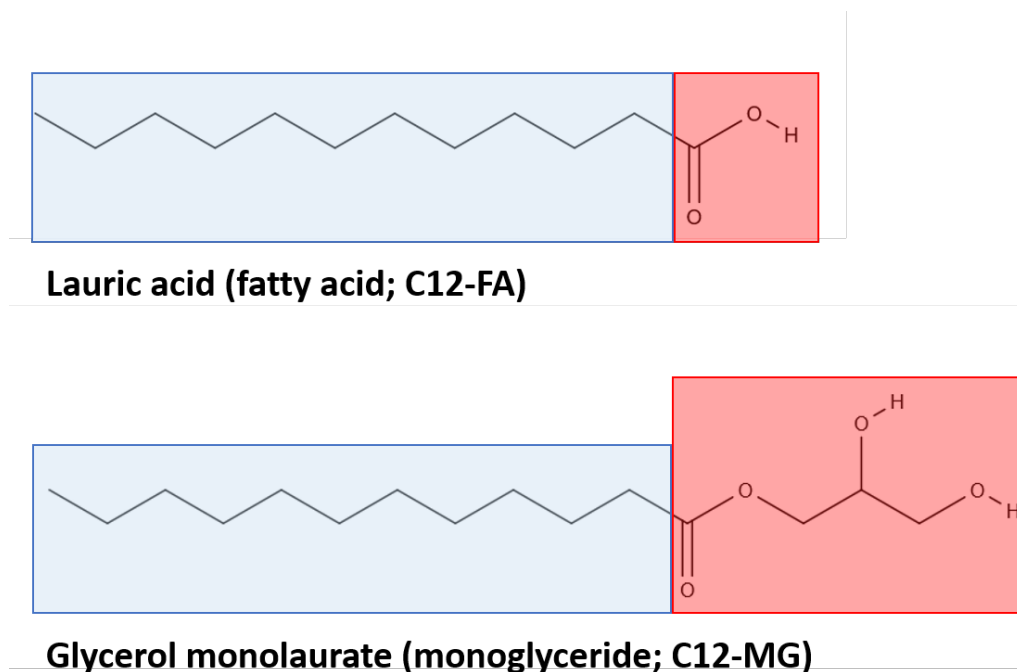


Figure 2.5: Structure of C12-FA lauric acid (top) and C12-MG glycerol monolaurate (bottom). Blue represents the hydrophobic tail region of the molecule. Red represents the hydrophilic headgroup region of the molecule.

FAs and MGs are diverse in both tail length and the degree of tail unsaturation. MGs can be referred to as 1-MGs if the ester link is on an end alcohol group, or 2-MGs if the ester link is on the middle alcohol group (Figure 2.6).

FAs have many roles and functions, being found ubiquitously in both eukaryotic and prokaryotic organisms. FAs and their derivatives or metabolites have key roles in the non-specific immune system found across both animal and plant immune systems [116,117]. Furthermore, the vast majority of fat molecules found in breast milk are FAs

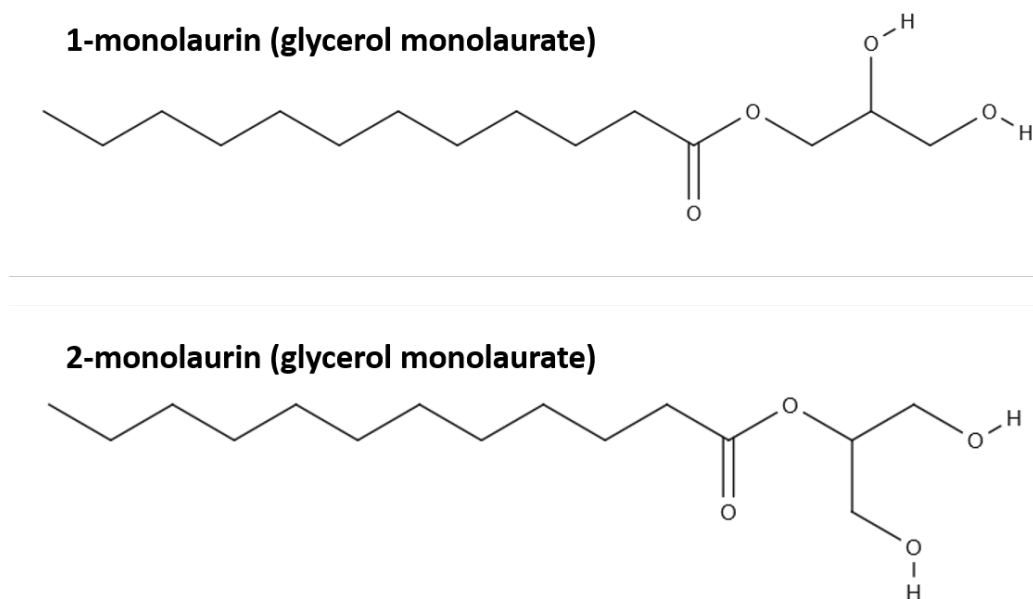


Figure 2.6: Structural differences between the 1- and 2- conformations of C12-MG. The acyl tail of 1-monolaurin is bound to the terminal alcohol group of glycerol. The acyl tail of 2-monolaurin is bound to the middle alcohol group of glycerol.

or triglycerides (TGs), which are believed to aid in preventing neonatal infection and assist in early immune system development [118]. Omega-3 FAs are common dietary supplements which are thought to promote cardiovascular health, and also act as a mammalian cell membrane component [119,120].

MGs similarly have a wide variety of functions and uses. Glycerol monolaurate (C12-MG) is another known antimicrobial lipid which has been identified in breast milk with known broad spectrum antimicrobial activity [121]. Long chain MGs such as glycerol monostearate (C18-MG) have reported functions as emulsifiers and stabilisers, and can be used as a non-toxic food additive and in formulations for skincare products [122,123].

2.5 Structure and Properties of Fatty Acids and Monoglycerides

FAs are hydrocarbon chains with an attached carboxyl group. FAs are considered short chain (SCFAs) if the hydrocarbon chain is fewer than 5 carbons in length; medium chain (MCFAs) if between 6 and 12 carbons in length, long chain (LCFAs) if between 13 and 21 carbons in length, and very long chain (VLCFAs) if 22 carbons or greater. Most naturally synthesised FAs have an even numbered carbon chain, and are not branched, though odd-numbered and branched FAs do exist [124].

FAs can be either saturated or unsaturated. Saturated FAs follow the structure of $\text{CH}_3(\text{CH}_2)_n\text{COOH}$, where n represents a number of CH_2 groups. For example, lauric acid (C12 FA), can be represented by $\text{CH}_3(\text{CH}_2)_{10}\text{COOH}$. Unsaturated FAs contain one or more $\text{C}=\text{C}$ double bonds. Cis double bonds introduce kinks into the chain, causing bending of the structure in comparison to those of saturated FAs. This bending is more pronounced in cis-polyunsaturated FAs, as more cis double bonds are present in the structure (Figure 2.7).

Trans-unsaturated FAs do not induce as significant changes to the chain structure. Cis-unsaturated FAs have lower melting points than trans-unsaturated FAs as the bends caused in their structure result in looser packing of molecules, thereby reducing intermolecular forces [125]. The looser packing of cis unsaturated FAs also increases structural fluidity more than saturated and trans-unsaturated FAs.

MGs are single tailed molecules which consist of a single FA and a single molecule of glycerol connected by an ester link. 1-MGs are formed between the FA and one of the primary alcohols of glycerol, and 2-MGs are formed between the FA and the secondary alcohol. MGs are part of the glyceride lipids class, which also includes the two tailed glycerol-FA esters known as diglycerides, and the three tailed triglycerides. FAs are considered hydrophobic molecules, with only the carboxyl group having hydrophilic properties, while MGs are amphiphilic. The degree of hydrophobicity increases in line with acyl chain length, which alters the ratio between the hydrophobic and hydrophilic regions of the molecules in favour of hydrophobicity. Neither MGs or FAs are water

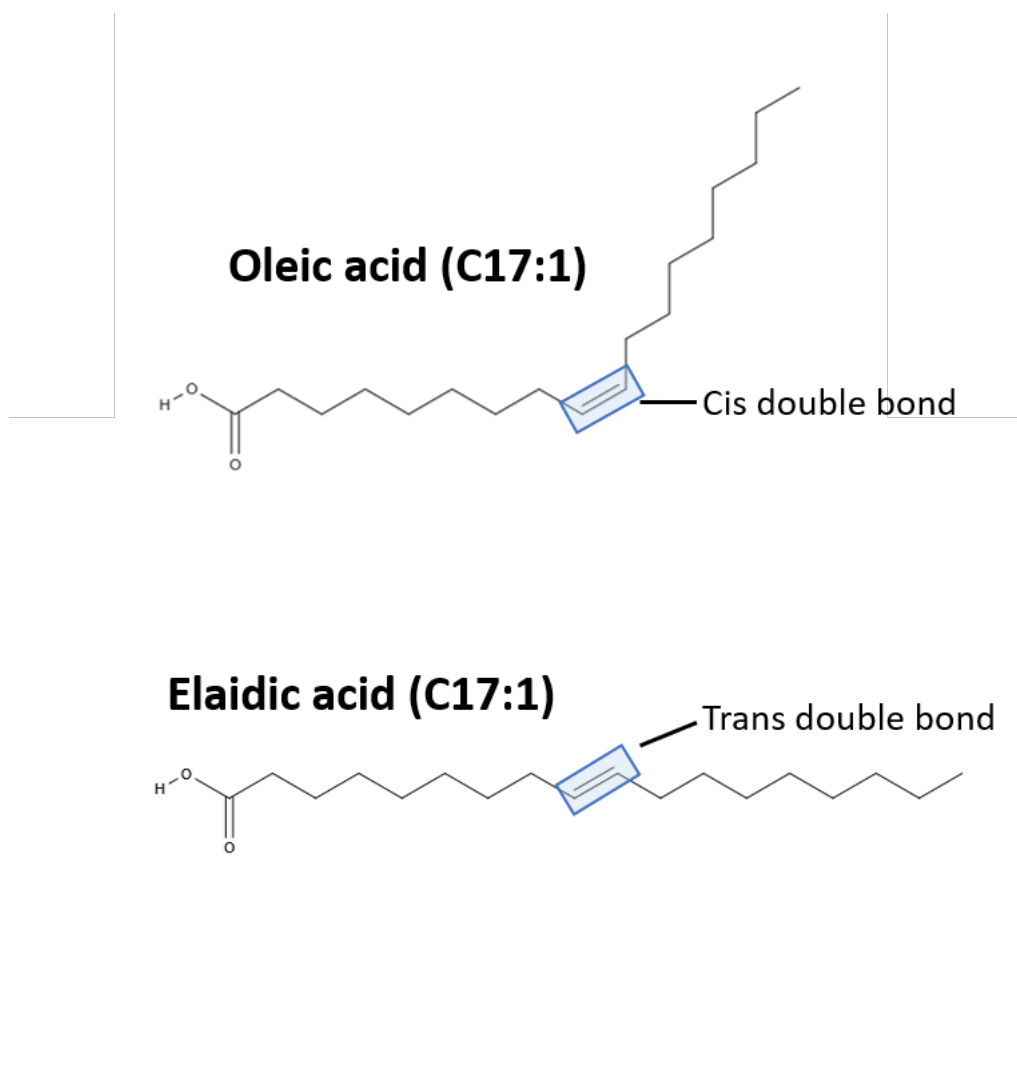


Figure 2.7: Comparison of how cis (top) and trans (bottom) double bonds affect FA structure when at the same position in the molecule.

soluble, however due to their amphiphilic nature MGs are surface active and can act as emulsifiers between oil and water phases [126].

MGs are generally more stable than FAs at an equal tail length and saturation, with MGs having consistently higher melting points than their FA counterparts. This can be a difference upwards of 20°C in some cases [127]. FAs, with a pK_a around 5, are typically deprotonated at laboratory and physiological conditions (pH 7.0 - 7.4) while MGs remain neutral with a pK_a around 14 [128]. The pK_a of both FAs and MGs is

higher for molecules with a longer tail length, and is not static [129].

FAs and MGs self-assemble into aggregate structures in water due to the hydrophobic effect. This is the tendency of nonpolar and hydrophobic molecules to cluster together in aqueous environments, maximising water-water interactions [130]. Structures include micelles, lamellar phases, and cubic phases (Figure 2.8). Micelles are relatively simple spherical NPs which are structured so that the hydrophilic head groups face outwards towards the water, while tails form a hydrophobic core. Nanomicelles are a subset of micelles which range in size from 10-100 nm and have lower critical micellar concentration (CMC) thresholds which need to be met for the FAs or MGs to assemble into the structure [131]. Lamellar phases feature a bilayer structure similar to that of a cell membrane. These bilayers are stacked on top of each other in a sheet like formation. Cubic phases are liquid crystal lipid structures which have a bicontinuous structure.

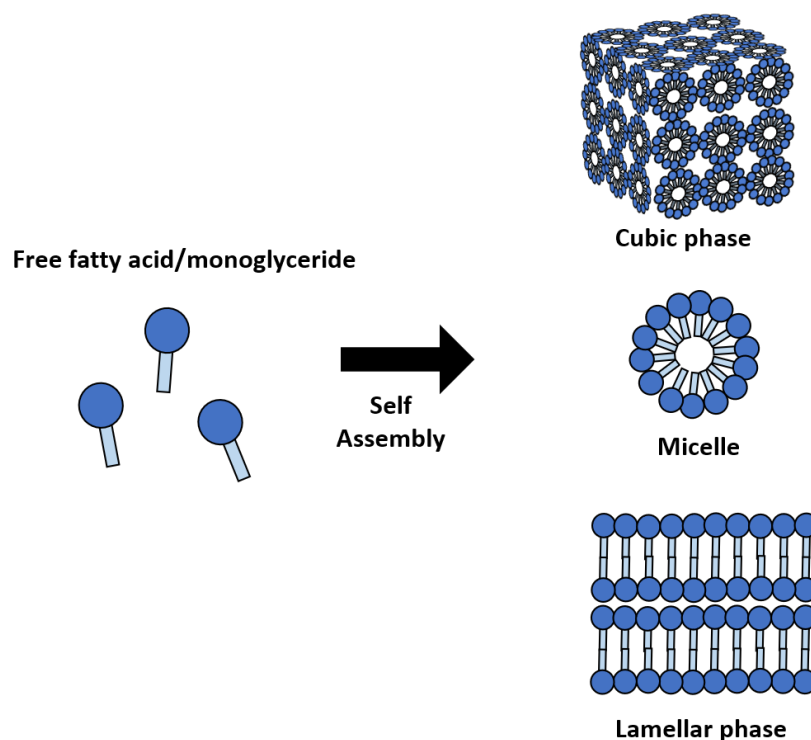


Figure 2.8: Example structures that can spontaneously form via FA and MG self-assembly.

The most significant structural difference between FAs and MGs at equal tail length is their headgroups. The headgroup of FAs is a negatively charged carboxylate (COO^-) at physiological pH. This causes FAs to have stronger electrostatic interactions than MGs and causes electrostatic repulsion between individual FA molecules. The MG headgroup, as a glycerol molecule, is physically larger than the FA carboxylate group and neutrally charged at physiological pH. Consequently, individual MG molecules do not experience electrostatic repulsion when near each other, which FAs molecules do. Instead, hydrogen bonding between molecules and water, facilitated by the two hydroxyl (OH) groups present on the glycerol are the main headgroup interactions seen in MGs. The lack of electrostatic repulsion and the presence of hydrogen bonding enables tighter MG packing and greater diversity in self-assembled NPs than FAs, forming the structures described above, while FAs most commonly form micelles. Electrostatic repulsion between FAs also influences the CMC in comparison to MGs of equal chain length as overcoming repulsion is an additional energetic cost to FA micellisation which MGs do not experience [39, 132].

2.6 Sources of Fatty Acids and Monoglycerides

FAs and MGs are naturally occurring molecules, and can be obtained from many different sources. FAs are produced naturally by both plants and animals through synthesis pathways and can also be synthesised using industrial processes. MGs are found in trace amounts in any source that typically contains FAs or TG [133, 134].

Animal FAs tend to be saturated, while plant FAs tend to be unsaturated [135, 136]. FAs and MGs can be found in many animal products, including milk, eggs, and meats. In meat, fats are primarily located in adipose tissue or in muscle tissue. The fat content of meat is predominantly made of TGs, which are common energy storage molecules in higher organisms [137]. TGs feature three esterified FA molecules, which tend to be unbranched molecules with a tail length of 12-14 carbons. FAs and MGs in eggs are localised almost entirely within the yolk, and primarily function to provide energy and nutrients to the embryo [138]. FAs in milk are suspended in hydrophobic globules which consist of a phospholipid membrane surrounding a core of FAs and TGs [139].

In most plants, oleic acid (C18:1-FA) and linoleic acid (18:2-FA), where CX:Y indicates a FA with X carbon atoms and Y double bonds, are the most common unsaturated FAs [140]. In plants, FAs and MGs can be stored in the cytoplasm of cells in the form of droplets or within plastids [141]. FAs and MGs can be found in most plant tissues, particularly high concentrations have been reported in fruits and seeds [142]. Due to plant FAs being more commonly unsaturated, plant fats tend to be oils at room temperature while animal fats are solid.

Some FAs and MGs are commonly produced by both plants and animals, while others can only be produced by specific sources. Both saturated and unsaturated C16- and C18-FAs are commonly found in plants and animals [143]. Because of this, MG esters of these FAs are also common within fats and oils. FA content can also differ between species, therefore also affecting MG content. For example, C12-MG, which can be found in both plants and animals, is abundant in human milk but not cows milk [121]. The synthesis of FAs and MGs is discussed further in Appendix A.4.

Microorganisms, like higher organisms, also produce FAs for a variety of functions. FAs are essential for the synthesis of membrane lipids and normal membrane function [144]. Some bacteria store FAs as TGs, while others store long chain FAs as esters with long chain alcohols [145]. FAs synthesised by bacteria tend to be shorter than eukaryotic FAs and are usually saturated or monounsaturated. Some bacteria can also produce branched FAs or FAs with hydroxyacyl groups [145]. MGs are not typically produced by bacteria, however some may produce MGs as an intermediate product during TG hydrolysis [146]. Bacteria have been utilised for biotechnological applications to produce FAs through genome editing [147].

2.7 Inherent Antimicrobial Efficacy of Fatty Acids and Monoglycerides

The ability to inhibit bacterial growth has been identified in a range of FAs and MGs. The potential for antimicrobial efficacy appears dependent on the structure of the MG, with medium-chain FAs and MGs reportedly having greater antimicrobial potency than

longer chain MGs. A summary of select medium and long chain saturated FAs and MGs can be seen in Table 2.2.

Table 2.2: Reported antimicrobial activity of select FAs and their MG esters.

Tail (name)	FA Antimicrobial Activity	MG Antimicrobial Activity	Ref
8:0 (caprylate)	Broad spectrum activity against bacterial mastitis pathogens; greater efficacy against G+ bacteria than G-, <i>E. coli</i> most tolerant.	Broad spectrum activity against bacterial mastitis pathogens; greater efficacy against G+ bacteria than G-, <i>E. coli</i> most tolerant.	[148]
10:0 (caprate)	Broad spectrum activity.	Broad spectrum; Significantly improved potency in comparison to longer chain MGs	[33, 35, 55, 149]
12:0 (laurate)	Broad spectrum activity; inhibition of superantigen production at sub-bactericidal levels.	Broad spectrum activity; inhibition of superantigen production at sub-bactericidal levels; two-hundredfold greater potential in comparison to lauric acid. No notable resistance after yearlong exposure of <i>S. aureus</i> to sub-inhibitory concentrations.	[35, 40, 121, 150–153]
14:0 (myristate)	Broad spectrum activity; particularly against Gram positive bacteria.	Some broad spectrum activity with greater potency against G+ bacteria.	[35, 154–156]
16:0 (palmitate)	Broad spectrum antibacterial and moderate antifungal activity.	Limited to no antimicrobial activity reported.	[156, 157]
18:0 (stearate)	Limited activity against Gram positive and negative bacteria.	No significant antimicrobial activity.	[35, 158]

Broadly, antimicrobial efficacy in FAs is inverse to acyl chain length [35]. The MCFAs lauric acid (LA; C12-FA) has been noted to have the greatest efficacy against G+ bacteria, while capric acid (CA; C10-FA) also has high efficacy against G- bacteria. The LCFAs oleic acid (C18:1-FA) and linoleic acid (C18:2-FA) display antimicrobial activity against G+ bacteria, despite the saturated C18 FA, stearic acid's (C18:0-FA) limited observed activity [159].

Among saturated MGs, the medium-chain MGs glycerol monocaprate (C10-MG) and C12-MG are the most consistently reported to display antimicrobial activity. Antimicrobial activity observed in MGs is usually greater than in the equivalent

FA, which is hypothesised to be attributed to the lower CMC required for MGs to spontaneously form micelle nanoparticles in comparison to FAs [160]. Additionally, MGs maintain potency at high pH as they lack the carboxyl group present in FAs which has a pK_a of around 5 [161].

C12-MG was reported to produce a more than two-hundredfold increase in bactericidal effectiveness in comparison to its FA equivalent, lauric acid in one study by Schlievert and Peterson (2012) [40]. Another study by Schlievert *et al.* (2019) assessed the effects of C12-MG in human milk identified antimicrobial activity against 4 of the 5 bacterial species tested, with the only uninhibited species, *Enterococcus faecalis*, producing a growth factor, reutericyclin, which acts as an analogue of C12-MG. This study further identified the antimicrobial properties of C10-MG, as removal of lipophilic molecules from human milk resulted in the loss of antimicrobial activity, which could then be restored through the re-addition of C12-MG to the milk [121]. Furthermore, C12-MG has also been proven to be active against other microbes, including enveloped viruses such as HIV-1 and Zika virus, and some fungi [162,163]. Whether C12-MG or C10-MG was determined to have greater potency varies depending on the study. Wang *et al.* (2020) reported C10-MG to be the most potent saturated MG against many foodborne bacteria [35]. A comparison of formulations containing C12-MG or C10-MG for treatment of *Neisseria gonorrhoeae* carried out in Bergsson *et al.* (1999) identified that while both inhibited bacterial growth, C10-MG produced a greater than 6-log reduction in viable bacteria at 1 mM with a 2 minute exposure time, while C12-MG produced a 3.4 log reduction, meaning C10-MG was significantly more effective at preventing *N. gonorrhoeae* growth [164].

FAs and MGs have known potency against most ESKAPE pathogens, which are a group of 6 MDR bacteria, which are of significant threat to public health (*E. faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp). Anacarso *et al.* (2017) identified that a blend of FA and MG, which included C10-MG and C12-MG, inhibited the growth of 37 strains of *E. coli* and 17 strains of *Enterococcus* spp., all of which displayed a MDR phenotype [43]. Furthermore, Schlievert *et al.* (2012) identified C12-MG to be 200 times more potent

against *S. Aureus* than LA. This study also tested the evolution of bacterial resistance against C12-MG and found no development of resistance to C12-MG in *S. aureus* after one year of exposure to the lipid at sub-inhibitory concentrations, which suggests the development of resistance against MGs may be slow [40]. There have been some reports that bacteria can develop tolerance against free FAs, however, with tolerant strains of *Listeria monocytogenes* isolated from a serial passage experiment with increasing concentrations of antimicrobial FAs. This isolate displayed glycosylated wall teichoic acids and a more hydrophilic cell surface interface, which likely exhibited repulsive effects on FAs from the cell envelope. An increase in cell surface hydrophobicity was also observed in a different tolerant *L. monocytogenes* isolate. This isolate had a mutated *ccpA* gene which was also shown to play a role in bacterial cell responses to other stresses, and this phenotype was not induced in response to C12-FA exposure [165].

2.8 Mechanisms of Antimicrobial Activity in Fatty Acids and Monoglycerides

While there are some gaps in knowledge within our understanding of precisely how FAs and MGs inhibit bacterial cells, it is largely agreed upon that antimicrobial FAs and MGs cause the disruption of aspects of the bacterial cell envelope. This mechanism is hypothesised to be shared with their antimicrobial activity against enveloped viruses [166]. While not all mechanisms are shared by all FAs and MGs, several bacterial pathways and organelles can be altered by interactions with FAs and MGs, including changes to membrane permeability, membrane lysis, or the inhibition of bacterial enzymes (Figure 2.9) [39].

The major method of bacterial inhibition recognised in FAs and MGs is the destabilisation of the bacterial cell membrane. As described in Section 2.1, the cell membrane is a vital cellular component which acts as an interface between the bacterium and the external environment, facilitating nutrient exchange, intercellular communication, and offering protection from environmental conditions. Treatment of bacteria with antimicrobial FAs and MGs has resulted in changes to the properties

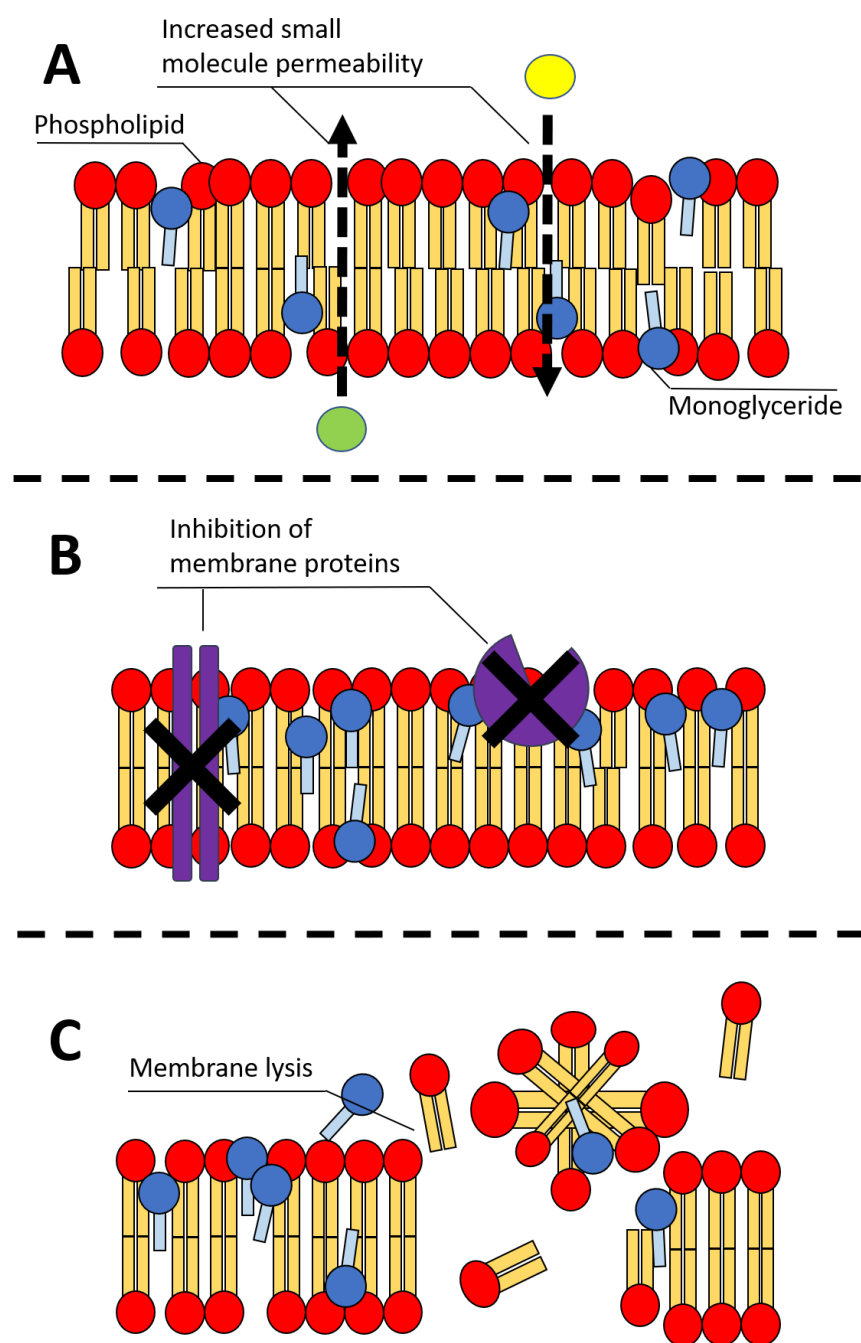


Figure 2.9: Hypothesised mechanisms of antimicrobial activity in MGs. Increased membrane permeability (A), inhibition of membrane-bound protein functions (B), and membrane lysis (C) are all hypothesised to contribute towards the antibacterial properties of MGs

of the cell membrane. FAs and MGs readily insert into phospholipid bilayers due to the hydrophobic effect, leading to such physical and mechanical changes. In *in vitro* settings, FAs and MGs usually interact with a region of the membrane at a high localised concentration as antimicrobial activity is generally reported above CMC, where nanostructures such as micelles spontaneously form [167]. These structures fuse with the cell membrane, causing a high concentration of FA or MG at the site of fusion, initially in the outer facing leaflet. Although activity in MGs is primarily reported above CMC, some disruption of model bilayer systems has been reported at lower concentrations by C12-MG [153, 168].

The insertion of antimicrobial FAs and MGs causes increased bilayer permeability by disrupting phospholipid packing and lipid organisation within the bilayer. FAs and MGs partition into the lipid bilayer from the aqueous external environment by orienting themselves with the outer leaflet such that the head and tail groups are oriented alongside the outer leaflet phospholipids and then inserts into the lipid bilayer between phospholipid molecules [169]. FAs and MGs above CMC exist as a micelle structure which maintains a low concentration of monomeric FA or MG due to a dynamic equilibrium with the external environment which causes molecules to continuously dissociate from and reinsert into the micelle [170]. This allows small concentrations of monomeric FAs or MGs in solution to partition into nearby lipid bilayers. Additionally, when micelles come in close contact with lipid bilayers FA or MG molecules can likely transfer from the micelle into the cell membrane as has been observed in other surfactant micelles [171, 172]. Rapid partitioning of FAs or MGs into the bilayer can result in transient regions of high localised concentrations within a region the bilayer before redistribution through lateral diffusion. These regions of high FA or MG content may disrupt bilayer organisation and disrupt bilayer properties [28].

The increase in membrane permeability from FA and MG insertion alters the selectivity barrier mechanism of the structure, allowing the diffusion of bacterial cytosolic contents through it, including ions and molecules required for normal functioning. This was reported to occur in *S. aureus* treated with antimicrobial FAs in Parson *et al.* (2012), where treatment with palmitoleic acid caused membrane

depolarisation, inhibition of molecular synthesis pathways, and the release of low molecular weight proteins from the cell [173]. Inhibition of bacterial growth has been observed in both G+ and G- bacteria, though FA and MG efficacy can differ across organisms. For example, C10-FA and C10-MG cause significantly more damage to *E. coli* membrane models than C12-FA and C12-MG respectively do, as reported in Tan *et al.* (2024) [28]. Similar findings were also found for C10-MG in Wang *et al.* (2020) where this was suggested to be because of the shorter acyl tail length in C10-MG than C12-MG [35].

Significant disruption of the cell membrane structure has also been observed from treatment with FAs and MGs. FA and MG insertion can cause pore formation in the membrane which have been correlated with the release of low molecular weight peptides from *S. aureus* [173]. Pores formed as a result of this may be permanent or transient. Antimicrobial FAs and MGs have also been shown to drive extensive remodelling of the cell membrane, as has been observed with other surfactants. Yoon *et al.* (2020) identified that interactions between *in vitro* model bilayers and C12-MG or C12-FA resulted in visible morphological changes to the lipid bilayers, with C12-MG causing the formation of tubules which rapidly collapsed into hemispheric caps, while lauric acid produced longer lasting tubules that eventually collapsed into hemispheric caps following the stopping of lauric acid flow and rinsing of the bilayer [27]. This study hypothesised that a significant factor which drives membrane deformation is the asymmetric stresses generated from the insertion of FAs and MGs into the outer leaflet of the membrane. As these stresses accumulate, it is hypothesised that they can induce curvature within the membrane, and it is attempted relaxation of this stress that drives the physical deformation of the bilayer [27, 174]. Furthermore, FAs and MGs have been reported to cause lysis of the cell membrane at high concentrations. This process is thought to occur due to the extensive membrane destabilisation and remodelling caused by FAs and MGs, which causes the formation of micelles that bud off from the membrane and eventually lead to the rupturing or solubilisation of the membrane as integrity is lost [39]. FAs and MGs can "flip-flop", a process which refers to the movement and subsequent realignment of these molecules to the inner-

facing leaflet of the bilayer, which may facilitate destabilisation within the cell [175]. The time required for FAs and MGs to complete this process is highly dependent on structure and especially molecule charge, and can vary from minutes to hours [176]. It has been suggested that G+ bacteria are primarily disrupted by antimicrobial FAs and MGs through disruption to the plasma cell membrane, while in G- bacteria they appear to degrade the LPS of the OM [35].

The destabilisation of the bacterial cell membrane can also disrupt membrane-bound processes [39]. The primary ATP production pathway in bacteria, the electron transport chain, is a multi-step, membrane bound process which utilises a series of carrier proteins to move electrons, generating a proton gradient which is used to produce ATP by ATP synthase in a process called oxidative phosphorylation [177]. The increase in bilayer permeability caused by FA and MG insertion inhibits this process indirectly by facilitating the easier diffusion of small molecules across the membrane. The electron proton gradient required for oxidative phosphorylation to occur is weakened by the enhanced ability for H^+ ions to move across the bilayer, preventing the electrochemical membrane potential required for enzyme function, thereby uncoupling the electron transport chain from oxidative phosphorylation [39]. Similarly, FAs have been reported to decrease oxygen uptake by the cell, which is essential for the final step in the electron transport chain. Bacteria treated with antimicrobial FAs have been reported to have both reduced oxygen uptake and reduced ATP production [178]. As ATP is an essential energy molecule for bacteria, the inhibition of any part of this process can prove fatal to a bacterium [179].

Not all mechanisms of antimicrobial in FAs and MGs occur via membrane destabilisation. FAs have been reported to bind several membrane-bound enzymes and inhibit their functions, as well as other crucial molecules inside the cell. Exogenous FAs have been reported to inhibit FA biosynthesis in bacteria by binding to the enzyme bacterial enoyl-acyl carrier protein reductase, inhibiting FA elongation [180]. Linoleic acid has been reported to inhibit peptidoglycan synthesis in G+ bacteria, which is a component essential to cell wall elongation [181]. Inside the cell, palmitic acid can also inhibit DNA replication by inhibiting the enzyme DNA gyrase, which is

essential for bacterial DNA replication by inducing negative supercoils into the strand during the process [182]. By inhibiting DNA replication bacteria are unable to divide and therefore population growth cannot occur.

As described above, headgroup structure differences between FAs and MGs cause these molecules to experience different intermolecular interactions. It is understood that the charged headgroup of FAs at physiological pH is likely to play a role in the antimicrobial differences between these molecules and MGs. As the headgroup of FAs is anionic, and bacterial lipid membranes are generally composed of a significant amount of negatively charged lipids, electrostatic repulsion means that FA monomers insert into bacterial membranes less readily than MGs. Due to the difference in FA and MG headgroups, FAs and MGs have different geometries, which is defined by the critical packing parameter $P = \frac{V}{a_0 l_c}$ where V is the volume of the lipid acyl tails, a_0 is the area per headgroup, and l_c is the length of the acyl tails [183]. This affects how FAs and MGs pack within the bilayer, as FAs are less conical than MGs, which is likely to present differences in the extent of phospholipid packing disruption caused by these molecules. It is thought that MGs destabilise lipid bilayers due to hydrophobic mismatch, which is an energetic mismatch within a bilayer leaflet caused by the insertion of molecules with hydrophobic regions that differ in length to a bilayer’s hydrophobic core, which causes surrounding lipids to bend in order to maintain the hydrophobic core, thereby generating curvature stress on the system [184].

Some evidence of synergy has been observed in antimicrobial MGs, both between different MGs and other molecules. Batovska *et al.* (2009) studied the antimicrobial activity of saturated MCMGs C10-MG and C12-MG, monomyristin (C14-MG) and C12-FA in various mixed ratios against G+ bacteria. This study identified that MGs were significantly more antimicrobial against the tested species than LA individually, but also that mixtures containing both C12-MG and C12-FA displayed much greater activity against *Staphylococcus pyogenes*, particularly when one was present in a 2:1 ratio with the other. Synergy between C10-MG and C12-MG was also observed against *S. pyogenes* and *S. aureus* [185]. Yoon *et al.* 2020 identified synergistic bilayer disruption on DOPC bilayers between C12-MG and C12-FA, being most disruptive

when mixtures containing equal concentrations of FA and MG were used [27]. It has also been identified that against *E. coli* bilayer models synergy between C10-MG and C10-FA were present [186]. Hess *et al.* (2014) reported potential synergies between MGs and antibiotics for use against bacterial biofilms, which are more difficult to treat than planktonic bacteria due to the extracellular matrix bacterial communities form. Combination treatments of either C12-MG or C12-FA with gentamicin and streptomycin on *S. aureus* biofilms significantly increased biofilm eradication compared to antibiotic alone using antibiotic concentrations closer to those normally effective against planktonic cells [152]. Additionally, synergy between the G- cell envelope targeting antibiotic polymyxin B and C12-MG was reported by Zheng *et al.* (2022) against isolates of *A. baumannii*, *E.coli*, *K. pneumoniae*, and *P. aeruginosa* both in *in vitro* experiments and *in vivo* using mouse models, with significant damage to the cell envelope and inhibition of biofilm formation being noted [187]. Consequently, antimicrobial MGs have significant promise not just as antibiotic alternatives and disinfectants themselves, but also for use with other treatments to enhance efficacy. Therefore, improving our understanding of how MGs produce antimicrobial activity may aid in the design of antimicrobial products in a variety of ways.

2.9 Approaches to Characterising Mechanisms of Lipid Bilayer Disruption in Fatty Acids and Monoglycerides

Our understanding of the antimicrobial mechanisms of MGs comes primarily from experimental approaches, which aim to understand these interactions in a biological or biophysical context.

2.9.1 Biological Approaches

For larger scale investigations into the understanding on antimicrobial MGs, biological approaches using living organisms can provide insight into how these molecules directly interact with cells.

As with antibiotics, the antimicrobial efficacy of FA and MG formulations can be

assessed via MIC assays, to determine the titre required to inhibit cellular growth [35]. The extent of bacterial inhibition from treatment can also be assessed by plating bacterial isolates in order to confirm viable bacteria, measured through colony forming units. Assays that utilise microorganisms can also give insight into how FAs and MGs inhibit biofilm production, which is highly relevant to real-life applications, as bacterial biofilms significantly improve the survival of bacteria and reduce antibiotic permeability. Ham and Kim (2016) reported that C12-MG and glycerol monobehenate (C22-MG) had strong inhibitory effects on biofilm formation in three bacterial species - *Yersinia enterocolitica*, *Streptococcus mutants*, and *Xanthomonas oryzae* - in continuous flow cells which were fed with media containing MG dissolved in ethanol for 24 hours [188].

2.9.2 Biophysical Approaches

Biophysical studies, which aim to better understand mechanical and structural alterations to the bilayer structure, are used alongside microbial studies in order to provide different insights into the mechanisms. Biophysical studies are often performed using model bilayer systems rather than living cells. This approach allows more control to understanding lipid bilayer dynamics, as these models can have precisely controlled variables, lacking membrane proteins and having precise phospholipid ratios to improve reproducibility of results. Model membranes used in biophysical studies vary in form depending on the properties to be studied.

Liposomes

Spherical liposomes of model membranes are classified by diameter as small unilamellar vesicles (SUVs; 20 to 100 nm), large unilamellar vesicles (LUVs; 100 nm to 1 μm) or giant unilamellar vesicles (GUVs; over 1 μm). GUVs have been used to study membrane morphology changes caused by MG micelles interaction, such as Yoon *et al.* (2020) which highlighted that C12-MG can produce fission, fusion, and pearling in the structure [27]. Phosphatidylcholine LUVs have been used in kinetic studies of FA interactions with lipid bilayers, and to assess how vesicle permeability can be altered by FAs and MGs [189]. GUVs have been used in similar studies using antimicrobial

proteins and peptides, where molecule penetration and leakage could be monitored to understand how pore formation was produced by these molecules [190]. GUVs can be formed with encapsulated contents such as calcein dye to facilitate these studies [191]. Additionally, GUVs can be used for microscopy to understand the morphological changes produced by FAs and MGs on the vesicle structure, making these a particularly good vesicle size to use for bilayer physiology studies [192]. GUVs are limited in their ability to model membranes that contain high asymmetry in lipid composition, such as the G- bacterial OM.

Supported Lipid Bilayers

Supported lipid bilayers (SLBs) are planar bilayer structures formed on solid surfaces which acts as a support. Between the surface and SLBs is a thin layer of water, and the whole system is immersed in water. SLBs are excellent models for mechanical studies into bilayer disruption as they are more stable than GUVs, allowing longer term experiments to be conducted. As SLBs are planar, they are compatible with surface-sensitive equipment which allows real-time measurement of changes to bilayer properties, giving insight into characteristics which are not easily able to be assessed using unilamellar vesicles. QCM-D, atomic force microscopy, and x-ray reflectivity can also be used to understand bilayer disruption. As with unilamellar vesicles, the membrane composition of SLBs can be controlled to mimic relevant biological membranes and models mimicking the complex G- bacterial OM have been successfully created for use in SLBs [193]. SLBs have been utilised to understand how C10-MG and C12-MG nanomicelles influence 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) bilayers by Yoon *et al.* (2020), which reported that mixed C10-MG and C12-MG micelles can induce pore formation and membrane budding. The extent of this disruption could also be controlled by altering the C10-MG:C12-MG, and C10-MG appeared to be the more membrane lytic of the two MGs [27].

Tethered Bilayer Lipid Membranes

Tethered bilayer lipid membranes (tBLMs) are a specific type of SLB which are tethered to the surface by a peptide or polymer, creating an aqueous reservoir between the IM leaflet and the surface the bilayer is attached to [194]. tBLMs are more stable than SLBs and are exposed to solution on both sides, creating an environment which is closer to a biological membrane [195]. tBLMs have been used for electrical impedance spectroscopy to determine the extent of disruption caused by C10-FA, C10-MG, C12-FA, and C12-MG on a *E. coli* bilayer model [196].

2.9.3 Computational Approaches

While experimental work has been the primary method of providing new information on the disruptive properties of FAs and MGs, computational studies in this field of research are an under explored, but upcoming, tool which can provide novel insight into aspects of the mechanism which experimental procedures can struggle to reliably analyse. MD focused approaches (expanded upon next chapter) allow the calculation of expected velocities of atoms over a timescale of nanoseconds, producing a trajectory which allows the visualisation of how molecule movement at an atomistic level of detail. This allows the behaviour of individual MG molecules within a bilayer to be observed, which is a level of detail that cannot be replicated experimentally. MD methodologies do have some limitations, however, as in all-atom simulations bonds cannot be broken or made. This means that while processes such as asymmetric stresses, membrane lysis, and MG flip-flop can be simulated, inhibition of ATP production cannot be. Due to the constraints of both timescale and system size, only parts of the mechanisms, even if often the most crucial, can be simulated in these systems.

Simulating Experimental Hypotheses

MD simulations can allow experimental hypotheses to be tested, such as the asymmetric stress hypothesis of bilayer destabilisation, which cannot be easily tested experimentally due to the inability to have an equal concentration of FA/MG interact with both the outer and inner leaflets of a GUV or SLB to create a system with

symmetrical amphiphile interactions. Drew Bennett *et al.* (2022) utilised C12-FA and its protonated form, laurate, alongside C16-diethyl ester dimethyl ammonium and C12-tri-methyl ammonium to show that systems with a symmetrical distribution of the amphiphiles exhibited lower perturbation than asymmetrically distributed systems at equal concentrations [197].

Control of Amphiphile Concentration

Akinshina *et al.* (2016) simulated the interactions of 18-carbon LCFAs and MGs at varying degrees of saturation with a ceramide bilayer, which observed unsaturated FAs and MGs to cause disintegration of the bilayer, while saturated FAs and MGs caused changes to lipid packing [169]. Experimentally, the concentration of FA or MG which interacts with the bilayer can be difficult to control, as FAs and MGs are administered at a set concentration in solution and not directly placed in contact with the cell membrane. Simulation approaches which test a range of FA or MG concentrations allow for a systematic, direct assessment of how amphiphile count in a bilayer may alter properties.

Furthermore, simulations can be created where FAs and MGs are in a position immediately preceding or immediately following the key initial interactions with a bilayer, skipping the initial wait period required for FAs/MGs to diffuse towards the membrane. Many bilayer properties which can be calculated experimentally, such as area per lipid, bilayer thickness, and phospholipid order parameters, can also be determined in simulations, allowing direct comparison to reported experimental values to ensure the models used, and therefore its findings, are valid. MD simulations are conducted at an atomistic scale, and therefore these simulations can provide insight at a very fine level of detail regarding how individual FA or MG molecules behave within a bilayer, and how this may lead to disruption.

Calculating Free Energy Barriers

Gahan *et al.* (2021) calculated the potentials of mean force (PMF) required for entry into, and movement across, a DOPC bilayer system for anionic C12-FA and C12-MG

alongside quorum sensing signal molecules, which indicated that C12-MG has lower energy barriers to translocation than C12-FA, and therefore more readily inserts into the model bilayer [191]. The calculation of free energy barriers for molecules is very difficult experimentally, and can only be estimated indirectly from other measurements [198]. Therefore, the use of molecular dynamics techniques such as umbrella sampling can provide valuable insight into molecule behaviour within a system.

2.10 Key Gaps in Literature

Overall, when attempting to characterise the lipid bilayer disruptive properties of MGs, antimicrobial or otherwise, although much is already known, there are still specific aspects of the mechanism which have not been fully elucidated by previously experimental, nor computational, research, particularly in regards to well known antimicrobial MCMGs C10-MG and C12-MG.

No computational approaches have been employed to screen a wide range of concentrations for these molecules to understand potential thresholds required for significant bilayer deformation to occur.

Additionally, there is a lack of understanding why significant disruption of lipid bilayers has been reported to be caused by C10-MG and C12-MG, but not shorter chain molecules such as C8-MG, which simulations may provide novel insight into, and free energy values have also not been reported for these molecules. Furthermore, there has also been no direct evidence reported in published literature regarding the role of MG-driven asymmetry in driving changes to bilayer properties for these molecules, and whether they are similar to the results of other FAs and amphiphiles reported by Drew Bennett *et al.* (2022) [197].

Finally, although a variety of models and microorganisms have been used to understand the efficacy of these molecules experimentally, no MD simulations have been performed using C8-MG or C10-MG for any membrane model, and C12-MG has only been modelled in a single study with a DOPC bilayer [191]. Therefore, little insight into the differences of how medium-chain MGs interact with mammalian and bacterial model systems is known at an atomistic scale.

Chapter 2. Bacteria, Antibiotic Resistance, and Antimicrobial Lipids

These specific gaps in literature will be the focus of this thesis, and will be approached using a methodology centred around MD simulations, outlined in Chapters 3 and 4.

The first results chapter, Chapter 5, aimed to examine experimental hypotheses regarding MG concentration, MG structure, and the importance of asymmetric stresses on a simple model bilayer system. This was done to assess whether MD simulations could be used to provide novel evidence for, or against, existing experimental hypotheses in the research field, proving their use as a tool which can be used alongside experimental approaches in this field. The bilayer system used for this chapter was a simple POPC bilayer model which is analogous to the POPC or DOPC bilayer systems frequently utilised in biophysical studies to research fundamental mechanisms of lipid bilayer disruption by FAs or MGs [29, 199–201].

The second results chapter, Chapter 6, follows a similar protocol to Chapter 5, utilising models better representative of the phospholipid bilayers present in bacteria in order to determine whether the behaviour observed in Chapter 5 could also be reflected in model bacterial bilayer systems and therefore could be tied to potential antimicrobial mechanisms of action. The bilayer models utilised in this chapter were strong representatives of the lipid bilayer compositions of the G⁻ inner membrane and G⁺ plasma membrane developed by Pogocheva *et al.* from experimental data on phospholipid headgroup ratios and tail structures. These models were developed to provide standardised benchmark systems for MD simulations and facilitate more consistent comparisons between simulation studies [1].

The final results chapter, Chapter 7, utilised umbrella sampling to calculate energy barriers to MG translocation for C8-MG, C10-MG, C12-MG, and C18-MG across the three lipid bilayer models used in Chapter 5 and 6. This was performed to further contextualise findings and MG behaviours in these chapters within the free energy landscape of these systems. In particular, differences in free energy barriers for movement into, out of, and across a leaflet for the four MG tail lengths and the three lipid bilayer compositions were the focus of Chapter 7.

Chapter 3

Molecular Dynamics Simulations

While often considered a gold standard for understanding biological systems, experimental procedures for understanding functions of systems have fundamental limitations when attempting to assess the changes exhibited at a molecular level. To overcome these limitations, computational techniques can be utilised to produce reasonable approximations of processes that occur at this scale.

Simulation methods used for this purpose vary depending on the level of accuracy required. The most precise simulation techniques, such as Density Functional Theory (DFT), utilise quantum mechanical calculations to produce output [202]. High accuracy, however, requires the trade-off of a high resource investment required to perform these simulations. Simulations that rely on quantum calculations have very high computational costs, and consequently are limited in size to around a thousand atoms [203].

Molecular Dynamics (MD) is a computational methodology which simulates the evolution of the behaviour of, and interactions between, molecules and atoms modelled within a created system [204]. This technique has been utilised to study many biological phenomena at atomistic levels of detail, including the behaviour of lipid bilayers, which can be directly compared to work presented within this thesis. MD systems allow the study of up to roughly a million atoms at a timescale of nanoseconds to around a microsecond at a reasonable length of real-world time [204]. Though the quantum-mechanical nature of atoms and molecules is discarded in systems which employ the

MD method, many interactions at a molecular level can still be observed within MD systems.

3.1 Chapter Aims

1. To describe the basic principles of MD simulations.
2. To describe the standard workflow for preparation of an MD simulation.

3.2 Atomic and Molecular Representations

Atomistic MD methodologies simulate the behaviour of atoms at an atomic scale. With this method each atom in a system is represented as vector:

$$r_i = (x_i, y_i, z_i) \quad (3.1)$$

where i represents the i th atom in the system.

A molecule within the system can be expressed as the geometry:

$$\vec{R} = \vec{r}_N \quad (3.2)$$

where N represents the vector describing the position of each atom of the molecule. For example, water is comprised of oxygen $\vec{r}_1$ and two hydrogen $\vec{r}_2$ and $\vec{r}_3$ (Figure 5.15). Atomic positions in 3D space across the whole system can be represented by the single vector $3N$.

MD systems typically utilise a simulation box (also known as a cell), which all atoms are contained within. The most commonly used cell shapes in biomolecular simulations are cubic, rectangular, octahedral, and hexagonal (Figure 3.2). While the cubic cell shape is the most commonly used, cell shape could be considered when setting up a simulation, as the box shape has been noted to influence the dynamics of the system in some instances [205].

The starting structures of molecules, especially proteins, used in MD simulations

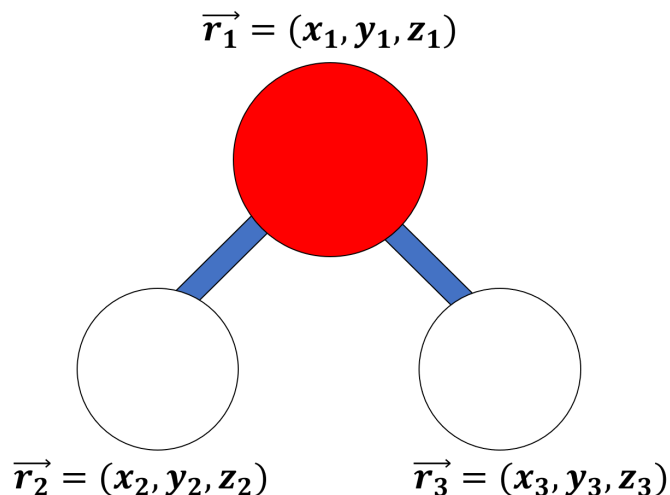


Figure 3.1: Atomic representation of a water molecule. The set of vectors of the molecule consist of the vectors of one oxygen atom ($\vec{r}_1$) and two water atoms ($\vec{r}_2, \vec{r}_3$)

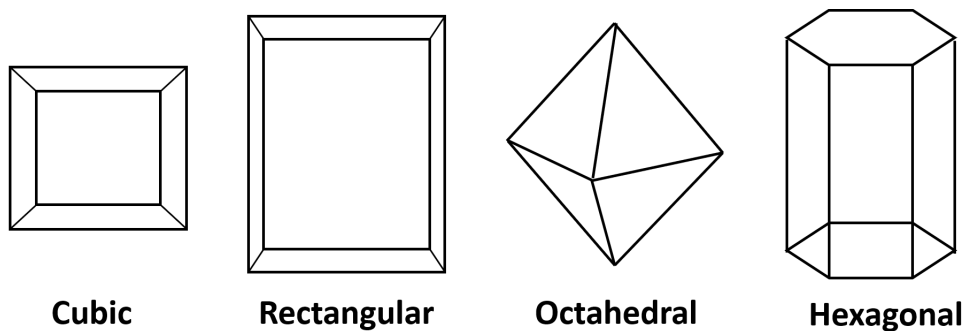


Figure 3.2: Common MD simulation box shapes.

are typically acquired from experimental data such as X-ray crystallography. To create a system ready for simulation, the molecular structure files are used, along with other components such as water and ions [206].

When modelling biomolecular systems accurately water must be included, as interactions in aqueous environments are a key part to many biological systems. There are various developed water models for MD simulations which are optimised for varying levels of computational efficiency or detail as required [207]. The water model TIP3P is utilised by the CHARMM36m force field (FF) used in this work (see Section 3.4) [208]. Restraint algorithms are typically used on bulk water molecules to speed up computational efficiency. NAMD (Nanoscale Molecular Dynamics) utilises

the SETTLE algorithm as the default [209]. SETTLE is non-iterative algorithm which constrains hydrogen-oxygen and hydrogen-hydrogen bonds to the parameters specified in the FF. While constraints require additional calculations to be performed, they significantly increase overall simulation speed.

3.3 Equations of Motion and Time Integration

The interactions between atoms described in simulations are generated by the integration of Newton's equations of motion. From the starting system configuration, successive configurations are obtained through integrating Newton's equations of motion for each atom:

$$\vec{F} = m \vec{a} \quad (3.3)$$

where $\vec{F}$ is the force acting on an atom, m is the mass of the atom, and $\vec{a}$ is the acceleration of the atom:

$$F_i(t) = m_i \frac{d^2 r_i}{dt^2} = -\nabla_r U(r) \quad (3.4)$$

where m_i is the mass of atom i , r_i is the position of vector i , r is the position of the system in a 3 dimensional space, and U is the total potential energy.

Due to the number of atoms in MD systems this equation is not analytically solvable, and therefore Newton's equations of motions are instead integrated using the finite difference method. This method divides the equation into very small timesteps. At each timestep Newton's equations of motion are solved simultaneously for each atom to calculate the force acting on each atom, and from the force, acceleration is then determined. From the current velocity and acceleration of each atom, the position and velocity of each atom at the next timestep can then be calculated and used for the next step until a stopping point is reached (Figure 3.3). MD simulations are performed with a timestep of 1 or 2 femtoseconds as standard, though larger timesteps can be used.

In an MD simulation the integration of Newton's equations of motion is carried out by an algorithm to determine the positions of all atoms at a given time ($\vec{r}_i(t)$).

Integration algorithms are iterative, calculating the positions and velocities of all atoms in the system at each specified time interval, as described in the finite difference method. There are several integration algorithms available for use in MD simulations however most modern algorithms utilise the leap-frog algorithm [210]. This algorithm calculates position and velocity at staggered intervals, calculating the positions of each atom r at time $t + \Delta t$ and velocities v for each atom at each timestep $t + \frac{1}{2}\Delta t$. The leap-frog algorithm is effective at conserving the energy of a system across a long simulation time and is also time reversible. In NAMD a variant of the leap-frog algorithm called the Velocity Verlet algorithm is used [211]. Velocity Verlet is similar to the leapfrog method, but calculates positions and velocities at the same timestep rather than staggered. The Velocity Verlet algorithm is typically implemented through the following steps:

Calculate velocity for the next half timestep:

$$\vec{v}_i(t + \frac{\Delta t}{2}) = \vec{v}_i(t) + \frac{1}{2}\vec{a}_i(t)\Delta t \quad (3.5)$$

Calculate positions for the next timestep:

$$\vec{r}_i(t + \Delta t) = \vec{r}_i(t) + \vec{v}_i(t + \frac{1}{2}\Delta t)\Delta t \quad (3.6)$$

Derive acceleration for the next timestep using the calculated positions:

$$\vec{a}_i(t + \Delta t) = f(\vec{r}_i(t + \Delta t)) \quad (3.7)$$

Calculate velocity for the next timestep:

$$\vec{v}_i(t + \Delta t) = \vec{v}_i(t + \frac{\Delta t}{2}) + \frac{1}{2}\vec{a}_i(t + \Delta t)\Delta t \quad (3.8)$$

3.4 Force Fields

FFs are required to simulate the evolution of an MD system. FFs are a collection of parametrised equations which describe the potential energy of atoms in the system,

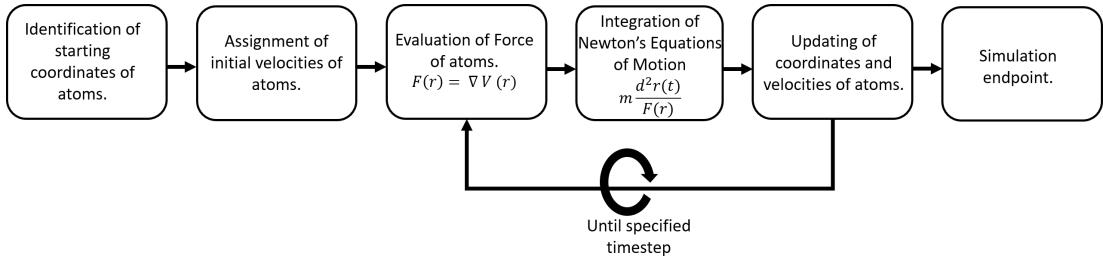


Figure 3.3: Simplified process of the finite difference method used in MD simulations.

allowing forces exerted on atoms to be calculated. This further allows the calculation of new atomic positions and velocities to the next timestep. The specificities of these calculations are highly dependent on the package selected, but at their basics, all FFs calculate the total potential energy of a system as:

$$U_{total} = U_{bonded} + U_{non-bonded} \quad (3.9)$$

Classical FFs can be separated broadly into different classes. Class I FFs model potential energy as the sum of bonds, angles, torsion, Lennard-Jones (LJ) potential, and electrostatic interactions: [212]

$$U_{classI} = U_{bonds} + U_{angles} + U_{torsion} + U_{LJ} + U_{ES} \quad (3.10)$$

Class II FFs additionally model bond-bond and bond-angle coupling on top of the Class I interactions, and Class III force fields further add polarisation [212]. Another key aspect to FFs are the parameters they use. These are provided in data files as part of the FF, describing the properties and inter-atomic interactions of atoms. Parameters are typically developed in accordance with experimental data or quantum mechanical calculations and specific to a respective FF's parameter optimisation protocol, therefore making them incompatible with other FFs. FFs are typically developed with a specific goal in mind, and therefore selecting the correct FF for a system is a very important step in the process of MD system setup. In terms of biomolecular simulations, which require large system sizes simulated over long periods of time, FFs such as CHARMM [213] and AMBER [214] are optimised for calculation of non-bonded potential and preservation of

chemical structure, while FFs such as GROMOS [215] and OPLS [216] are the favoured FFs for systems where liquid properties are of particular interest.

3.4.1 CHARMM Force Field

All work carried out in this thesis was performed using the CHARMM36m All-Atom Force Field, which has been validated for use in lipid simulations [217]. The rationale for selecting this FF is detailed in the next Chapter (Section 4.2.2). The CHARMM FF is a class I additive FF which models bonded (U_{bonded}) and non-bonded ($U_{nonbonded}$) potential energy of atoms in the systems. Bonded potential models bond stretching, angle torsion, and proper/improper dihedral torsion. Non-bonded potential accounts for van der Waals (vdW), which is represented empirically by LJ potential, and electrostatic interactions (Figure 3.4).

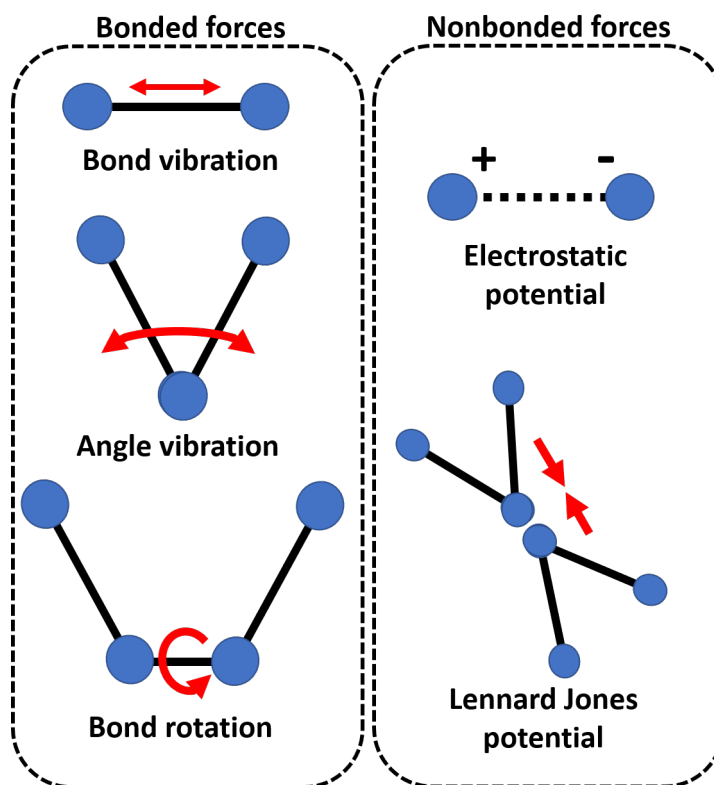


Figure 3.4: Bonded and non-bonded forces considered in MD simulations.

CHARMM36 calculates the full potential energy as the sum of bonded (bonds,

Chapter 3. Molecular Dynamics Simulations

angles, dihedrals, improper dihedrals), and non-bonded interactions (Van der Waals and electrostatics):

$$U(r) = U_{bonds} + U_{angles} + U_{torsion} + U_{improper} + U_{non-bonded} \quad (3.11)$$

where U_{bonds} represents the sum of bond stretching potential, U_{angles} represents the sum of angle potential, $U_{torsion}$ represents the sum of dihedral angle potential, $U_{improper}$ represents the sum of improper dihedral angle potential, and $U_{non-bonded}$ represents the sum of non-bonded potential at a given point in time during a simulation.

When calculating bonded potential, bond stretching is the most heavily weighted bonded interaction, followed by angles, dihedrals, and improper dihedrals in that order.

Covalent bonds between atoms are represented as a spring which has a harmonic (or spring) potential:

$$U_{bonds}(r_{i,j}) = \sum_{bonds} K_b(r_{i,j} - r_0)^2 \quad (3.12)$$

where

$$r_{i,j} = ||\mathbf{r}_i - \mathbf{r}_j|| \quad (3.13)$$

represents the current bond distance between the atoms, r_0 is the equilibrium bond length, and K_b is the bond force constant.

Angles between atoms are described through an angular harmonic potential:

$$U_{angles}(\theta) = \sum_{angles} K_\theta(\theta - \theta_0)^2 \quad (3.14)$$

where θ represents the angle between atom vectors, θ_0 is the equilibrium angle, and K_θ is the force constant.

Dihedral potential is described through through:

$$U_{torsion}(\varphi) = \sum_{dihedrals} K_\varphi(1 + \cos(n\varphi - \delta)) \quad (3.15)$$

where K_φ is the force constant, δ is the phase shift, φ is the dihedral torsion angle, and n is the multiplicity, which defines how many rotamers there are. Dihedrals define

the energy of bond rotation between atoms and therefore the geometry of a molecule. Dihedral angles are specified through four atoms, where each atom is attached to the next, and are the angles between the planes formed by these atoms.

Improper dihedrals are described as:

$$U_{improper}(\vec{R}) = \sum_{improper} K_{\varphi}(\varphi - \varphi_0)^2 \quad (3.16)$$

where φ is the current angle between the two planes, φ_0 is the equilibrium angle, and K_{φ} is the multiplicative force constant. Improper dihedral potential describes geometry which cannot be described by dihedral potential - primarily planarity and chirality. Improper dihedral angles are also angles between planes formed by four atoms, however these planes are the atoms around a group of 4 atoms where three are bonded to a single central atom.

Non-bonded interactions model interactions between atom pairs which are not covalently bound and are primarily contributed by van der Waals (vdW) and electrostatic interactions [212]. Non bonded interactions are expressed as:

$$U_{non-bonded} = \sum_{non-bonded} \epsilon_{ij} \left[\left(\frac{R_{min,ij}}{r_{ij}} \right)^{12} - 2 \left(\frac{R_{min,ij}}{r_{ij}} \right)^6 \right] + \sum_{non-bonded} \frac{q_i q_j}{4\pi D r_{ij}} \quad (3.17)$$

where $r_{i,j}$ represents the distance between atoms i and j , $\epsilon_{i,j}$ represents the LJ potential well depth, $\sigma_{i,j}$ represents the LJ potential minimum radius, D is the dielectric constant, and q_i and q_j is the partial charge of the respective atom.

The former part of this equation corresponds to LJ which is used as an approximation for the attractive and repulsive forces of vdW. vdW interactions are short range forces between molecules which are driven by dipole interactions, including forces between permanent dipoles, the forces between induced dipoles, and the forces between permanent and induced dipoles [218]. vdW is repulsive at very short ranges, while attractive forces occur at longer ranges. The former term represents with the repulsive forces of vdW in the form of the Pauli exclusion principle and the second term represents the attractive forces through London dispersion forces.

Electrostatic potential is expressed through Coulomb's Law. The potential energy of electrostatic interactions is greatest at short distances due to the high degree of repulsion of atoms with the same charged state, or attraction between opposites charged atom, at this length scale.

Non-bonded potential calculations are applied to all intramolecular non-bonded interactions between atoms separated by at least 4 bonds and never to those separated by 1 to 2 bonds. Interactions between atoms separated by exactly 3 bonds (1-4 interactions) are treated with separate parameters by the FF to prevent overestimation of non-bonded potentials. 1-4 interactions in CHARMM FFs utilise a scaling factor which allows the adjustment of vdW and electrostatic interactions strength. Additionally, non-bonded potential exhibits decay towards zero as distance between atoms increases, with vdW interactions decaying at a more rapid rate than electrostatics. To reduce the computational cost of these calculations a cut-off distance is typically set, usually 12 Å, after which the potential is automatically set to zero.

While a similar method can be used for electrostatic interactions, the Ewald summation is more commonly used for the calculation of long range electrostatic potential in MD simulations [219]. The Ewald summation calculates short range and long range electrostatic potential separately, with short range interactions being calculated for interactions which occur in the primary system and long range interactions calculated across periodic images. Long range electrostatic potential is calculated using the Fourier Transform algorithm [220]. The Particle Ewald Method (PME) is most commonly used for MD simulations as the Ewald summation is computationally intensive. PME approximates long range electrostatic potential by creating a 3D grid which it assigns charges to. The Fourier transformation is used in PME to convert the charge distribution to reciprocal space, from which electrostatic potential is calculated and then converted to real space using an inverse Fourier transformation and combined with the short-range electrostatic potential to obtain the full electrostatic potential of the system at each timestep throughout the simulation (Figure 3.5).

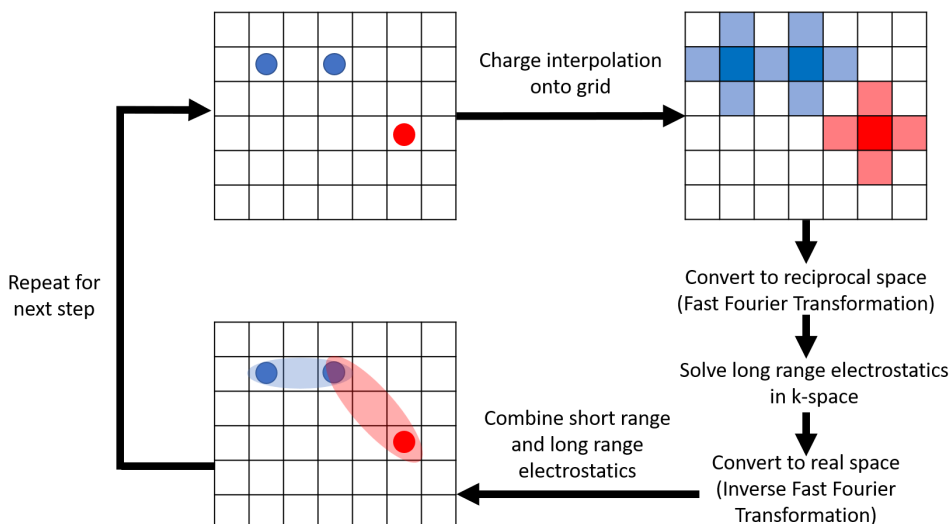


Figure 3.5: Steps for calculation of long-range interactions using the PME method.

3.5 Periodic Boundary Conditions

A key goal of MD simulations is to mimic the real-world conditions a system would appear in it order to prevent artefacts caused by the finite size of the system from occurring. This is commonly referred to the boundary effect and is caused by atoms at the edges of the simulation box having fewer neighbouring atoms than those in the bulk conditions of the box, altering their behaviour. Due to the computationally heavy nature of MD simulations it is not directly possible to simulate bulk conditions large enough to mitigate the boundary effect, however periodic boundary conditions (PBC) allow a small section of a much larger system to be simulated instead by creating an infinitely repeating system composed of periodic images of the primary system box. When a periodic image is created, the positions of all atoms in the simulation box are copied in the x , y , and z dimensions dimensions, forming a repeating lattice structure (Figure 3.6). Cubic periodic boxes are the most straight-forward to visualise as they have the most simple packing, however periodic boundaries can be applied to most box shapes.

Across all periodic images, movement of molecules in the primary cell is mirrored,

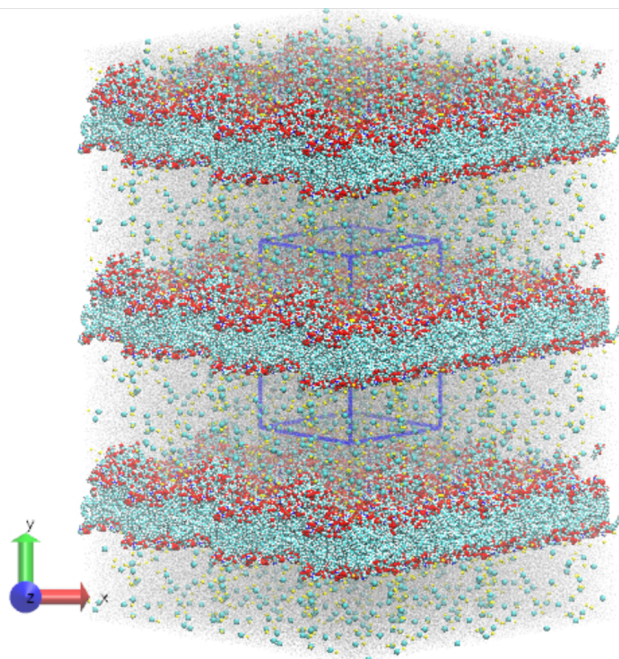
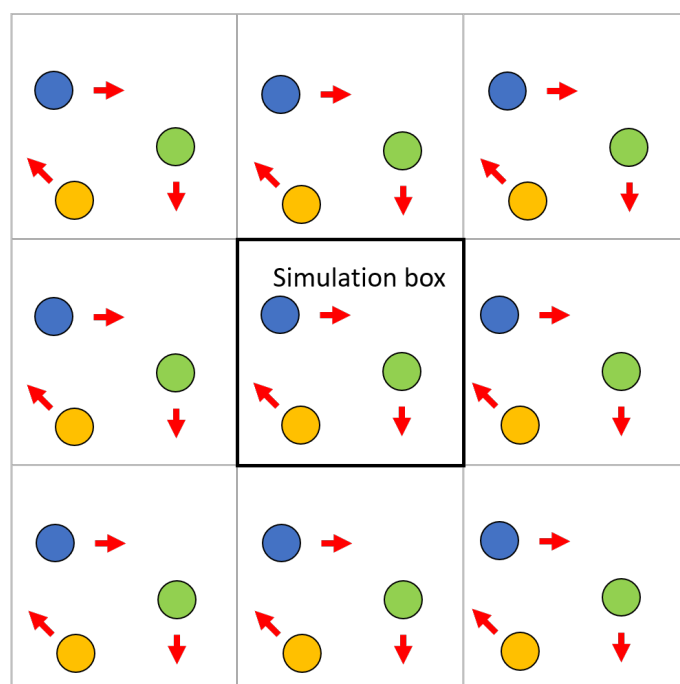


Figure 3.6: Top: 2D representation of periodic boundary conditions. Bottom: 3D representation of periodic images centred around the simulation box (blue).

meaning each periodic box experiences the same interactions. This also means that the number of atoms in the box is conserved as each atoms that passes a period boundary

reappears on the opposite side of the cell. PBC allow interaction of atoms at the edge of system to interact with neighbouring molecules at the other side of the simulation box, preventing the boundary effect from occurring. Although PBC prevent some finite size artefacts, PBC still produce artefacts of their own, however these are considered to be less severe than finite size effect. Furthermore, applying PBC to a system also allows the calculation of long range non-bonded interactions, namely vdW and PME electrostatics, across periodic boundaries to calculate more accurate non-bonded potential.

3.6 Energy Minimisation

Energy minimisation is a precursor step to performing MD simulations which allows a system to reduce potential energy to a minimum. This is a necessary step in MD as algorithms utilised for generating the starting structure of a system often causes a high initial potential energy, leading to unphysical behaviour in the system. The two main methods of energy minimisation used in MD are Steepest Descent (SD) and Conjugate Gradient (CG). The SD algorithm is more straight forward calculating force and potential energy for a starting position, by moving atoms in proportion to the forces acting on them and repeating the process until the maximum potential energy is below a specified threshold. The CG method functions similarly to SD, however it also accounts for previously sampled directions and selects an orthogonal direction for the next set of displacements. (Figure 3.7). The CG method is less computationally intensive than SD and requires fewer iterations to reach the energy minimum [221].

3.7 Temperature and Pressure Control

In order to maintain ideal thermodynamic conditions within a system, NAMD has built-in algorithms to maintain temperature through a thermostat and pressure through a barostat [211]. MD systems employ ensembles which allow certain conditions to remain constant within the system box (Figure 3.8). The most basic ensemble, microcanonical (NVE) keeps the number of atoms in the system, the volume of the system, and the energy of the system constant, meaning the system is entirely isolated from the external

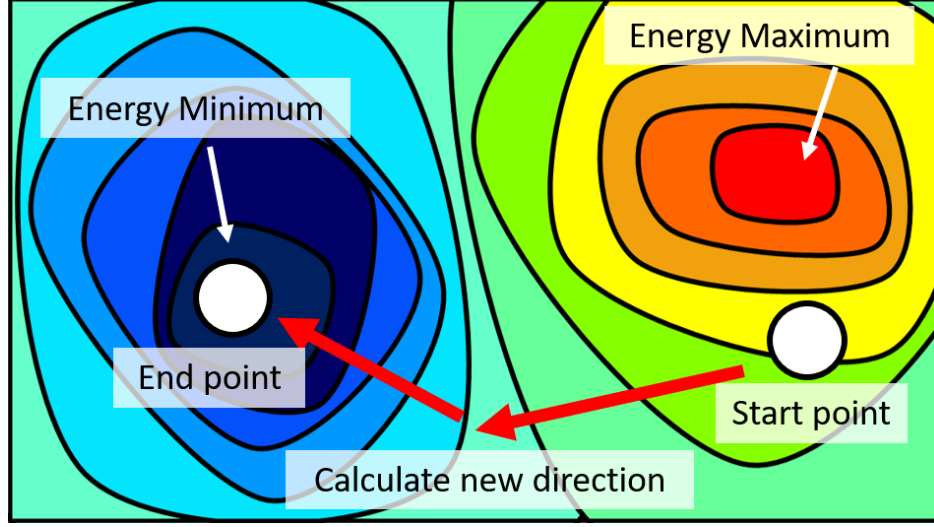


Figure 3.7: 2D schematic of conjugate gradient method of energy minimisation.

environment [222]. The canonical ensemble (NVT) allows the exchange of heat with the external environment to maintain a constant temperature within the simulation box [222]. Temperature controlled ensembles are often utilised in the simulation of systems designed to be directly comparable to laboratory experiments which keep temperature constant as a variable. Furthermore, allowing exchange of energy with the external environment can prevent unphysical behaviour if high potential energy is not sufficiently reduced during equilibration.

Thermostat algorithms function through adjusting the velocities of atoms in a system to maintain a constant temperature if they are outside the specified temperature range. Temperature is estimated from the average kinetic energy of the system:

$$\left\langle \sum_{i=1}^N \frac{1}{2} m_i \mathbf{v}_i \cdot \mathbf{v}_i \right\rangle = \frac{1}{2} N_f k_B T \quad (3.18)$$

where N_f is the number of degrees of freedom in the system containing N atoms, K_B is the Boltzmann constant, and $\mathbf{v}_i$ is the velocity of atom i .

NAMD simulations typically use the Langevin thermostat for its temperature control algorithm [223]. The Langevin thermostat is a stochastic method which calculate system dynamics by replaces Newton's equations of motion with the

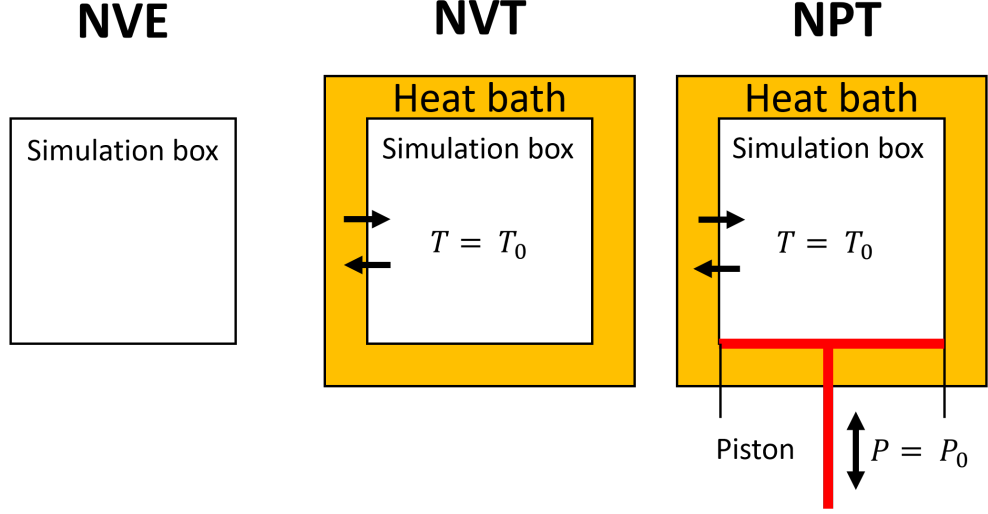


Figure 3.8: Representation of NVE, NVT, and NPT ensembles. $T = T_0$ represents the controlled reference temperature. $P = P_0$ represents the controlled reference pressure.

Langevin equation:

$$\dot{\mathbf{p}}_i = \mathbf{F}_i - \gamma_i \mathbf{p}_i + \mathbf{f}_i \quad (3.19)$$

where $\mathbf{F}_i$ is the force acting on atom i , γ_i is the friction coefficient, $\gamma_i \mathbf{p}_i$ is the momentum of atom i , and $\mathbf{f}_i$ is a stochastic force chosen from a Gaussian distribution with mean zero and unknown variance:

$$\sigma_i^2 = \frac{2m_i\gamma_i k_B T}{\Delta t} \quad (3.20)$$

where Δt is the timestep used to integrate the equations of motion.

The isothermal-isobaric ensemble (NPT) is a commonly used ensemble in biomolecular simulations, especially when simulating lipid bilayers. The NPT ensemble conserves a constant number of moles, pressure, and temperature. This ensemble utilises both a thermostat to maintain temperature, and a barostat to maintain constant pressure.

Barostats function through scaling the simulation box volume to account for changes

in pressure across the trajectory. The compressibility of the system differs across dimension, and consequently the simulation box can change in shape as well as size. The work carried out in this thesis utilises the Nosé-Hoover Langevin piston barostat [224]. The Nosé-Hoover Langevin Piston method combines the Nosé-Hoover thermostat with fluctuations created using Langevin dynamics [225].

The Nosé-Hoover Langevin Piston method calculates box change as

$$\frac{1}{V} \frac{dV}{dt} = \eta \quad (3.21)$$

where $\dot{V}$ is the rate of volume change, V is the box volume, and η is the expansion and compression rate, where $\eta < 0$ indicates a box shrinking and $\eta > 0$ indicates a box growing, and piston motion as

$$\dot{\eta} = \frac{1}{W} (P_{\text{int}} - P_{\text{ext}}) - \gamma\eta - \eta^2 + R(t) \quad (3.22)$$

where $\dot{\eta}$ is the time derivative of the piston velocity, W is the piston mass, P_{int} is the internal pressure of the system, P_{ext} is the target pressure of the system, γ is the friction coefficient, and $R(t)$ is the stochastic random force.

3.8 Enhanced Sampling Methods

According to ergodic theory, if an MD system is allowed to run for enough time, it will be able to explore all potential states. As the nanosecond-to-microsecond scale of all-atom MD simulation is generally insufficient to explore the entire energy landscape of the system, and consequently the space around the energy minimum sees high sampling while high energy regions are poorly sampled - particularly in regions where the energy barriers are greater than $k_B T$.

Enhanced sampling techniques are designed to overcome energy barriers and allow the sampling of high energy regions of the phase space.

3.8.1 Steered Molecular Dynamics

Steered Molecular Dynamics (SMD) is an enhanced sampling technique which can be applied to MD simulations to facilitate the sampling of energetically unfavourable events on MD accessible timescales. SMD works through applying either a constant external force or a constant external pressure onto a specific atom, or group of atoms, which causes it to be pulled in a specified direction to overcome energy barriers [226]. Constant velocity SMD was used in Chapter 7 of this thesis, with the rationale for choosing this technique over constant force SMD detailed in Section 4.4.1 of the next chapter. In constant velocity pulling the chosen SMD atoms are attached to a dummy atom via springs. The dummy atom is pulled via the spring, which the constant velocity is applied to (Figure 3.9). From the output of the pulling, the difference in free energy between the two states can be calculated using Jarzynski equality if sufficient statically independent repeats are performed [227].

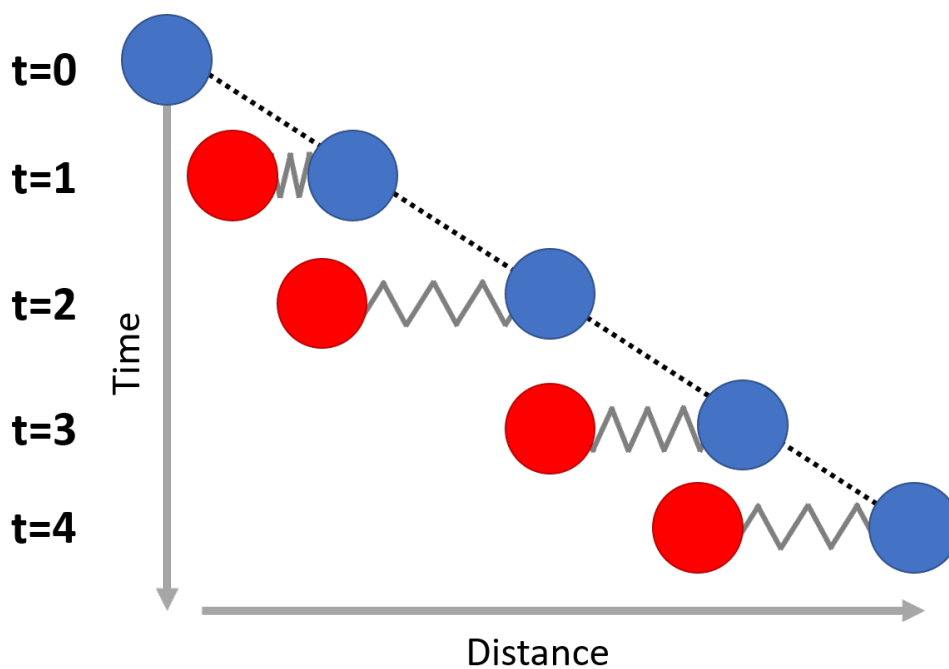


Figure 3.9: 1D representation of constant velocity SMD. As the dummy atom (blue) is pulled at a constant velocity by a spring (grey), the steered atom (red) experiences differing steering force between timesteps, dependent on the distance between the two atoms.

3.8.2 Umbrella Sampling

Umbrella sampling (US) is an enhanced sampling technique which utilises a series of parallel MD simulations referred to as windows which are restrained using harmonic bias potentials to explore a small section of the phase space of the system in each simulation, from which data can be combined to calculate the free energy of different conformational states [228]. The collective variable of the US windows is the reaction coordinate they are placed along, and it is along this collective variable that phase space is explored. Each window has a harmonic bias of K used:

$$\omega_i(\xi) = \frac{K}{2}(\xi - \xi_i^{ref})^2 \quad (3.23)$$

where ξ is the reaction coordinate, ξ_i^{ref} is the reference value for the i th window, K is the harmonic bias force constant strength, and $\omega_i(\xi)$ is the bias potential applied to the system at the i th window. Harmonic bias potential is applied to encourage sampling of phase space near each window's reference position. A higher number of windows is correlated with reduced error, however this increases the required computational resources required to carry out an US. US windows are independent and therefore can be ran in parallel to reduce the physical time required to complete a sampling. US windows are analysed using the Weighted Histogram Analysis Method (WHAM), which produces an unbiased potential of mean force (PMF) from the windows by removing biasing potential and combining data from the windows (Figure 3.10) [229].

3.9 Limitations of MD

Although it is an important and highly useful method of understanding molecular interactions at the nanometre scale, particularly for the work carried out in this thesis, MD is not without limitations and cannot be used indiscriminately. The most obvious limitation of MD is the timescale of the simulation. MD simulations have a high computational cost due to the complex calculations required to simulate the trajectories within the system, often requiring the use of a High Performance Computer to be

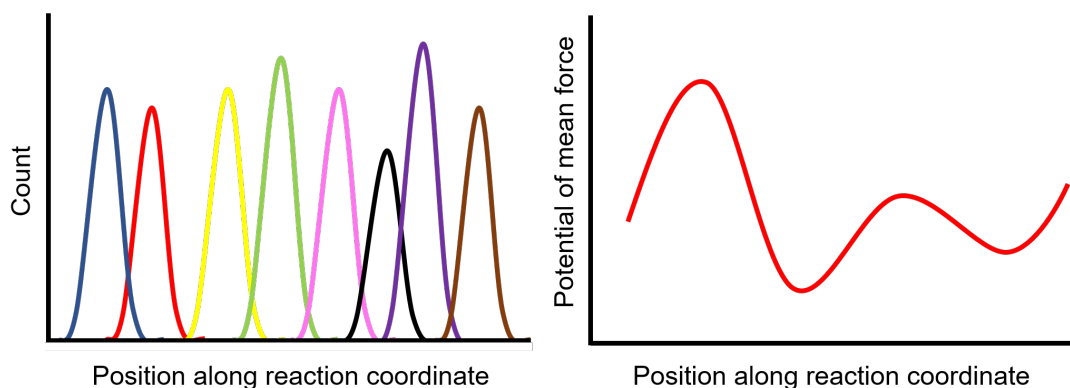


Figure 3.10: Simplified figure representing Umbrella Sampling output. Left: Overlap of histograms produced by WHAM. High overlap represents low bias across the reaction coordinate windows. Right: unbiased PMF curve indicating changes in free energy along the reaction coordinate.

carried out. The extent of the computational cost rises dramatically with increased size of the system, and therefore simulating large systems, such as those in this thesis which typically sit at around one hundred thousand atoms, past a maximum of one microsecond is difficult to justify. Many processes in biomolecular systems happen over longer timescales and therefore cannot be appropriately sampled using atomistic MD methodologies [204].

The nature of MD also leads to slight differences in comparison to physical systems which may make the desired simulation unfeasible. In MD systems used in this work, the bonds between atoms are fixed and therefore cannot be broken or made over the course of a simulation, which excludes the study of reactions that synthesise or degrade molecules from this methodology. Modelling such reactions can be done by employing reactive force simulations or DFT, however DFT simulations have an even larger computational cost and are therefore further limited in system size and simulation duration [230]. Quantum mechanics/molecular mechanics (QM/MM) methods have been developed which allow small sections of the system to be calculated using QM methods while the bulk is calculated using MM methods. This allows the modelling of reactions in a small chemically active region of the system and has applications in biomolecular systems [231]. QM/MM methods have been used in study of certain cell membrane characteristics, however still remain more computationally intensive

than running classical MD simulations [232, 233]. Relatedly, MD also does not model quantum mechanics of atoms, instead relying on approximations which may lead to unphysical behaviour. Because of this, it is essential to validate findings with experimental values where possible. The FF utilised in the work carried out for this thesis also does not allow for the polarisation of atoms within the system, highlighting another deviation from their physical nature. While covalent bonds cannot be created or destroyed in an MD simulation non-covalent bonds such as hydrogen bonds can still be created and monitored across the trajectory in addition to the electrostatics and vdW interactions modelled in the system, allowing detailed insight into systems to still be obtained. Furthermore, the creation of covalent bonds can also be predicted to an extent over the course of a simulation based off the positioning of atoms that are known experimentally to produce covalent bonds.

Some limitations of all-atom MD simulations, particularly those related to system size and time constraints, can be addressed through the use of coarse-grained simulation techniques. Coarse-grained simulations group small clusters of atoms into pseudoatoms, reducing the computational cost by representing molecules with fewer pseudoatoms. While coarse-graining is widely utilised in lipid bilayer simulations, the use of coarse-grained models should not be done indiscriminately [234, 235]. For small molecules, such as the MGs investigated in this thesis, unphysical interactions can be introduced into simulations due to the loss of atomic detail in the pseudoatoms. Consequently, validation against pre-existing reference data is a vital step in these simulations. In the context of this thesis, the MGs C8-MG, C10-MG and C12-MG were constructed specifically for this project and therefore no existing reference parameters were available for coarse-graining. Therefore, all-atom simulations were chosen for this work over coarse-grained simulations to accurately capture MG monomer behaviour.

3.10 NAMD and Running MD Simulations

There are several software packages which have been developed to produce MD simulations. Arguably the most well known software packages are AMBER, [214]

LAMMPS, [236] GROMACS, [237] and NAMD, [211] though for the simulation of large biomolecular systems the gold standard of these are GROMACS and NAMD. This is because AMBER is incompatible with the CHARMM36m FF, which is favoured for biomolecular simulations, and LAMMPS is significantly less computationally efficient when simulating large systems. When comparing GROMACS and NAMD there is arguments to be made for the use of either when running biomolecular simulations as both are computationally efficient and compatible with relevant FFs. For the work presented in this thesis NAMD was selected as the simulation engine. The rationale for the choice of NAMD is described in the following chapter in Section 4.2.1.

Nanoscale Molecular Dynamics (NAMD) is a MD software package developed by the University of Illinois Computational Biophysics group. While primarily developed for use with protein and DNA simulations, NAMD has been proven effective at simulating lipid bilayers [238]. NAMD is built off Charm++, a modification of the C++ coding language. NAMD is parallelisable and can split calculations across different compute nodes to improve efficiency. NAMD additionally supports GPU acceleration for calculations, though this is a feature limited in use to NVIDIA GPUs which support CUDA integration. GPU acceleration using NAMD can significantly increase simulation speeds in comparison to CPU-only NAMD simulations, however the standard method of GPU acceleration, GPU-offload mode, also utilises CPU for part of the calculation and therefore speeds may be limited by the number of both GPU and CPU cores available for the simulation.

To obtain an analysable output from a NAMD trajectory a specific workflow needs to be followed (Figure 3.11).

The first step of the simulation procedure is to prepare a system to simulate. As mentioned earlier in this chapter, biomolecule structures are typically obtained from either data banks, such as the Protein Data Bank (<https://www.rcsb.org/>) or the CHARMM-GUI small molecule library (<https://charmm-gui.org/?doc=archive&lib=csml>) and then solvated with water and ions. Addition of water and ions are required to model realistic physical conditions as otherwise the system would exist in a vacuum with unphysical electrostatic interactions as the system has not been

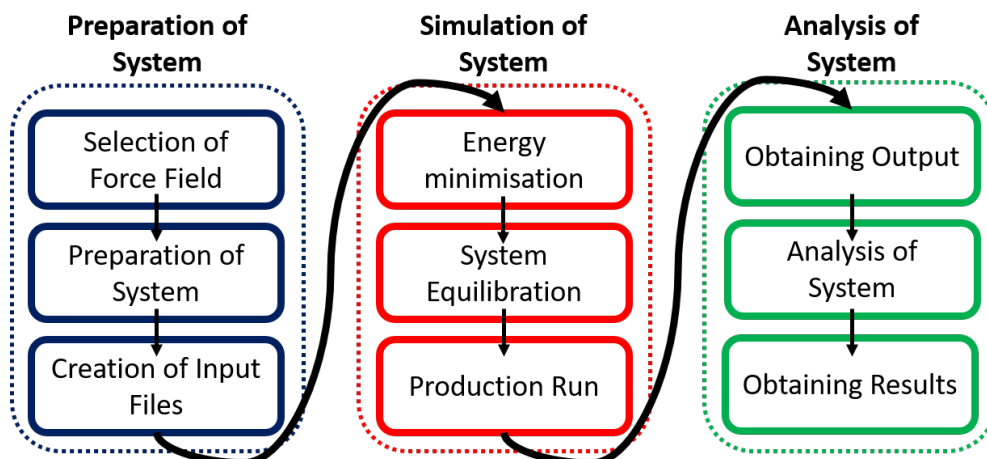


Figure 3.11: Standard workflow for MD simulation.

neutralised. As lipid bilayers are complex structures the CHARMM-GUI Membrane Builder online platform is often used to automate the modelling of realistic bilayer systems and has been utilised heavily in the work presented in this thesis [239]. CHARMM-GUI can also be used to produce various other structures in addition to bilayers such as nanomaterials, ligands, micelles, and glycolipids. CHARMM-GUI also acts as a platform for assisting in the preparation of MD simulations by simplifying molecule parameterisation, generating input files compatible with multiple different FFs and MD software packages, combining molecules into one single system, and converting necessary files between all-atom MD, and polarisable FFs. NAMD requires two key files to simulate a system. The pdb file contains the three-dimensional structure of the system by specifying the coordinates and names of each atom contained within. The protein structure file (psf) contains the topology of all molecules within the system, specifying the mass and charge of each atom in the system in addition to the bonds between atoms. NAMD can create psf files for a system through psfgen if the FF parameters and coordinate file are available. Additionally, the parameter files required for all molecules in the system must be available for the system to allow the calculation of non-bonded interactions between molecules. Parameters for molecules already included in a FF are often readily accessible. Parameters for CHARMM FFs can be obtained from the CHARMM-GUI Ligand Reader and Modeller [240].

The MD simulation itself has three key stages to it. The first stage of the simulation is energy minimisation of the system, which has been discussed in more detail early in this chapter, to prevent unphysical behaviour from occurring due to high potential energy. Once sufficiently minimised, the simulation must next be equilibrated to ensure the force exerted on each atom is appropriate. Equilibration of the system is performed in stages using NVT then NPT simulations, beginning with non-solvent molecules in the system restrained to enable equilibration of the solvent only. This is followed by a series of short NPT simulations one after another which progressively loosen the restraints on the non-solvent molecules before eventually being removed entirely. NAMD uses collective variable (colvars) restraints to do this.

Once equilibration of the system is finished the production run for the system can begin. The production run sees no restraints put on molecules in the system, unless utilising an enhanced sampling method. For biomolecular simulations the production run is typically run using an NPT ensemble to create conditions comparable to experimental conditions with a temperature of 298.15 Kelvin (room temperature) and a pressure of 1.013 bar (1 atm) though this will depend on the environment the system would physically exist in [241]. The duration of the production run selected should be sufficient to appropriately sample the phase space of the system. The length of this will vary by the type of system, and should be evaluated on a case-by-case basis. Even after the dedicated equilibration step of the simulation the properties of the whole system may not have fully equilibrated, and in such situations early sections of the production run trajectory may need to be discarded to allow analysis of only a fully equilibrated system. Some systems also may never fully equilibrate due to the nature of the system. As a NAMD simulation runs, checkpoints files are created at specified time steps, which can allow the extension of a trajectory if runtime is found inefficient.

When a production run has finished, the trajectory produced is analysed to understand how the system changes across the simulated timescale. There are multiple routes to analyse a trajectory, and typically a combination is used. Visual Molecular Dynamics (VMD) allows the visualisation of atom positions calculated at each timestep as individual frames to allow the visualisation of a trajectory and also has in-built

Chapter 3. Molecular Dynamics Simulations

analysis functions [242]. Scripts can also be written in various languages such as Tcl, which VMD is built off, and Python. Python libraries such as MDAnalysis are also available specifically developed for analysis of MD trajectories [243–245].

A more detailed explanation of the parameters selected for the MD simulations presented in this thesis is discussed in the next chapter.

Chapter 4

Methodology

4.1 Rationale

This chapter aims to act as a guide for the methodologies employed to generate the MD simulations necessary to carry out the work required for this thesis. To do this, detail will be provided on the technical aspects of this thesis, including the protocols necessary to run simulations and analyse the outputs generated from them, in order to provide transparency and facilitate any potential repetition or further work based on the work conducted in this thesis in the future. Furthermore, this chapter aims to provide insight into why specific methodological choices were made in this work, in order to explain the decision-making processes behind this work and aid researchers with similar research interests in applying similar simulation methodologies. Specific details for the work carried out in each chapter will be provided in a short methodology chapter at the beginning of Chapters 5, 6, and 7, respectively.

4.2 System Creation

4.2.1 Simulation Software Selection

MD simulations can be performed at various scales, depending on the scale of the desired system to be studied and the level of detail required. Biological membrane simulations are primarily carried out at an all-atom MD or a coarse-grained scale. For membrane

simulations which aim to study larger-scale behaviour of the membrane, such as in the case of membrane proteins, coarse grained simulations can be preferable, as they allow a larger surface area of a lipid bilayer can be simulated. All-atom MD can be preferable for studying how small molecules interact with a bilayer system, as the trade-off for the increased scale in coarse-grained simulations is a decrease in detail. All-atom MD was selected as the simulation approach for the simulations carried out as MGs are small molecules, and a lack of simulation work in the field using MGs meant that validation for coarse-grained systems could not be performed, which may have risked unphysical behaviours occurring in these systems. Therefore, it was deemed preferable to sacrifice the potential for a larger lipid bilayer surface area in the systems in order to generate higher detail information about the molecules.

All-atom MD simulations of cell membranes are often performed using one of two MD simulation software packages. These are GROMACS and NAMD [211, 237]. AMBER can also be used for lipid bilayer simulations [246]. Both packages are viable for producing high-quality MD simulation files, and for the system size simulated, there was no significant difference in estimated time required to complete the simulation. Due to this, there was no significant disadvantages to selecting one software over the other, so NAMD was chosen as the simulation software for this thesis due to better familiarity within the research group. NAMD 3 was selected for the generation of trajectories, as all simulations were carried out using the ARCHIE-WeSt supercomputer, based at the University of Strathclyde (<https://www.archie-west.ac.uk/>). NAMD 3 supports the use of GPU nodes on ARCHIE-WeSt via offloading to increase simulation speed [247].

4.2.2 Force Field Selection

The correct selection of a FF for a simulation is essential to producing simulations with realistic results which can be used to complement and be compared to experimental data. For all-atom simulations, the CHARMM FFs are widely used for biomolecular simulations, as it has been optimised for use with bio-organic molecules such as lipids [213, 248]. CHARMM36 FF has been recognised as highly accurate for the simulation

of lipid bilayers and is compatible with NAMD [249]. This FF has also been selected for use in several publications which simulate lipid bilayers [217, 249]. In addition, all phospholipids which were planned to be used in lipid bilayer models had topology and parameter information included within the FF, so no parameterisation or the molecules used to construct the control lipid bilayer systems was necessary when using CHARMM36m. The CHARMM36m all-atom FF is an updated variant of the CHARMM36 FF, which is further optimised for proteins while retaining parameter information for lipids, so this FF was selected for the simulations carried out in this thesis [208].

4.2.3 Lipid Bilayer Model Selection

To produce results that are comparable to experimental findings, the correct lipid bilayer compositions must be chosen so that a relevant cell membrane model can be simulated. Ideally, these ratios would be previously reported, to allow direct comparison for validation. Three phospholipid bilayer compositions were selected for this work, and the lipids used to construct the membrane models for this study were as follows:

For the **POPC membrane model system**:

- 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (**POPC**)

For the **Gram-positive plasma membrane model system**:

- 1-myristoyl-2-anteisopentadecanoyl-sn-glycero-3-phosphoglycerol (**MAIPG**)
- 1-(12S-methylmyristoyl)-2-(13-methylmyristoyl)-sn-glycero-3-phosphoglycerol (**AIPG**)
- 1-palmitoyl-(12S-methylmyristoyl)-sn-glycero-3-phosphoglycerol (**PAIG**)
- 1,2-dipalmitoyl-rac-glycero-3-phosphoglycerol (**DPPG**)
- 1-myristoyl-2-anteisopentadecanoyl-sn-glycero-3-phosphoethanolamine (**MAIPE**)
- 1-(12S-methylmyristoyl)-2-(13-methylmyristoyl)-sn-glycero-3-phosphoethanolamine (**AIPE**)

- 1-palmitoyl-(12S-methylmyristoyl)-sn-glycero-3-phosphoethanolamine (**PAIE**)
- 1,2-dipalmitoyl-rac-glycero-3-phosphoethanolamine (**DPPE**)
- Tetra-(hexadecanoyl/anteiso-pentadecanoyl)-cardiolipin (**PAICL2**)

For the **Gram-negative inner membrane model system**:

- 1-palmitoyl-2-cis-9,10-methylene hexadecanoyl-phosphatidylethanolamine (**PMPE**)
- 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (**POPE**)
- 1-pentadecanoyl-2-cis-9,9R,11S-methylene-hexadecanoyl-sn-glycero-3-phosphoethanolamine (**QMPE**)
- 1-oleoyl-2-(9Z-hexadecenoyl)-sn-glycero-3-phosphoethanolamine (**OYPE**)
- 1-palmitoyl-2-(9R,11S-methylene-hexadecanoyl)-glycero-sn-3-phosphoglycerol (**PMPG**)
- 1-palmitoyl-2-(9Z-hexadecenoyl)-sn-glycero-3-phosphoglycerol (**PYPG**)
- 1,3-bis(sn-3'-phosphatidyl)-sn-glycerol cardiolipin (**PVCL2**)

The structures of all these lipids are shown below in Figure 4.1. The exact ratios of these lipids in each model are shown in Tables 4.1, 4.2, and 4.3.

The simplest lipid bilayer model (Table 4.1) consists solely of the zwitterionic POPC. The POPC membrane is one of the most well-characterised membrane systems, and is most closely similar to eukaryotic cell membranes, however they can also be utilised as a simple system which can provide general, fundamental information about membrane properties which can be applied to a variety of more complex phospholipid bilayer systems. POPC bilayers have many publications describing their behaviour [250–252]. The POPC model membrane is the focus of the simulations conducted in Chapter 5 of this thesis.

In addition to the POPC bilayer system, two further models were selected to produce more realistic simulations of bacterial cell membranes, by representing the G+ plasma

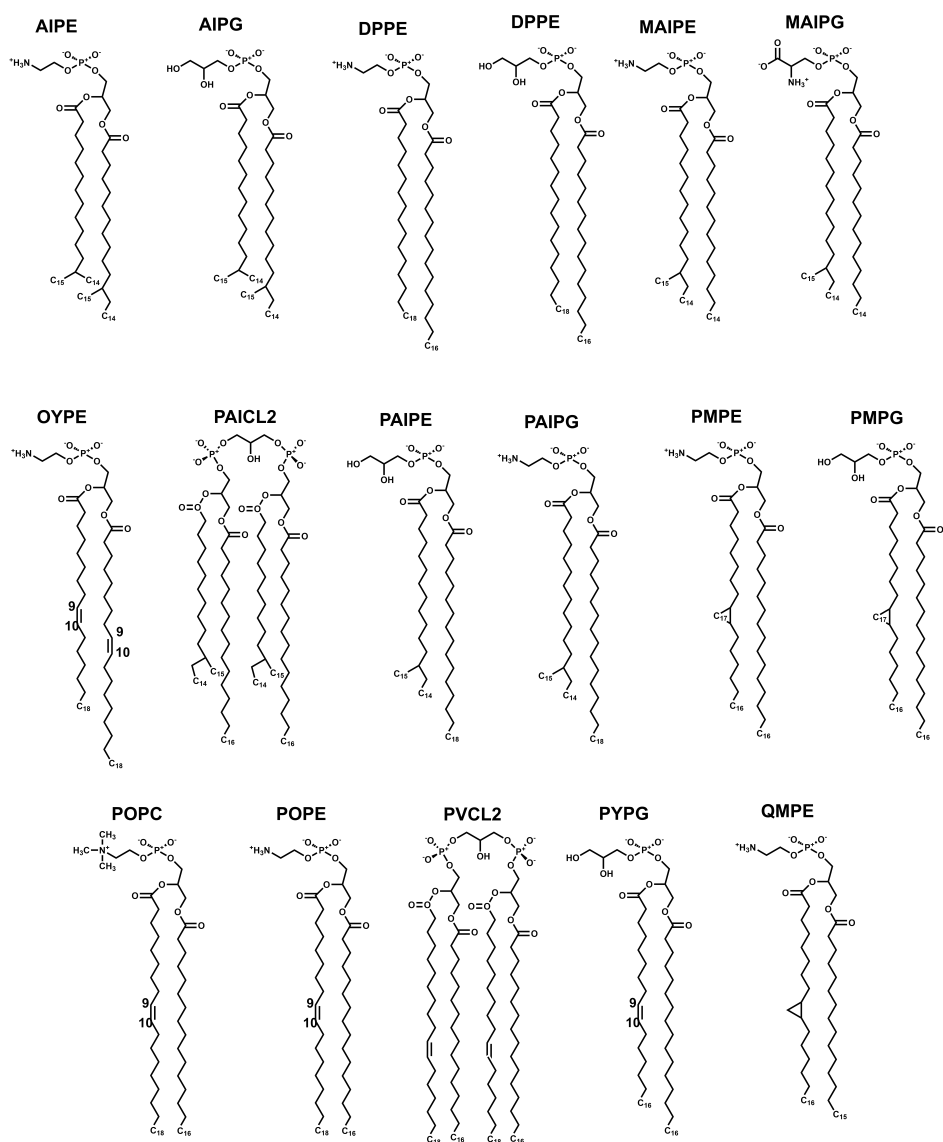


Figure 4.1: Phospholipids used to construct membrane models used in this thesis.

Table 4.1: Bilayer composition of the POPC model system. OL and IL represent the number of molecules of POPC in the outer and inner membrane leaflets respectively. X(Y/Z) represents the structure of the head group (X) and the Sn1 (Y) and Sn2 (Z) lipid tails, respectively. N:1(9Z) represents a double bond present in the acyl chain at the C9 position. PC = phosphatidylcholine.

Lipid Name	Lipid Head/Tails	OL count	IL count	Charge
POPC	PC(16:0/18:1(9Z))	100	100	0

membrane (Table 4.2), and the G- inner membrane (Table 4.3) respectively. Both these models were composed entirely of phospholipids.

Table 4.2: Bilayer composition of the Gram-positive bacterial plasma membrane model system (G+PM). OL and IL represent the number of molecules for each lipid species in the outer and inner membrane leaflets, respectively. X(Y/Z) represents the structure of the head group (X) and the Sn1 (Y) and Sn2 (Z) lipid tails, respectively. N:1(XZ) represents a double bond present in the acyl chain at the carbon X along position. PVCL2 has two acyl tails at the 1' and 3' positions PG = phosphoglycerol, PE = phosphatidylethanolamine. CL = cardiolipin.

Lipid Name	Lipid Head/Tails	OL count	IL count	Charge
MAIPG	PG(14:0/a15:0)	22	22	-1
AIPG	PG(a15:0/i15:0)	27	27	-1
PAIPG	PG(16:0/a15:0)	9	9	-1
DPPG	PG(16:0/16:0)	7	7	-1
MAIPE	PE(144:0/a15:0)	9	9	0
AIPE	PE(a15:0 /i15:0)	11	11	0
PAIPE	PE(16:0 /a15:0)	4	4	0
DPPE	PE(16:0 /16:0)	3	3	0
PAICL2	CL(1'-[16:0 / a15:0],3'- [16:0 / a15:0])	8	8	-2

Table 4.3: Bilayer composition of the Gram-negative inner membrane model system (G-IM). OL and IL represent the number of molecules for each lipid species in the outer and inner membrane leaflets, respectively. X(Y/Z) represents the structure of the head group (X) and the Sn1 (Y) and Sn2 (Z) lipid tails, respectively. N:1(XZ) represents a double bond present in the acyl chain at the carbon X along position. cy indicates the presence of a cycloacyl group in the acyl chain. PVCL2 has two acyl tails at the 1' and 3' positions PG = phosphoglycerol, PE = phosphatidylethanolamine. CL = cardiolipin.

Lipid Name	Lipid Head/Tails	OL count	IL count	Charge
PMPE	PE(16:0/cy17:0)	46	46	0
POPE	PE(16:0/18:1(9Z))	13	13	0
QMPE	PE(15:0/cy17:0)	12	12	0
OYPE	PE(18:1(9Z)/16:1(9Z))	8	8	0
PMPG	PG(16:0/cy17:0)	10	10	-1
PYPG	PE(16:0/16:1(9Z))	9	9	-2
PVCL2	CL([16:0/18:1(11Z)], [16:0/18:1(11Z)])	2	2	-2

The lipid ratios of these models were taken from a study by Pogozeva *et al.* (2022), which performed MD simulations of 18 realistic biological membranes, including the two bacterial membrane compositions listed above [1]. Importantly, this study was

performed using the CHARMM36 FF, and utilised lipids which were parameterised, validated, and added to the FF to be used in the CHARMM-GUI bilayer builder in this study, allowing the data published in this study to act as a direct comparison to ensure the validity of the simulations performed in Chapter 6 of this thesis. The lipid ratios and species for the systems in Pogozheva *et al.* were determined through a literature review of experimental analysis of real bacterial membranes, from which information on phospholipid headgroup ratios and tail structures across G+ and G- bacterial species was accumulated. From this information, simplified lipid ratios that represented the correct lipid headgroup ratios and representations of average tail length and saturation were created and the lipids built and implemented into CHARMM-GUI. Although the systems from this paper were designed to act as benchmark systems, these models have not been widely adopted in lipid simulations, as often complex bacterial bilayer models are tailored towards representing a specific organism. Despite this, these models are still widely referenced in literature and are still representative of G+ and G- bacterial membranes, so were selected due to being easy to construct in CHARMM-GUI. It should be noted that although these models are representative of bacterial bilayers, the full complexity of bacterial membrane physiology cannot be captured through MD simulations. Because of this, some compromises must be made to allow simulations to be performed. For these model systems the most important aspects of bacterial membranes lost are the lack of curvature, which has been shown to possibly play a role in MG mechanisms of action, and the lack of membrane proteins which are normally distributed throughout the membrane [27].

4.2.4 Monoglyceride Structure Creation

The MGs selected for this thesis were the saturated 1-monoglycerides 1-monocaprylin (C8-MG), 1-monocaprin (C10-MG), 1-monolaurin (C12-MG), and 1-monostearin (C18-MG) (Figure 6.10). In the CHARMM36m FF, the parameter and topology information for C18-MG already existed, under the ID GMS1. The structure of GMS1 was also freely downloadable in the form of a PDB file from the CHARMM-GUI Small Molecule Library (<https://charmm-gui.org/?doc=archive&lib=csml>). As

C8-MG, C10-MG, and C12-MG were not pre-existing in the FF, parameters for these molecules were created in line with the information contained in the FF for C18-MG and the FA equivalents to C8-MG, C10-MG, and C12-MG; caprylic acid, capric acid, and lauric acid. The PDB and PSF files for these molecules were initially generated using the CHARMM-GUI Ligand Reader & Modeller. These files were then manually checked and compared to similar files existing in the FF to ensure consistency in partial charges. [240] Parameters for these molecules can be found in Appendix B. In the CHARMM36m FF molecules are divided into groups of small atom clusters which are individually balanced in electrostatics and are connected together to construct a molecule. Each atom group required construction for C8-MG, C10-MG, and C12-MG was already present in the CHARMM36m FF, and therefore each of these groups had already been optimised against experimental and quantum mechanical data. Due to this, rigorous validation of the generated parameters was not required for these molecules, as no new atom groups had to be developed for the construction of these molecules.

4.2.5 System Generation

Starting structures for each MD simulation were created using the CHARMM-GUI Bilayer Builder [253]. CHARMM-GUI is a web-based graphical user which acts as a platform for the creation of MD simulation inputs, and provides several useful features, including the Ligand Reader & Modeller mentioned above, and the ability to build lipid bilayers with specific lipid ratios [254]. As CHARMM-GUI is automated, streamlined, and reliable at generating accurate lipid bilayers, this tool was selected for use in this thesis. Bilayers were created by specifying lipid species and the exact number of molecules required for the creation of a system containing 100 membrane lipids in each leaflet. For the phospholipid bilayer models, 100 membrane lipids were in each leaflet and symmetrical between leaflets. TIP3P water was added to each system 40 Å along the Z axis on each side of the bilayer. TIP3P was selected as the water model for this study as it is optimised for use with the CHARMM36 FF, and has been used in previously published literature [217, 249]. Rectangular Periodic Boundary Conditions

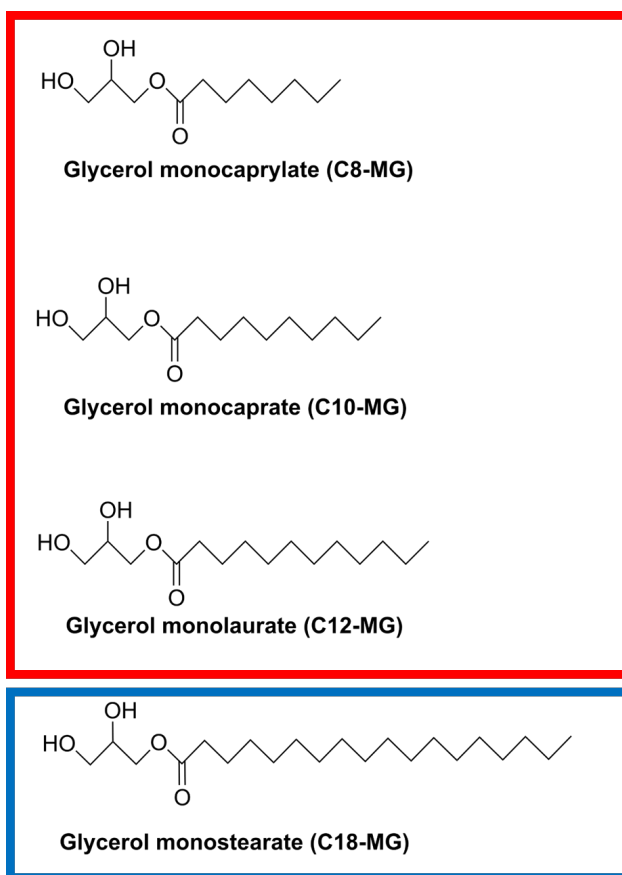


Figure 4.2: Chemical structures of lipids used in this work: 1-monoglycerides 1-monocaprylin (C8-MG)), 1-monocaprin (C10-MG), 1-monolaurin (C12-MG), and 1-monostearin (C18-MG). Red represents medium-chain saturated MGs. Blue represents long-chain saturated MGs.

were employed. Na^+ and Cl^- ions were used to neutralise systems where relevant, and set the sodium chloride concentration to 0.15 M. This concentration, alongside the simulation temperature of 298.15 Kelvin, was selected to reflect laboratory conditions. Each system underwent further CHARMM minimisation for 100 steps during output generation, and outputs were generated in NAMD format [255].

As GMS1 is pre-existing in the CHARMM36m FF and CHARMM-GUI molecule library, systems which were intended to contain MGs were generated with GMS1 molecules pre-inserted. A Tcl script was developed in-house in order to reduce the length of GMS1 acyl tails to the MG required for the system, and alter the residue name of the PDB and PSF to one relevant to the new molecule (i.e. GML1). This

script was executed in VMD, and is available in Appendix C. This process required the isolation of the membrane and MG components from the surrounding water box, so once MG acyl chain length had been adjusted, the system was re-solvated in VMD with TIP3P water, and ions were re-added to the system. Following this, a CHARMM CRD format file was created from the NAMD PDB and NAMD PSF of the final system, and fed into the CHARMM-GUI Force Field Converter alongside parameters for C8-MG, C10-MG, or C12-MG as required, in order to create fresh NAMD input files for running the simulation. An example of the typical system generated from this process can be found in Figure 4.3.

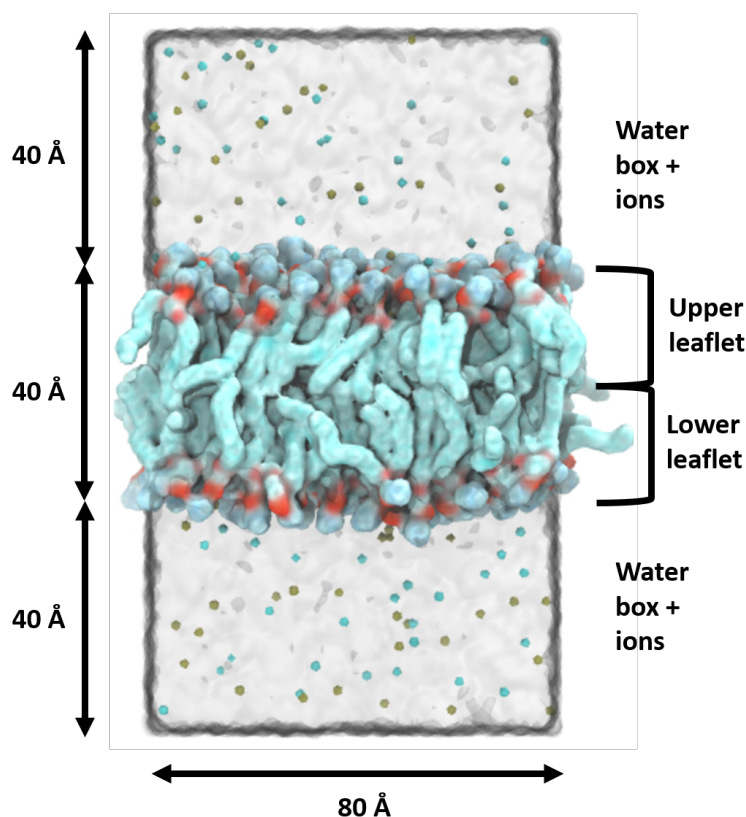


Figure 4.3: Annotated example system set-up. Lipid bilayer is positioned at the centre of the system with a 40 Angstrom water box on either side along the Z axis. MGs were added to systems in alignment with POPC headgroup positioning. Control system without MG inserted pictured.

The choice to pre-insert MG into the bilayer prior to the simulation as opposed to a simulation setup which studied initial MG insertion dynamics and then bilayer property

destabilisation was made for three reasons. Firstly, pre-insertion of MG into the bilayer allowed for direct control of MG count within the bilayer, which is a useful variable for understanding the extent of bilayer disruption differences between MGs with varying tail lengths. This is difficult to control experimentally for a similar reason to placing MG in the water box - namely that you cannot directly control how MGs partition into the bilayer, so the count of MG molecules within the bilayer cannot be controlled. This was especially important when comparing systems loaded fully asymmetrically or fully symmetrically with MG in Chapter 5. Secondly, placing MG in the water box while also employing PBCs in initial test simulations caused instances where some monomeric MG would diffuse across the PBCs and insert into the lower leaflet while others would insert into the upper leaflet, which is behaviour that could not happen in real-world conditions as MGs cannot access the inner facing leaflet of a cell membrane without first diffusing through the outer facing leaflet, so pre-inserting MGs guaranteed that MGs would interact with only the upper leaflet when inserting into the bilayer. Finally, additional simulation time was required for MGs to partition from the water box into the bilayer before any changes to properties could be analysed, which would have been a significant computational cost due to the large number of simulations conducted in this thesis.

Other approaches have been utilised in literature for simulating the behaviour of similar amphiphilic molecules when interacting with lipid bilayers. Kang *et al.* successfully studied uptake dynamics of permeation enhancers salcprozate sodium and sodium caprate by creating a micelle structure in a water box between two phospholipid bilayers and simulating direct interactions between the micelle and lipid bilayer [256]. A similar methodology to the pre-insertion of molecules utilised in this thesis was performed in Drew Bennett *et al.* to study the impact of asymmetric stress on bilayer perturbation by other neutral or charged amphiphiles [197]. Therefore, there are several methodologies which can be utilised for the study of bilayer disruption by lipids which differ in appropriateness for different research aims. Pre-inserting MGs into the bilayer prior to the simulation was the most time efficient approach to address the research objectives of this thesis and allowed the most effective variable control for the

experimental hypotheses it aimed to examine.

4.3 Molecular Dynamics Simulation Protocol

All-atom MD simulations in NAMD have two main phases. Firstly, an energy minimisation and equilibration phase is carried out to ensure that minimum energy is reached in the system to prevent unphysical behaviour from occurring. Following this, a production run is performed, which produces the main simulation trajectory.

As the water box and ions had been replaced following MG tail adjustment, an initial water equilibration step was performed. In this step, all non-water atoms were fixed, and minimisation was carried out for 10^3 steps in an NPT ensemble. Pressure was controlled by semi-isotropic pressure coupling at 1.01325 using the Langevin piston Nosé-Hoover method. Following this, an equilibration protocol was followed, using adapted input files generated by the CHARMM-GUI Force Field Converter. Equilibration was conducted in 6 steps with progressively reducing restraint strength in each step (Table 4.4). Collective variable restraints (colvars) were used for restraints. Planar restraints (restraining atoms within a plane), dihedral restraints (restraining bond angles between atoms), and carbohydrate restraints (restraint of glycosidic linkages) were all employed.

Table 4.4: Equilibration protocol parameters. PR represents the planar restraint; DR represents the dihedral restraint; CR represents the carbohydrate restraint. Restraint potential given in $\text{kcal mol}^{-1} \text{\AA}^{-1}$. Brackets in run column indicate timestep in fs

Step	Minimisation (steps)	Run (steps)	PR	DR	CR
1	10^4	1.25×10^5 (1)	5	500	500
2	-	1.25×10^5 (1)	5	200	200
3	-	1.25×10^5 (1)	2	100	100
4	-	2.5×10^5 (2)	1	100	100
5	-	2.5×10^5 (2)	0.2	50	50
6	-	2.5×10^5 (2)	0	0	0

The production run of each system was performed using an NPT ensemble for 600 ns with the first 100 ns discarded as additional equilibration time, and the final 500 ns used for analysis. Pressure was controlled by semi-isotropic pressure coupling at 1.01325 using the Langevin piston Nosé-Hoover method, and temperature

was controlled by a Langevin thermostat. PME was employed for the calculation of long-range electrostatics with a PME cut-off of 1.0 Å. Output trajectories were updated every 5×10^5 steps. A 2.0 fs timestep was used for the production simulations. Due to the 48-hour maximum runtime limit for jobs running on the ARCHIE-WeSt GPUs, the production run was completed in 4 identical steps, each simulating 150 ns and restarting from the final checkpoint of the previous step. Each input file can be found in Appendix C.

4.4 Enhanced Sampling

In addition to the classical all-atom MD simulation utilised in this thesis, enhanced sampling techniques were utilised to further explore the interaction of MGs with lipid bilayer systems. The two techniques used in this work were SMD and US, which were utilised to better understand the long-time-scale movement of MGs through a lipid bilayer.

4.4.1 Steered Molecular Dynamics

SMD simulations can be performed in NAMD and are performed by one of two methods. These are Constant Force (CF) pulling and Constant Velocity (CV) pulling. See Chapter 3.8.1 (Steered Molecular Dynamics) for the theory underpinning the CF- and CV-SMD procedure. CV-SMD was selected for the simulations carried out in this thesis as CV-SMD allows a steady rate of movement through the lipid bilayer. These SMD simulations were carried out primarily for the purpose of having a range of MG positions within the simulation box available as reference points for producing the US windows detailed in the next section, and therefore producing trajectories which provide all necessary conformational states of the molecule along a reaction coordinate was important for future simulations.

The initial starting structures for SMD simulations featured a single MG molecule in the water box above a lipid bilayer, which was then pulled through the membrane. The membrane structure for each system was taken from the final frame of the

relevant classical MD trajectory was saved as a PDB format file, as these bilayers had already undergone the necessary energy minimisation from the initial CHARMM-GUI generated outputs. The systems used for this purpose are highlighted in Table 4.5.

Table 4.5: Components of lipid bilayers used in the creation of starting structures for the SMD. MG Count represents monoglyceride concentration in the bilayer system the SMD MG was pulled through. SMD MG represents MGs dummy atoms were attached through for pulling in individual systems

System Name	Bilayer Model	MG Count	SMD MGs
POPC_CTRL	POPC	0	C8-MG, C10-MG, C12-MG, C18-MG
POPC.44GMCY	POPC	44 C8-MG	C8-MG
POPC.44GMC	POPC	44 C10-MG	C10-MG
POPC.44GML	POPC	44 C12-MG	C12-MG
POPC.44GMS	POPC	44 C18-MG	C18-MG
G+PM_CTRL	Gram-positive bacterial plasma membrane (G+PM)	0	C10-MG
G-IM_CTRL	Gram-negative bacterial inner membrane (G-IM)	0	C10-MG

One additional molecule of C8-MG, C10-MG, C12-MG, or C18-MG, which was to be the molecule pulled through the membrane, was added to the simulation box approximately 35 Å above the membrane centre (0 Å) (Figure 4.4) To prevent overlap of atoms causing issues with the simulations, all water molecules and ions within 4 Å of the added MG molecule were deleted using the TopoTools VMD Plug-in [257]. Any ions removed during this step were re-inserted into the simulation box to maintain the desired ion concentrations. As the water box is altered during this process, a water minimisation step with MG, membrane lipids, and ions fixed identically to the step used prior to the equilibration steps of the classical MD simulation was performed prior to the SMD simulation.

All atoms except the water box MG were fixed with an occupancy of 0 as a reference file for the SMD harmonic constraints. SMD dummy atoms were attached to each atom of the SMD molecule, so that the constant velocity was applied equally across the whole molecule. This was done rather than pulling by headgroup or centre of mass in an attempt to preserve the shape of the MG. Centre of mass pulling was chosen rather than a head group pulling due to the assumption that pulling the molecule

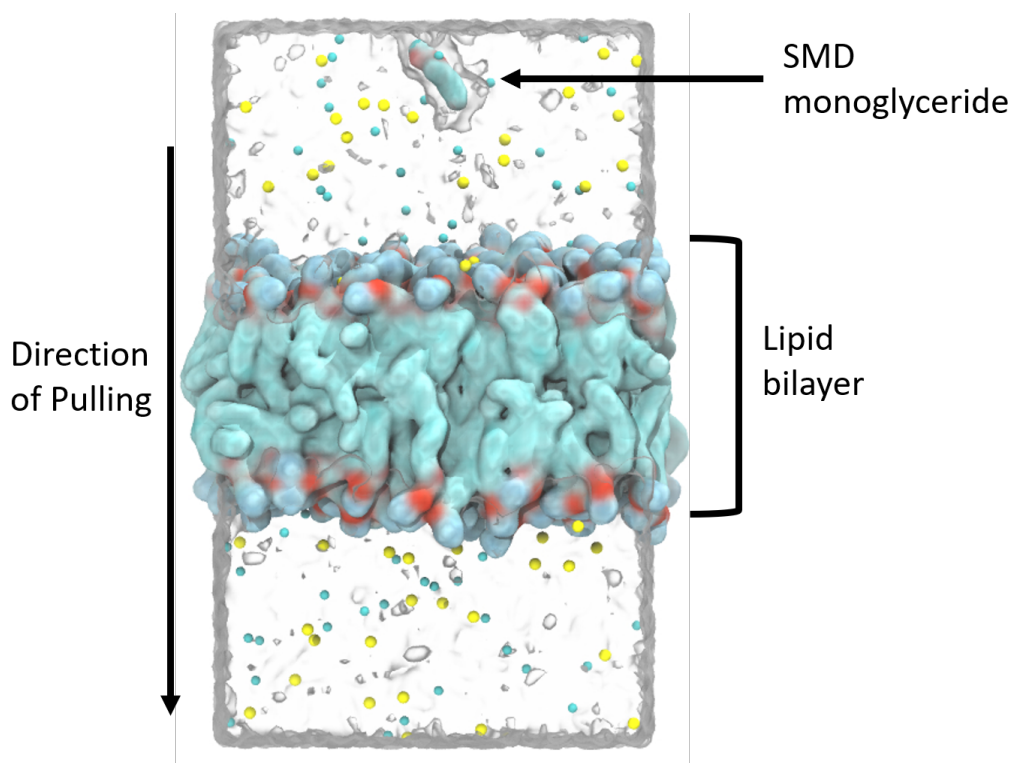


Figure 4.4: Example system set-up for SMD simulation. Lipid bilayer is positioned at the centre of the system with a 40 Å water box on either side along the Z axis. POPC membrane without pre-inserted MG, and a single molecule of GMCY in the water box pictured.

through the membrane from its centre would allow the MG to more easily reorient itself in line with the other molecule in the upper and lower leaflets of the bilayer as it moved through the structure, which would both be more realistic, and provide a better reference point for umbrella sampling windows. A SMD harmonic force constant of $k=1$ and a constant velocity of 5×10^{-6} Å/fs was applied to each SMD, though additional velocities of 2.5×10^{-6} Å/fs and 1×10^{-5} Å/fs were also tested in some SMD simulations to determine the most suitable velocity for MG pullings. This is discussed in further detail in Chapter 7. Each SMD was conducted for 10^7 steps, which was an appropriate time for each MG to be pulled through the entirety of each phospholipid bilayer system, but not the Gram-negative outer membrane model. SMD simulation trajectories were output every 5×10^4 fs. A 1.0 fs timestep was used for the SMD simulations.

4.4.2 Umbrella Sampling

US was used to obtain the free energy profile of MG movement through a lipid bilayer along the Z-axis. The collective variable used for US in this thesis was the reaction coordinate. All atoms of the MG pulled in the previous SMD simulations was selected to define the molecule, while the lipid bilayer boundaries were defined by the phosphorus atoms of each phospholipid in the upper and lower leaflets.

US simulation windows were obtained from the SMD pulling trajectories for relevant systems. US windows were spaced with 1 Å intervals between them. All US used at least 35 windows, from 0 Å to +34 Å along the Z axis. Starting structures to run the simulation of each window were also taken from the respective SMD trajectory and input files. Only the upper leaflet of each bilayer was sampled due to time constraints. The harmonic constant used was $K = 0.025 \text{ kcal mol}^{-1} \text{Å}^{-2}$. Each window was simulated for 40 ns. The free energy curve was calculated using the Weighted Histogram Analysis Method (WHAM) developed by the Grossfield Laboratory to remove biasing potential [229].

The protocol used for performing US simulations in NAMD in this thesis was developed previously in-house by Dr Magdalena Hudek. The templates for files used for US in this thesis are available online at https://github.com/mhudek/MD_utilities/tree/main/umbrella_sampling/NAMD.

4.5 System Analysis

4.5.1 VMD Analysis Tools

In addition to the manipulation of MD systems, VMD contains a variety of built-in features for the analysis of trajectories. VMD can allow manual visual analysis of trajectories displayed in its molecule viewer window, has several tools for analysing specific properties of a system, and is also compatible with Tcl-code-based analysis scripts [242].

Root Mean Square Displacement

Root mean square displacement (RMSD) was calculated using the VMD RMSD Visualizer Tool. RMSD is calculated by aligning each frame of a trajectory to the reference position of a selected set of atoms, then measuring the positional deviation of these atoms over each frame. The formula for calculating RMSD is $RMSD = \sqrt{\frac{1}{N} \sum_{i=1}^N (r_i)^2}$ where N is the number of atoms, and r_i is the distance between atoms after alignment.

Partial Density Profile

One-dimensional partial atomic density profiles were calculated using the VMD Density Profile Tool [258]. Profiles were calculated by the Density Profile Tool by equally dividing the Z axis into equally sized 1 Å bins along the Z-axis. From this, the density of the selected atoms was calculated from each frame for each bin, and then averaged. Density was finally calculated for each bin by dividing the averaged mass by the volume of the bin.

4.5.2 MEMBPLUGIN

MEMBPLUGIN is an optional VMD plug-in which further enhances the analytical capabilities of VMD by allowing the analysis of several properties important to characterising the behaviour of lipid bilayers. MEMBPLUGIN version 1.1.32 was used for all work carried out in this thesis [259].

Membrane Thickness Across the z-Axis

Membrane thickness along the Z-axis was measured using the Membrane Thickness tool function. Z-axis thickness was calculated as the distance between density peaks in the upper and lower leaflets. MEMBPLUGIN calculates density peaks by first calculating a density profile along the Z axis for specified atoms, which are expected to produce peaks at the lipid:water interface of each leaflet, usually the phosphorus atoms in phospholipid head groups. The peak at each leaflet was then identified, and the membrane thickness was calculated as the distance between the two. This process was repeated for each

frame and then averaged. Outputs contained both the average membrane thickness and the thickness for each frame.

Membrane Thickness Across the xy Plane

Thickness across the xy -plane was also calculated using the Membrane Thickness tool function. As above, the thickness per frame was calculated, and the average positions of the lipid heads are interpolated onto a 2D grid, which represents the xy -plane. This produces a contour map of Z-axis thickness variation across the xy -plane, enabling the visualisation of local variation in thickness within sections of the bilayer surface.

Area Per Lipid

Area per lipid (APL) was calculated using the Area Per Lipid tool function. APL was calculated using Voronoi tessellation, where the xy plane of the membrane was divided into local regions representing each lipid by the construction of Voronoi cells around each lipid, identified by the atom identifier provided to the tool. The area of each Voronoi cell was then calculated for each frame and then averaged. Outputs contained the average APL for each lipid species in the membrane and the overall APL, which was defined as the average area of each lipid in the membrane.

Lipid Tail Alignment

Lipid tail alignment, or lipid tail deuterium order parameters (S_{CD}), was measured using the Tail S_{CD} tool function. S_{CD} was calculated using MEMBPLUGIN with the deuterium order parameter formula $S_{CD} = \langle \frac{3\cos^2\theta - 1}{2} \rangle$ where θ represents the angle between a specific C-H bond vector and the membrane normal (z -axis) where 0° is perfectly parallel to the membrane normal, and 90° is perpendicular to the membrane normal. θ was calculated for all C-H bonds on the acyl tails of selected lipid species and averaged to produce an order parameter profile, which gave the average S_{CD} value for each carbon on an acyl tail, where 0.5 is complete alignment of acyl tails with the membrane normal, and 0 is complete acyl tail disorder. MGs were excluded from tail analysis as MEMBPLUGIN did not support these molecules in its pre-defined

Chapter 4. Methodology

templates.

Chapter 5

The Impact of Monoglycerides on Model POPC Lipid Bilayer Structure and Properties

5.1 Rationale

As discussed in previous chapters, although there are strong hypotheses regarding the antimicrobial effects of MGs, the work performed to investigate the antimicrobial activity of MGs has primarily been performed experimentally. Consequently, our understanding of these mechanisms is inherently limited to the insight which can be gained through laboratory based approaches. In particular, these approaches do not allow comparison of MGs at equal concentrations within a bilayer, as delivery of MGs into the structure via micelle cannot be precisely controlled, and there can be difficulty highlighting interactions that occur at a molecular level using experimental models.

This chapter presents a study which utilises MD simulations to study how the presence of MGs can disrupt the highly regulated properties of a lipid membrane by modelling a section of a model lipid bilayer containing randomly distributed MGs in varying concentrations across leaflets to investigate how the bilayer responds to high localised MG concentrations, and investigate associated hypotheses regarding MG disruption mechanisms.

5.2 Chapter Aims

1. Assess to what extent the concentration of MG within the membrane is correlated to disruption with membrane characteristics.
2. Determine whether differences in MG structure influence the disruption of membrane properties.
3. Determine whether MG-generated asymmetry in membrane composition plays a role in destabilisation of membrane characteristics.

5.3 Methodology

5.3.1 System Setup

The initial bilayer structure of each membrane system was generated using the CHARMM-GUI membrane builder. [239] The starting membrane structures were entirely composed of POPC with 100 molecules in each leaflet. To create membranes with MG inserted into the membrane, C18-MG was added into the upper leaflet at a count of 12, 28 or 44, or 100 molecules. For symmetric membrane systems, 50 molecules of the MG were placed into both the upper and lower leaflet of the membrane. In systems designed using C8-MG, C10-MG, or C12-MG, a Tcl script was developed in-house to modify the structure of C18-MG to the necessary chain length, which was executed in VMD (available in Appendix C). [242] Table 5.1 reports the lipid distributions of each system. Figure 6.10 displays the structures of each lipid used across the systems. The systems relevant to each research aim are described in Table 5.2

The CHARMM36m all-atom forcefield was used in the simulations. [208] Additional parameters for C8-MG, C10-MG, and C12-MG were generated as these were not provided through CHARMM-GUI. Parameters were generated based on the existing GMS1 parameters in the forcefield and validated by comparison to the CHARMM36m FF and parameters generated by the CHARMM-GUI automatic ligand reader [240] to

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Table 5.1: Initial MG count present in the upper leaflet (UL) and lower leaflet (LL) in the initial conformation of each system. "MG_A" represents an asymmetric bilayer system. "MG_S" represents a symmetrical bilayer system. All bilayers contained 100 molecules of POPC in each leaflet.

System Name	UL MG Count	LL MG Count
12GMC_A	12 C10-MG	0
28GMC_A	28 C10-MG	0
44GMC_A	44 C10-MG	0
100GMC_A	100 C10-MG	0
100GMCY_A	100 C8-MG	0
100GML_A	100 C12-MG	0
100GMS_A	100 C18-MG	0
100GMCY_S	50 C8-MG	50 C8-MG
100GMC_S	50 C10-MG	50 C10-MG
100GML_S	50 C12-MG	50 C12-MG
100GMS_S	50 C18-MG	50 C18-MG
200GMCY_A	200 C8-MG	0
200GMC_A	200 C10-MG	0
200GML_A	200 C12-MG	0
200GMS_A	200 C18-MG	0
200GMCY_S	100 C8-MG	100 C8-MG
200GMC_S	100 C10-MG	100 C10-MG
200GML_S	100 C12-MG	100 C12-MG
200GMS_S	100 C18-MG	100 C18-MG

ensure compatibility with the CHARMM36m forcefield. Parameters are available in Appendix B.

All models were solvated by adding 40 Å of TIP3P water either side of the membrane and NaCl ions were added to a concentration of 0.15 mol/L. The net charges of the POPC bilayer and all MGs were 0, so no neutralisation was required for the systems. All systems underwent an initial NVT simulation for energy minimisation for 10 ns using collective variable restraints which were reduced in strength as the simulation continued. The CHARMM-GUI equilibration protocol was followed, conducting the equilibration in 6 steps with progressively reducing restraint strength in each step (exact restraints detailed in Chapter 4). Following this, an NPT ensemble was used for 100 ns of equilibration and 500 ns of production runs. The production trajectories were executed at laboratory conditions with constant temperature (298.15 K) and pressure (1.01325 bar) with a 2 fs timestep. A 12 Å vdW cut-off and PME were employed together.

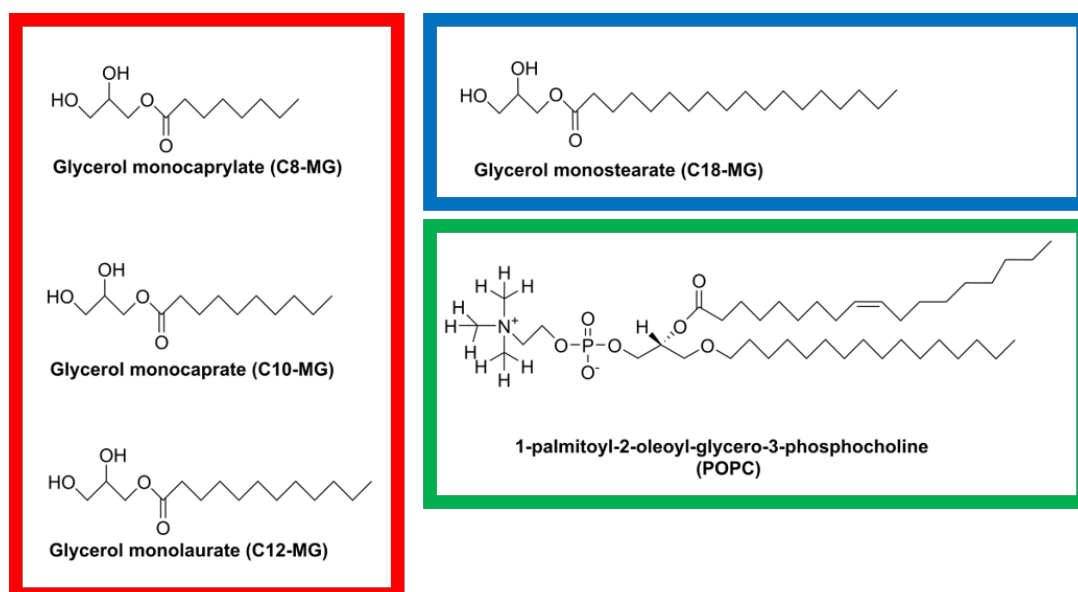


Figure 5.1: Chemical structures of lipids used in this chapter: glycerol monocaprylate (C8-MG), glycerol monocaprate (C10-MG), glycerol monolaurate (C12-MG), glycerol monostearate (C18-MG), and POPC. Red represents medium-chain saturated MGs. Blue represents long-chain saturated MGs. Green represents membrane phospholipids.

All models were simulated for 600 ns each with the first 100 ns used for equilibration and to allow relaxation of the membrane system. This 100 ns period was excluded from data analysis presented below, and further explanation of why this cut-off was selected is described in Section 5.4.1. All simulations were performed using NAMD3 CUDA version. [211]

5.3.2 Parameterisation of Monoglycerides

While the medium-chain saturated MGs relevant to this study were selected based from existing experimental literature on the subject, parameters for the selected molecules were not readily available in the selected CHARMM36m FF and therefore had to be created for the purpose of this study. The initial coordinate and topology files for each of the MCMGs (C8-MG, C10-MG, C12-MG) were obtained using the CHARMM-GUI ligand reader and modeller. While the CHARMM-GUI ligand reader and modeller provides the necessary files to use these molecule in simulations, the outputs generated by it often require manual tweaking to ensure accuracy before use in MD simulations.

Chapter 5. The Impact of Monoglycerides on Model POPC Lipid Bilayer Structure and Properties

Table 5.2: Systems relevant to each of the key research aims investigated in this chapter. "Local MG" represents investigation of how the number of MG monomers in the bilayer relates to the extent of bilayer disruption. "MG Tails" represents investigation of how MG tail length influences the degree of lipid bilayer disruption. "MG Symmetry" represents how important asymmetric stress generated by MG insertion into only one leaflet of the bilayer influences lipid bilayer disruption. "200 MG" represents investigation into how an extreme number of MG molecules in a bilayer system affects bilayer properties.

System Name	Local MG	MG Tails	MG Symmetry	200 MG
12GMC_A	X			
28GMC_A	X			
44GMC_A	X			
100GMC_A	X	X	X	X
100GMCY_A		X	X	X
100GML_A		X	X	X
100GMS_A		X	X	X
100GMCY_S			X	X
100GMC_S			X	X
100GML_S			X	X
100GMS_S			X	X
200GMCY_A				X
200GMC_A				X
200GML_A				X
200GMS_A				X
200GMCY_S				X
200GMC_S				X
200GML_S				X
200GMS_S				X

The changes made to molecule parameters to increase accuracy were to the distribution of partial charges across atoms. The partial charge distribution across MG was relatively simple as the only structural variance between the relevant MG is the length of the acyl tails. This allowed the pre-validated parameters for C18-MG found in the CHARMM36m FF (GMS1) to be used as a reference point to ensure accurate charge for each molecule. Furthermore, when setting up a system using these MGs, a Tcl script was used to calculate the overall charge of the system (which should be zero) to ensure these parameters were successfully integrated following the protocol used to shorten the tail length of GMS1.

5.3.3 Analysis

Pre-preparation of trajectories for analysis was performed using a Tcl script developed in house which centred the bilayer in the middle of the simulation box to remove movement of the membrane within the water box from the system. Area per lipid (APL) was calculated using VMD membrane analysis tool MEMBPLUGIN version 1.1.32. [242, 259] using the atoms C2, C1 and C3 for POPC and C3 for each MG (Figure 5.2). Root mean square displacement (RMSD) of the bilayer was calculated using the RMSD Visualiser Tool in VMD for each frame using the phosphorus atoms of the POPC lipid head groups in the upper and lower leaflets. To calculate the average membrane thickness, the difference in average z-coordinate of the phosphorous atoms in the upper and lower leaflets respectively was used. The 2D thickness across the XY plane was calculated every 20 steps of the trajectory using the membrane thickness map function of MEMBPLUGIN version 1.1. Lipid tail order parameters (S_{CD}) were calculated for each carbon of the Sn1 chain of POPC (Figure 5.2) every 50 steps of the trajectory. Partial density profiles for each system were calculated using the Density Tool plugin in VMD. [258] Partial density profiles were calculated for the water, ions, POPC and MG for the upper and lower leaflets individually, and the whole membrane (combined POPC and MG) for each system along the z-axis with 1 Å bin spacing and were calculated every 50 steps of the trajectory. All data was processed and visualised using Python.

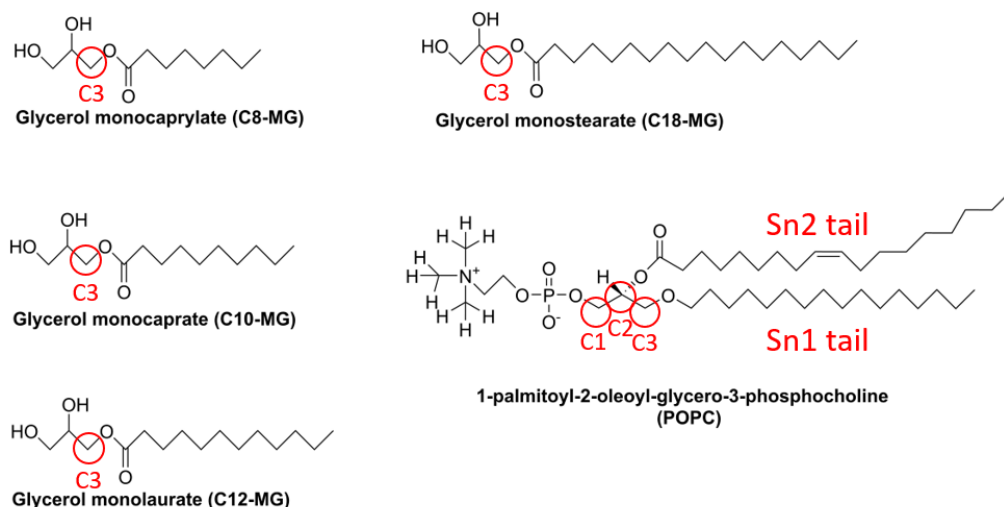


Figure 5.2: Annotated chemical structures of C8-MG, C10-MG, C12-MG, C18-MG and POPC. Red circles indicate the carbons used as reference for APL calculation. Sn1 and Sn2 indicate which tail was used for S_{CD} calculation.

5.4 Results and Discussion

5.4.1 Equilibration of Systems

As discussed previously (Chapter 3), proper equilibration of a system is vital to obtaining an accurate representation of its characteristics. Given enough time, MD systems will tend towards a stable state. [204] The asymmetrically loaded systems presented in this chapter, as they are designed to model the disruption of a stable membrane model, do not exist in a state of mechanical equilibrium and therefore determining the time point at which a system has been sufficiently equilibrated is vital to obtaining accurate results. To determine this cut-off, the equilibration of systems were assessed by two variables: Simulation box dimensions and phospholipid head group root mean square displacement (RMSD).

Phospholipid Head Group RMSD

RMSD is a measure of the average displacement of a group of atoms across a simulation. Plotting calculated RMSD over time for systems 12GMC_A, 28GMC_A, 44GMC_A,

100GMC_A, 100GMCY_A, and 100GMS_A (Figure 5.3) saw a rapid increase in RMSD over the first 100 ns, after which the RMSD begins to level off. Levelling off occurs at approximately 50 Å, which is around half the xy box dimension. Due to PBCs an atom cannot appear to move more than this size, and therefore a value at this range indicates that POPC lipids have explored the full extent of this plane, indicating equilibration of these lipids. Though fluctuation is still observed after this point, the significant reduction of variability in RMSD suggest relative stability of the systems.

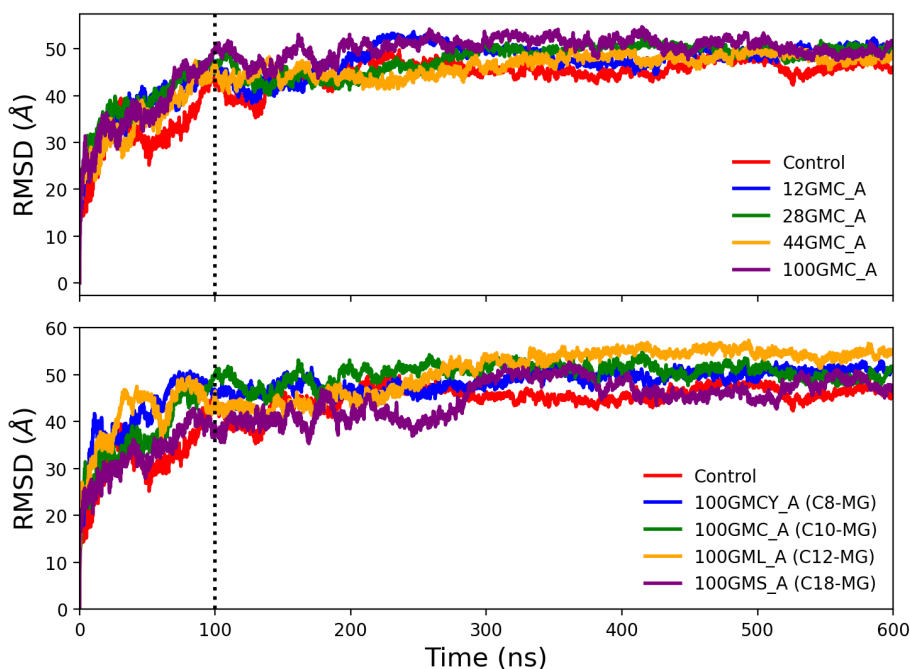


Figure 5.3: RMSD over time for different concentration GMC systems (top) and 100 MG loaded systems (bottom).

Box Dimension Fluctuation

The NPT ensemble used for the production runs of each system allow for variance in the xyz dimensions of the simulation box as the time step proceeds to maintain constant pressure. As lipid bilayers are more akin to a fluid than a solid they naturally vary in thickness along each dimension over the course of a simulation, which directly influences box dimensions. [260] Due to PBC the x , y , and z dimensions of the box are directly correlated, with an expansion in the xy dimensions of the membrane tending

to cause a decrease in the size of the box in the z dimension, and vice versa.

Figure 5.4 A shows the calculated lengths of each axis for the control system. This highlights the low variance of the membrane system over the entire production run, suggesting the systems were fully equilibrated from the CHARMM-GUI equilibration protocol used. xy dimensions were identical due to the anisotropic pressure scaling used in the simulation protocol.

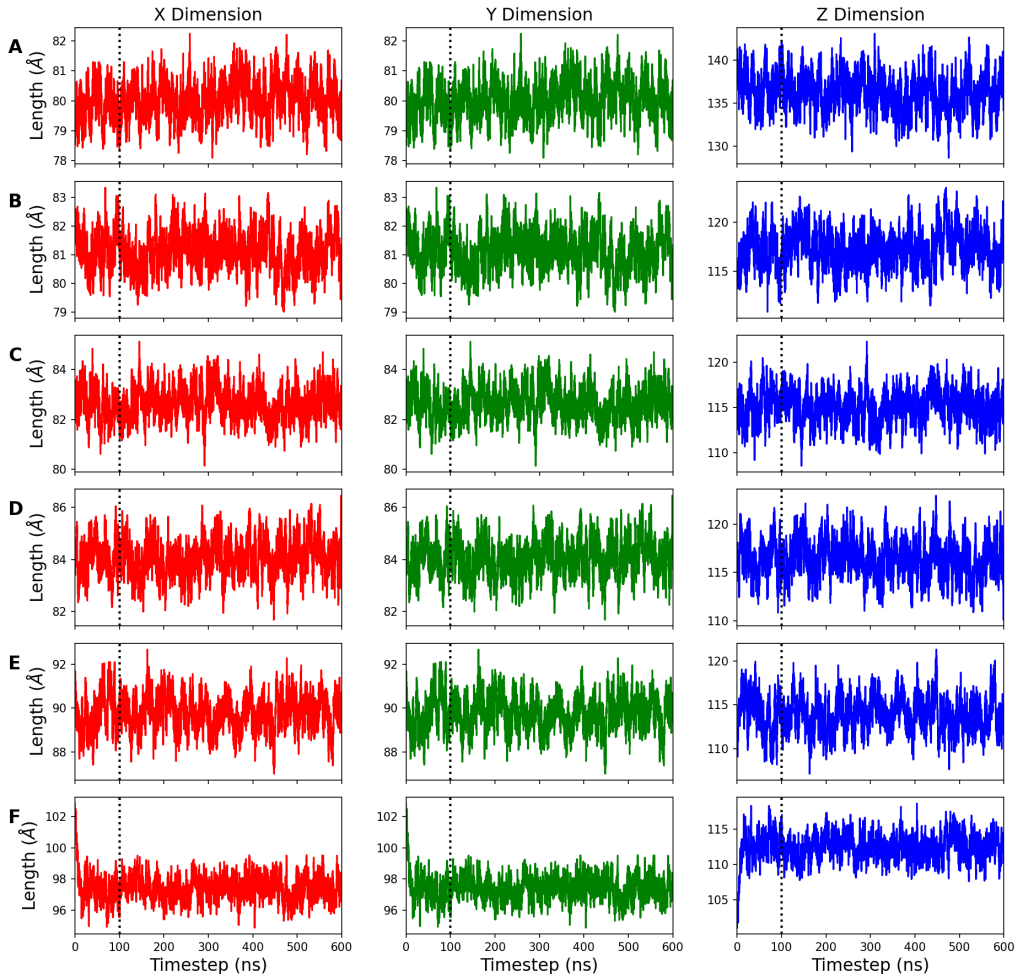


Figure 5.4: Size of xyz dimensions for C10-MG loaded systems over the production trajectory. A represents the control system. B-F represent 12/28/44/100/200 molecules of C10-MG respectively.

From the control, comparisons can be made to others to assess if similar levels of equilibration has been reached. C10-MG containing systems with varying concentration used in this study (Figure 5.4 B-F) saw similarly quick equilibration times, particularly

at lower MG concentrations. The 200GMC_A system saw a significant drop in xy size and rise in z axis thickness over the first 20 ns of the production run, which was presumed to be caused by the membrane relaxing into its optimal conformation once restraints were relaxed due to the significant change to free space in the upper leaflet caused by reduction of C18-MG tails into C10-MG. Due to this, the dimensions for all systems loaded with 200 MGs were also plotted. (Figure 5.5).

For MCMGs loaded systems, all systems saw normalisation of box dimensions within 100 ns. The significant changes to box dimensions observed in the 200GMCY_A and 200GML_A systems were due to the formation of aggregate structures on the upper leaflet of the membrane, discussed in section 5.4.5 which caused a portion of the bilayer to be taken up, therefore reducing the xy dimensions of the membranes. While the 100GMS_A system equilibrated quickly, the symmetric 100GMS system did not equilibrate over the course of the simulation, which aligns with the high instability of the bilayer (section 5.4.5).

RMSD values in Figure 5.4 suggest that the majority of structural changes to the membrane have occurred by the 100 ns mark, which is backed up by the stabilisation of simulation box dimensions by the same time. Therefore, to maintain consistency between all systems, the first 100 ns was discarded from each trajectory prior to analysis to account for equilibration of bilayer structure. Systems where the bilayer structure was compromised (section 5.4.5) were considered outliers from this due to the rapid speed that bilayer integrity was lost, and this protocol was not followed.

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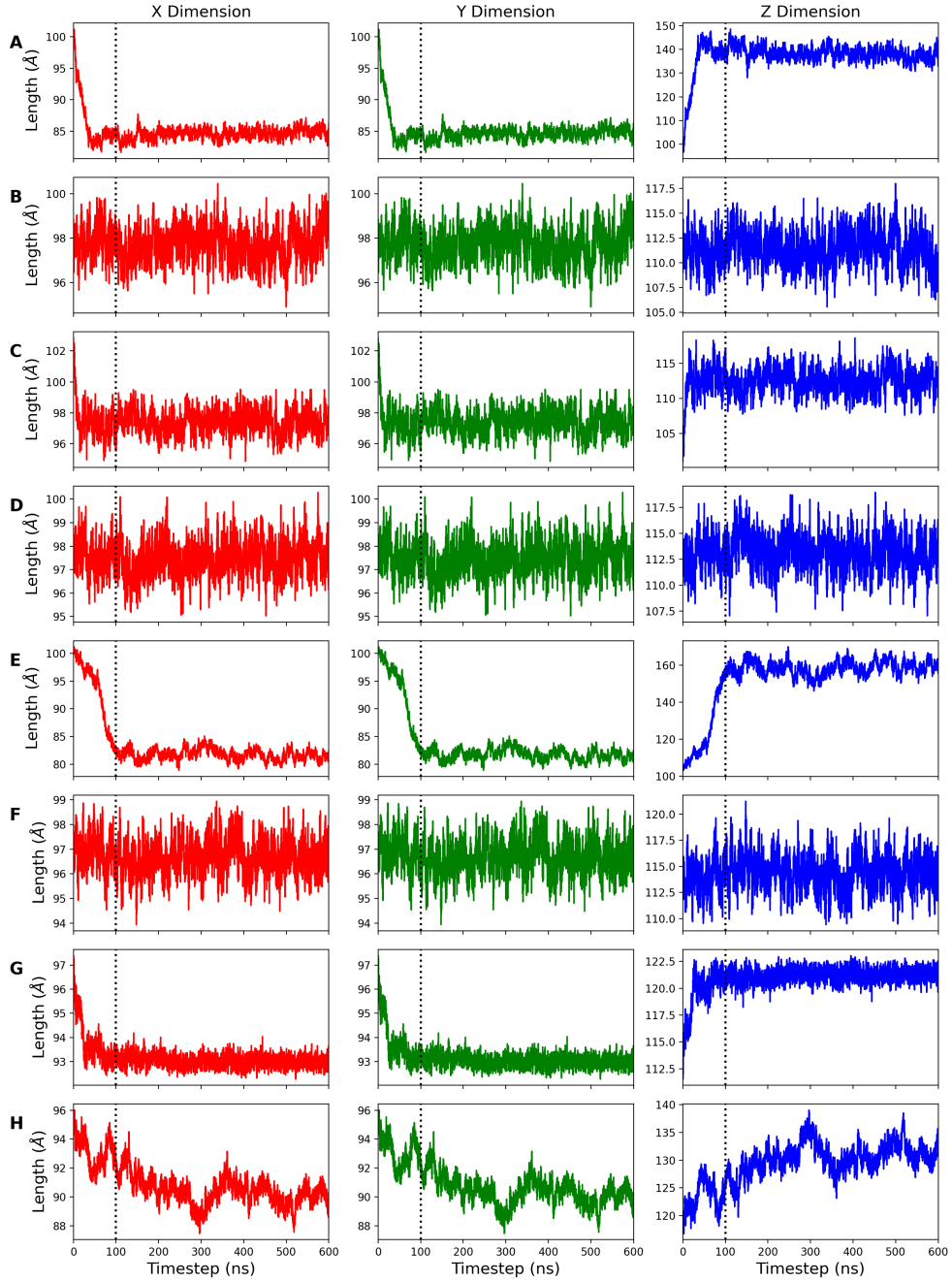


Figure 5.5: Size of xyz dimensions for systems loaded symmetrically and asymmetrically with 200 MG molecules. A, C, E, G represent systems 100GMCY_A, 100GMC_A, 100GML_A, and 100GMS_A respectively. B, D, F, H represent 100GMCY, 100GMC, 100GML, and 100GMS respectively.

5.4.2 Disruption of Membrane Thickness

Influence of MG Concentration Within Bilayer

To assess how the concentration of MG molecules within a leaflet influenced membrane properties, POPC membranes with 12/28/44/100 molecules of C10-MG inserted asymmetrically into the upper leaflet were simulated. C10-MG was selected for this purpose due to having among the greatest reported antimicrobial efficacy of the selected MGs experimentally. [35] Therefore, it was expected that this would show the most obvious changes to membrane properties. C12-MG was also considered for this reason and is used in simulations later in this chapter. Table 5.3 indicates membrane thickness, RMSD, and APL values for the control POPC system, which are in good agreement with similar literature. [261,262]

Table 5.3: Averaged bilayer thickness, lateral area of the bilayer, and APL for asymmetrically loaded C10-MG systems. APL represents average area per POPC molecule. Brackets show standard deviation to 1 decimal place. All values given in Å except for XY area, which is given in nm².

System	APL (upper)	APL (lower)	APL (whole)	Thickness	XY Area
Control	64.1 (1.1)	64.1 (1.1)	64.1 (1.1)	39.3 (0.5)	640.5 (10.0)
12GMC_A	58.7 (0.9)	65.8 (1.2)	62.0 (0.3)	39.1 (0.5)	657.6 (10.6)
28GMC_A	53.4 (1.5)	67.8 (1.1)	59.9 (0.9)	38.9 (0.5)	682.9 (10.5)
44GMC_A	49.5 (0.9)	69.7 (1.3)	57.9 (1.0)	38.6 (0.5)	706.6 (11.9)
100GMC_A	42.2 (2.1)	73.2 (8.5)	53.7 (0.9)	37.4 (0.6)	805.8 (13.6)

Each C10-MG-loaded system saw a reduction in average bilayer thickness in comparison to the control system. Although not linear, the reduction in membrane thickness is consistently increased by C10-MG concentration, with a reduction of 0.5%, 1%, 1.8%, and 4.8% for the 12/28/44/100GMC systems respectively. The observed system behaviour of a decrease of APL in the upper membrane and the increase in APL upon the asymmetric loading of amphiphilic molecules has been reported in previous literature, as has the observed changes to bilayer thickness. [197]. It is important to note that the decrease in APL in the upper leaflet results from the presence of MG in the upper leaflet taking up space space between phospholipids in the leaflet, and the area occupied by each phospholipid is similar for both leaflets towards the bilayer centre, as C10-MG is significantly shorter than POPC. As will be discussed

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in more detail later in this chapter, this likely contributes to the reduction in overall bilayer thickness across MCMG systems, as it allows regions of the POPC tails close to the bilayer centre to splay more. As the presence of C10-MG disrupts phospholipid packing, the area per POPC molecule was similar across both leaflets if not accounting for the presence of MG in the upper leaflet during APL calculations. The lateral area of the bilayer expanded as MG count increased within these systems, though this was largely due to the insertion of more molecules into the bilayer increasing the overall surface area, and not solely attributed to lateral expansion from the reduction in bilayer thickness.

The lateral distribution of average thickness showed greater variation across the simulated region of the membrane in C10-MG loaded systems. Figure 5.6 shows a very consistent distribution of thickness across the whole membrane in the control. Figure 5.7 shows the development of regions of lower average thickness in the membrane, particularly at the highest concentration of C10-MG within the membrane. In living organisms the thickness of the cell membrane is tightly regulated as changes to bilayer thickness can alter the properties of the membrane, and as such the induction of regions with reduced thickness as observed in MG loaded systems may inhibit required membrane functions. [263,264] This change to the bilayer structure could be associated with increased hydrophobic mismatch within a region of the bilayer, caused by regions of low thickness relative to the surrounding bilayer due to the tail mismatch of C10-MG with the surrounding POPC. Hydrophobic mismatch disrupts lipid packing and increases bilayer stress, which may promote the structural deformation observed in these systems.

Influence of MG structure

To explore the dependence of MG chain length on bilayer destabilisation a separate set of POPC membranes loaded with 100 molecules of C8-MG, C12-MG, and C18-MG were created. C12-MG is also a well known antimicrobial MG, while C8-MG and C18-MG have little reported evidence antimicrobial efficacy in literature. [35] Based on reported experimental findings, it was expected that C12-MG would produce slightly

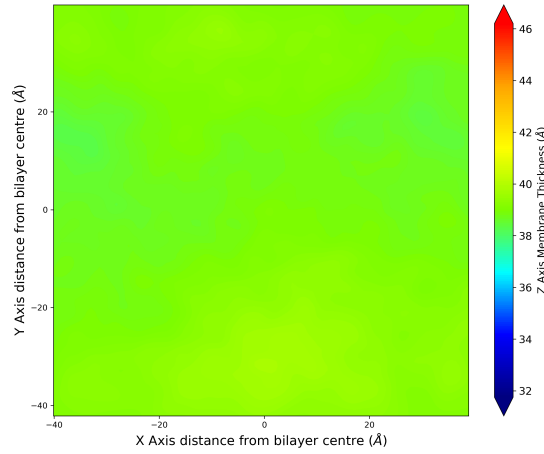


Figure 5.6: 2D average membrane thickness maps across the xy plane for Control POPC bilayer system.

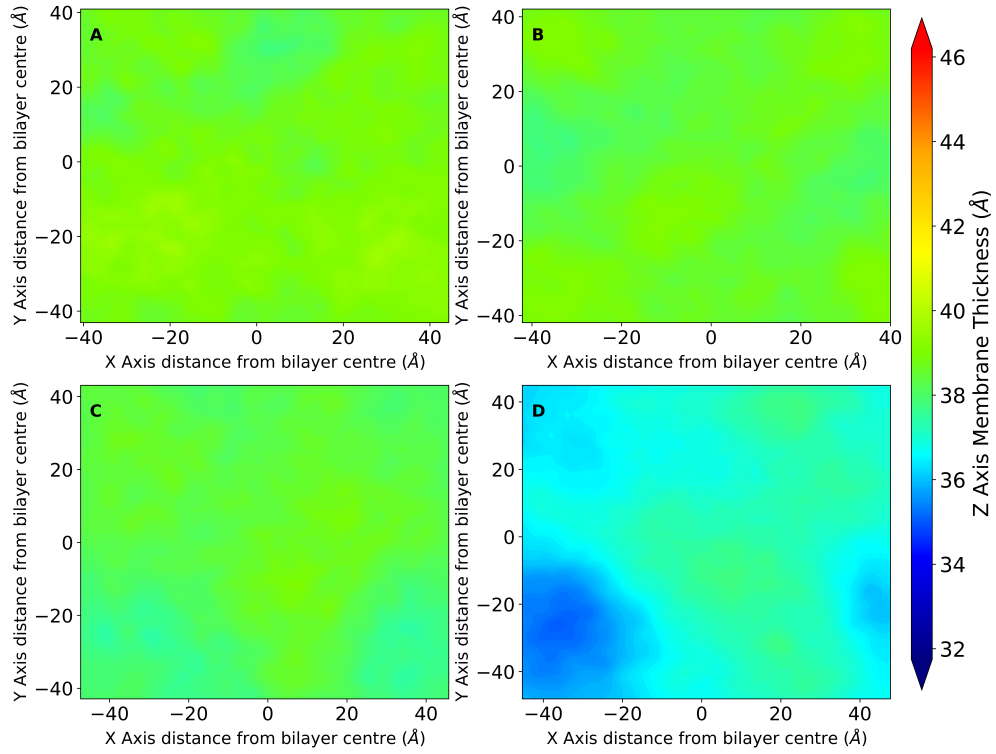


Figure 5.7: 2D average membrane thickness maps across the xy plane for asymmetrically loaded C10-MG systems 12GMC_A (A), 28GMC_A (B), 44GMC_A (C), and 100GMC_A (D).

more disruption than C10-MG, while C8-MG and C18-MG would produce low levels of bilayer disruption. [39]

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Each MCMG-loaded membrane displayed a decrease in average bilayer thickness, as displayed in Table 5.4. The reduction in bilayer thickness was inversely correlated with the MCMG acyl chain length. C12-MG insertion resulted in the smallest deviation from the control with a 2.8% decrease in thickness. In contrast, C18-MG loading resulted in a significant 13.2% increase in bilayer thickness. Additionally, while a reduction in overall area per lipid was observed in each system, the C18-MG loaded system the most significant drop in APL at 26.7%. This could be attributed to the tighter packing of POPC in the bilayer, induced by the increased order (discussed in Section 5.4.3) within the bilayer due to C18-MG taking up physical space that does not cause significant hydrophobic mismatch as C18-MG is roughly the same size as the Sn1 tail of POPC. Average lateral area of the bilayer did not significantly differ between MCMGs despite the observed differences in bilayer thickness between each other, however the area of C18-MG was significantly reduced in comparison to the MCMG systems. This indicates therefore that increased bilayer thickness likely caused compaction of the bilayer as packing becomes tighter between leaflets, whereas decreases in bilayer thickness likely caused expansion of lateral area as packing becomes looser, though this cannot be confirmed due to the absence of an appropriate control for this variable.

Table 5.4: Averaged bilayer thickness, lateral area of the bilayer, and APL for asymmetrically loaded C10-MG systems. APL represents average area per POPC molecule. Brackets show standard deviation to 1 decimal place. All values given in Å except for XY area, which is given in nm².

System	APL (upper)	APL (lower)	APL (whole)	Thickness	XY Area
Control	64.1 (1.1)	64.1 (1.1)	64.1 (1.1)	39.3 (0.5)	640.5 (10.0)
100GMCY_A	43.4 (0.7)	71.0 (1.0)	53.3 (0.8)	36.9 (0.6)	799.2 (12.8)
100GMC_A	42.2 (2.1)	73.2 (8.5)	53.7 (0.9)	37.4 (0.6)	805.8 (13.6)
100GML_A	41.4 (1.1)	74.4 (3.5)	53.1 (0.9)	38.2 (0.5)	797.4 (13.1)
100GMS_A	36.7 (1.5)	65.2 (2.9)	47.0 (1.2)	43.3 (0.7)	697.1 (12.2)

Thickness maps shown in Figure 5.8 A-C demonstrate that C8-MG induced the most significant lateral fluctuation of membrane thickness across the membrane of the MCMGs, having large regions of low thickness at around 35 Å and sections of higher thickness at around 38 Å. This represented the induction of curvature in the bilayer structure. The opposite can be seen in the thickness map for C18-MG (Figure

5.8 D), which highlights a significant increase in thickness across a large portion of the membrane surface area around 45 Å in height, complemented by lower thickness regions to each side with a similar thickness at 39 Å. The induction of curvature in bilayer systems could be related to phase-change behaviour in the membrane. At room temperature lipid bilayers typically exist in the liquid crystal state, but can transition to a gel-like state, where the membrane becomes more rigid and has reduced fluidity, or a fluid-like state which becomes more disordered. [265] Intermediate states between these three categories also exist such as the ripple phase, where the membrane simultaneously experiences gel phase and liquid crystal phase behaviour causing curvature in the membrane. Importantly, curvature induction in lipid bilayers has been observed in experimental models treated by MGs, which is proposed to be due to the hydrophobic mismatch caused by the differing length of MG tails to those of POPC causing the POPC molecules to bend in order to maintain hydrophobic interactions, so this curvature was not unexpected to see in systems containing high MG concentrations [27]. Potential factors that can additionally be associated with changes to membrane phase are deuterium order parameters, area per lipid, and bilayer thickness. Decreased in S_{CD} , increased APL, and decreased bilayer thickness are associated with more fluid membrane states, while the inverse is an indicator of more solid, gel-like phase states. Given these parameters, it appears consistent that MCMGs cause bilayer properties to tend towards a liquid-disordered bilayer phase, while C18-MG caused the bilayer to display gel-phase like behaviour, though systems containing all MGs still experienced local deformation from the presence of MG in the structure, primarily associated with the formation of curvature or thinning in regions of the bilayer. It should be noted, however, that phase transition behaviour could not be directly observed in these simulations, as each simulation was observed at a constant temperature. Confirming differences in phase-change behaviour caused by MG insertion would require simulations to be performed at additional temperature ranges to assess whether incorporation of MGs alters the bilayer's phase transition temperature [266].

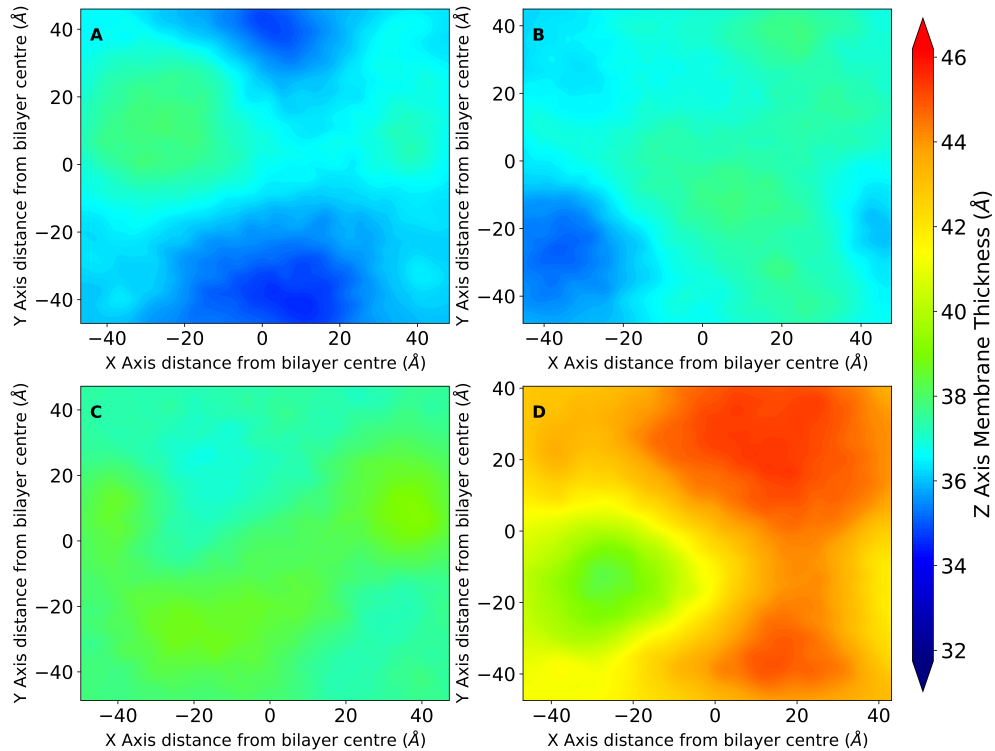


Figure 5.8: 2D average membrane thickness maps across the xy plane for the production runs of 100GMCY_A (A), 100GMC_A (B), 100GML_A (C), and 100GMS_A (D) systems.

Influence of MG Loading Symmetry Across Leaflets

It is hypothesised that asymmetric stress is an important driving force in bilayer deformation. [39, 191] Literature has previously assessed the formation of tubule structures on the membrane surface, and their subsequent collapse, to be correlated with the accumulation and relief of asymmetric stresses across the membrane in the context of other amphiphilic molecules. [191, 197] To assess the importance of asymmetric stress in the context of MGs, a series of symmetrically loaded systems were created. As in asymmetric systems, 100 MG molecules were inserted into the membrane, this time split evenly between the two leaflets, to mimic an ideal stable state where all MG-induced asymmetry within the system had been relieved.

Comparison between asymmetrically and symmetrically loaded systems indicated differences in bilayer thickness between the two states. Average thickness comparison

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between systems (Table 5.5) highlighted a consistently larger reduction in thickness in asymmetric MCMG systems than symmetric ones, although the difference was not significant. In contrast, the asymmetric C18-MG system produced a significant increase in membrane thickness in comparison to the symmetric system. This suggested that a higher number of MG molecules within a single leaflet correlates with greater disruption of overall bilayer properties, as shown in 5.4.2. This also suggests the relief of leaflet asymmetry reduces the impact of MG-induced disruption within the system. In terms of lateral area, all MCMGs saw a small reduction in average area in symmetrical systems, which may be because fewer MG monomers present in an individual leaflet required lesser expansion of surface area to accommodate the added molecules. For C18-MG a significant increase in lateral area was instead observed in this system. This is likely due to the POPC bilayer in this system being significantly less perturbed than the bilayer exposed to C18-MG asymmetrically, and therefore lesser lateral compaction occurred.

Table 5.5: Averaged bilayer thickness, lateral area of the bilayer, and APL for asymmetrically loaded C10-MG systems. APL represents average area per POPC molecule. Brackets show standard deviation to 1 decimal place. All values given in Å except for XY area, which is given in nm².

System	APL (upper)	APL (lower)	APL (whole)	Thickness	XY Area
Control	64.1 (1.1)	64.1 (1.1)	64.1 (1.1)	39.3 (0.5)	640.5 (10.0)
100GMCY_A	43.4 (0.7)	71.0 (1.0)	53.3 (0.8)	36.9 (0.6)	799.2 (12.8)
100GMCY_S	52.7 (0.1)	54.0 (0.8)	53.1 (0.9)	37.0 (0.7)	796.5 (13.5)
100GMC_A	42.2 (2.1)	73.2 (8.5)	53.7 (0.9)	37.4 (0.6)	805.8 (13.6)
100GMC_S	52.6 (0.1)	52.8 (0.9)	52.8 (0.8)	37.8 (0.5)	791.6 (12.4)
100GML_A	41.4 (1.1)	74.4 (3.5)	53.1 (0.9)	38.2 (0.5)	797.4 (13.1)
100GML_S	52.1 (0.8)	52.1 (0.8)	52.1 (0.8)	38.9 (0.6)	791.5 (12.1)
100GMS_A	36.7 (1.5)	65.2 (2.9)	47.0 (1.2)	43.3 (0.7)	697.1 (12.2)
100GMS_S	51.2 (0.9)	51.2 (0.9)	51.2 (0.9)	41.4 (0.5)	767.6 (12.2)

Despite the relief of asymmetry appearing to reduce the extent of disruption, there were still changes to average membrane thickness observed across the whole membrane structure. Average 2D thickness maps calculated for symmetric systems (Figure 5.9) highlighted that the xy thickness distribution is more even in symmetrical systems than in asymmetric ones. Notably, the symmetrical C18-MG system did not display the induced curvature observed in the asymmetric system. A region of particularly low thickness remained observable in the 100GMCY_S system (Figure 5.9A), though this

region is only a difference of around 1 Å in comparison to the 3 Å seen in 100GMCY_A. Moreover, the reduction in thickness variance across the xy plane was more similar to those of the control system than the asymmetric systems, therefore indicating greater stability of the bilayer structure in systems where MGs are not exerting asymmetric stresses onto the bilayer.

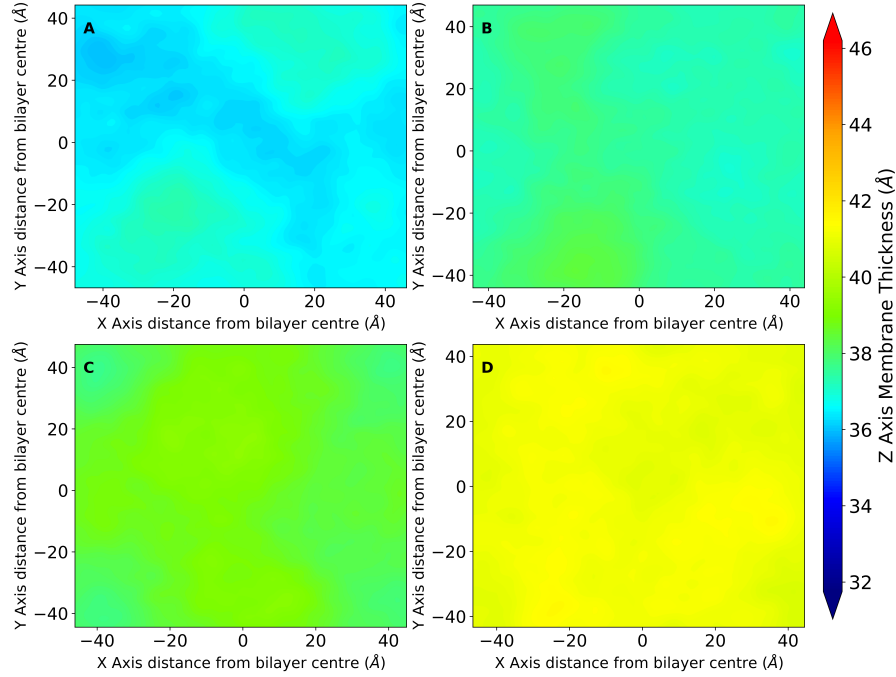


Figure 5.9: 2D average membrane thickness maps across the xy plane for the production runs of symmetrical C8-MG (A), C10-MG (B), C12-MG (C), and C18-MG (D) systems. All systems initially contained 50 MG in each leaflet.

5.4.3 Disruption of Membrane Structure and Properties

Influence of MG Concentration Within Bilayer

Mass density profiles, shown in Figure 5.10 A, indicated a reduction in the width of bilayer density at higher concentrations of C10-MG within the membrane in comparison to the control. This appeared to be in line with the reported average membrane thickness for each system (Table 5.3). This reduction in thickness was driven primarily by the reduction of thickness in the lower leaflet, while the upper leaflet remains largely unchanged. The density profile for the control also agrees with literature which has simulated POPC bilayers. [267] As C10-MG concentration increased, the mass at the centre of the hydrophobic core where the acyl tails of molecules meet also increased. This reduction in depth indicated a change to the positioning of lipids within the two leaflets.

The individual component breakdowns of the mass density profile for each system in Figure 5.10B and C showed this to be related to the positioning of C10-MG within the membrane. C10-MG density peaks at around +12 Å, indicating the preferential position for the molecules within the membrane, though a very shallow secondary peak also formed at around -12 Å. The primary peak was located in the upper leaflet, where all 100 molecules were initially inserted, while the second peak indicates a small amount of C10-MG movement to the lower leaflet. At 100 molecules inserted, where the most significant changes to the membrane structure are observed, the overall reduction in membrane thickness was reflected in the compaction of both leaflets, but more significantly in the lower leaflet. This indicated that the changes to membrane dynamics could be seen across the bilayer structure and not confined to leaflets with a high MG concentration.

Additionally, in C10-MG loaded systems there was an increase in water permeation within the system in comparison to the control (Figure 5.11, Table 5.6). The upper leaflet of the native POPC membrane showed that the water reached a negligible density (density $<0.01 \text{ g/mol}/\text{\AA}^3$) at +10.5 Å from the membrane centre, while this did not occur until +5.5 Å in the 100GMC_A system. This shallower slope indicated greater

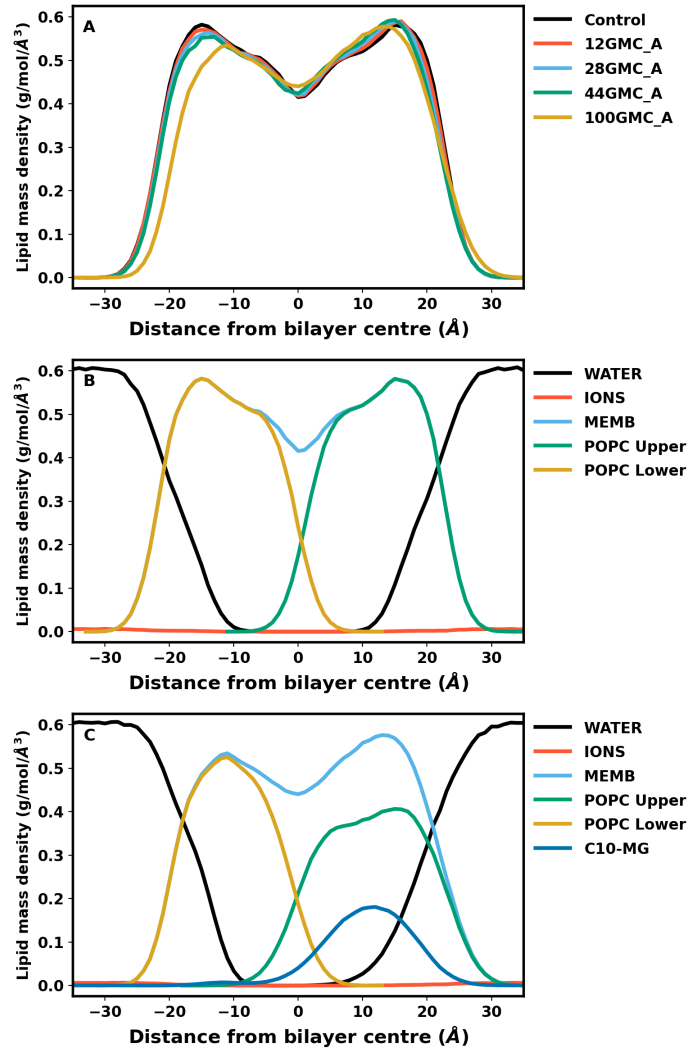


Figure 5.10: (A) Average 1D mass density profiles along the z-axis of the MEMB segment of POPC membranes with 12/28/44/100 molecules of C10-MG inserted into the upper leaflet. (B). Average mass density profile of the control system. (C) Average mass density profile of 100GMC_A. "MEMB" represents the total mass density of POPC and C10-MG within the system. "POPC upper" and "POPC lower" represent the mass density of POPC in the upper and lower leaflet of the membrane. "IONS" represents Na⁺ and Cl⁻ ions combined.

permeation of water molecules closer into the hydrophobic core of the membrane, and was also observed in the upper leaflet of the 28 and 44 C10-MG systems to a much lesser extent, with a negligible density being reached at -9.5 and +8.5 Å in the lower and upper leaflets respectively. This was a 1 Å increase in upper leaflet permeation and

no change to permeation for 12-44 molecules of C10-MG in relation to the control. The increase in water permeation across the bilayer was slightly higher than the decrease in bilayer thickness observed in 12-44 C10-MG systems, but was significantly higher than then 2 Å decrease in bilayer thickness observed in 100GMC_A. This increase in bilayer permeation at high concentrations therefore suggests that significant changes to water permeation is associated with a high localised concentration of C10-MG within that section of the membrane. This behaviour would align with C10-MG's reported ability to function as a permeation enhancer. [35,268].

For the 12-44 MG series of systems the head group positions indicate slight expansion of the upper leaflet by up to 1 Angstrom when these concentrations were added. The lower leaflet of the POPC bilayer in 12GMC_A was not affected, while at 28 and 44 headgroups were positioned 1.5 to 2.5 Å closer to the bilayer centre, suggesting compaction of the lower leaflet caused by the addition of MGs, despite no molecules being initially inserted into the lower leaflet. This therefore suggested that even at comparatively low concentrations MGs can still affect properties across the bilayer. At a high concentration of 100 molecules not only did further compaction of the lower leaflet occur, but also produced a decrease in upper leaflet thickness.

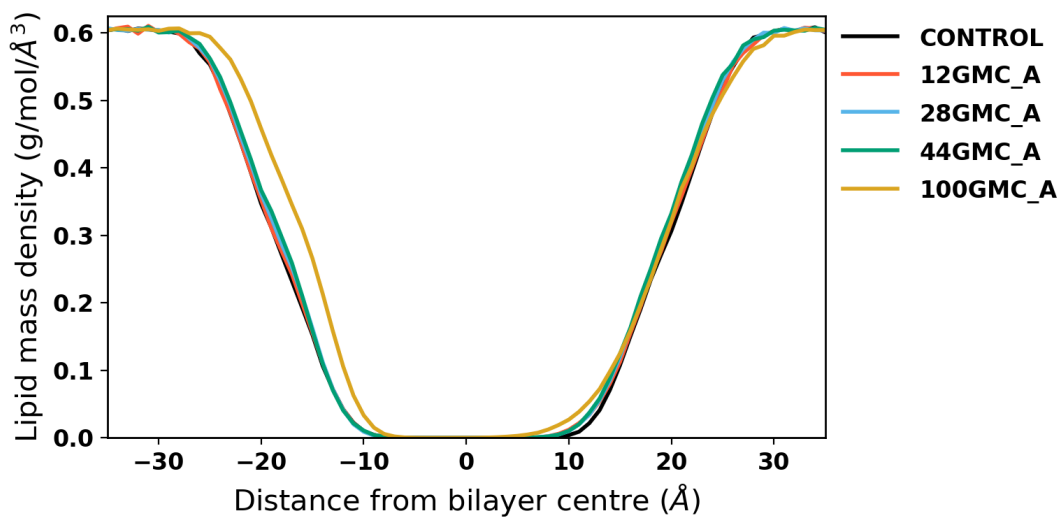


Figure 5.11: Average 1D mass density of water along the z-axis for systems Control (black), 12GMC_A (orange), 28GMC_A (blue), 44GMC_A (green) and 100GMC_A (yellow).

Table 5.6: Positions of membrane headgroup peaks and depth of water penetration (both given in Å) for Control and C10-MG loaded bilayer systems. All values were rounded to 1 decimal point.

System	HG Position (U)	HG Position (L)	Water Depth (U)	Water Depth (L)
Control	14.5	-15.5	9.5	-9.5
12GMC_A	15.5 (+1)	-15.5 (0)	+8.5 (+1)	-9.5 (0)
28GMC_A	15.0 (+0.5)	-14.0 (-1.5)	+8.5 (+1)	-9.5 (0)
44GMC_A	15.0 (+0.5)	-13.0 (-2.5)	+8.5 (+1)	-9.5 (0)
100GMC_A	13.0 (-1.5)	-11.0 (-4.5)	+4.5 (+5)	-8.5 (+1)

Further indication of changes to membrane permeability was also observed in calculated membrane tail order parameters (S_{CD}). S_{CD} is a measure of alignment between a lipid tail group and the membrane normal (z axis) [259]. Figure 5.12 displays the alignment of each carbon along the POPC Sn1 tail with the bilayer normal, where 0.5 represents perfect alignment with the bilayer normal, and 0 represents no preferred orientation. As fewer atoms are present near the hydrophobic core of the bilayer, terminal carbons typically see much more movement and so S_{CD} naturally tends to decrease towards 0, as seen in all lines. S_{CD} profiles reveal an increase in ordering of the upper leaflet and decrease of ordering in the lower leaflet, but overall only the 100 C10-MG system displayed a difference in order in comparison to the control. Membranes with reduced ordering typically see more fluid-like behaviour as tails become more disordered, causing a decrease in membrane thickness as observed in Table 5.3. An increase in phospholipid order was observed in upper leaflet systems, which is associated with reduced membrane fluidity and therefore permeability despite the observed increase in water penetration. The increase in order, however, was primarily seen in Sn1 tail carbons 4 to 10 and a negligible increase was observed beyond this, suggesting only the region of the tail around where C10-MG sits within the leaflet became more aligned with the membrane normal. C10-MG was not analysed for deuterium order parameters due to limitations of MEMBPLUGIN being unable to recognise the molecule, or the other MGs studied in this thesis, for automated analysis. As the primary justification for the calculation of S_{CD} was to assess how phospholipid tail packing was altered by MG insertion, this data would have been excluded from these graphs regardless.

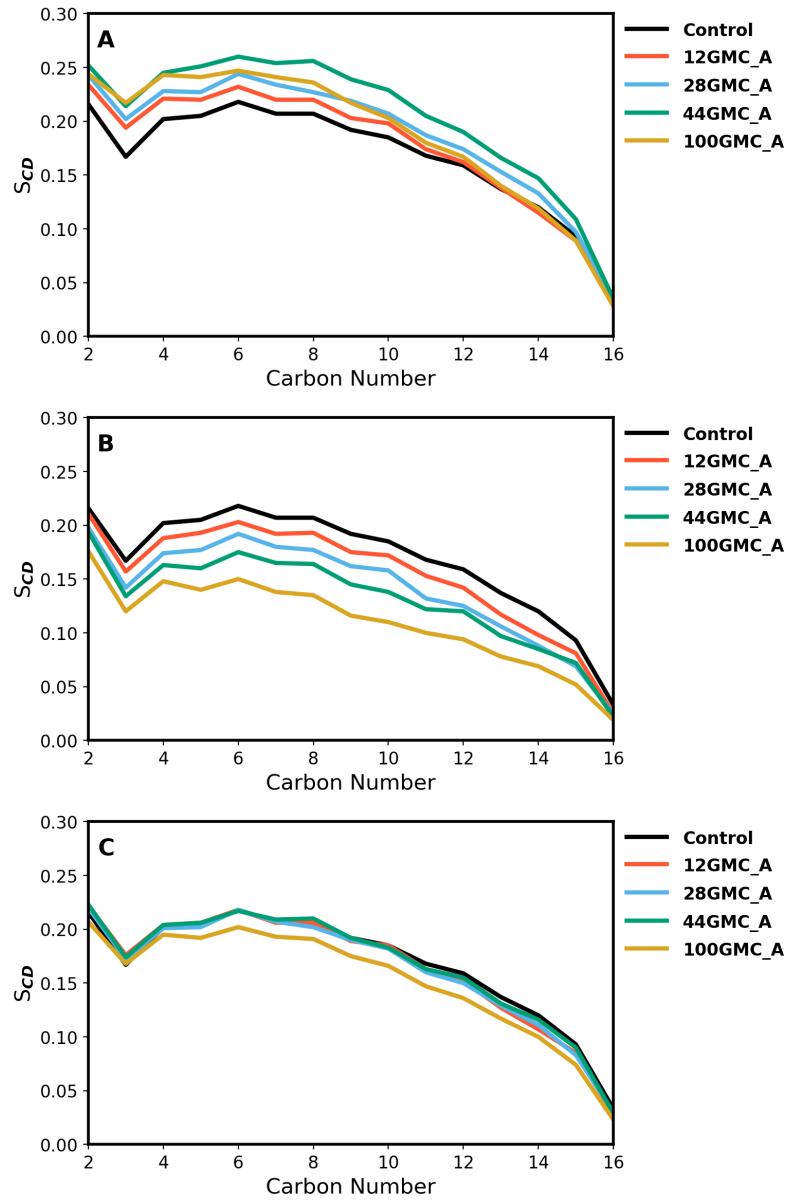


Figure 5.12: Average bilayer deuterium order parameter profiles for 100GMCY_A, 100GMC_A, 100GML_A, and 100GMS_A systems for the Sn1 palmitoyl tail of POPC in the upper membrane leaflet (A), lower membrane leaflet (B) and whole membrane (C). High S_{CD} (0.5) represents strong alignment with bilayer normal. Carbon number represents the carbon position along the sn1 palmitoyl acyl chain.

Influence of Inserted MG Structure

In the bilayer density profiles calculated for 100 MG systems (Figure 5.13) all MCMGs saw a reduction in the overall width of headgroup distribution of the bilayer, aligning

with the decrease in bilayer thickness discussed in the above section. However, how the three MCMGs affected the two leaflets differed somewhat (Table 5.7). C8-MG had the most equally distributed effects across the two leaflets, causing a 2 Å reduction in the upper leaflet and 3 Å reduction in the lower leaflet.

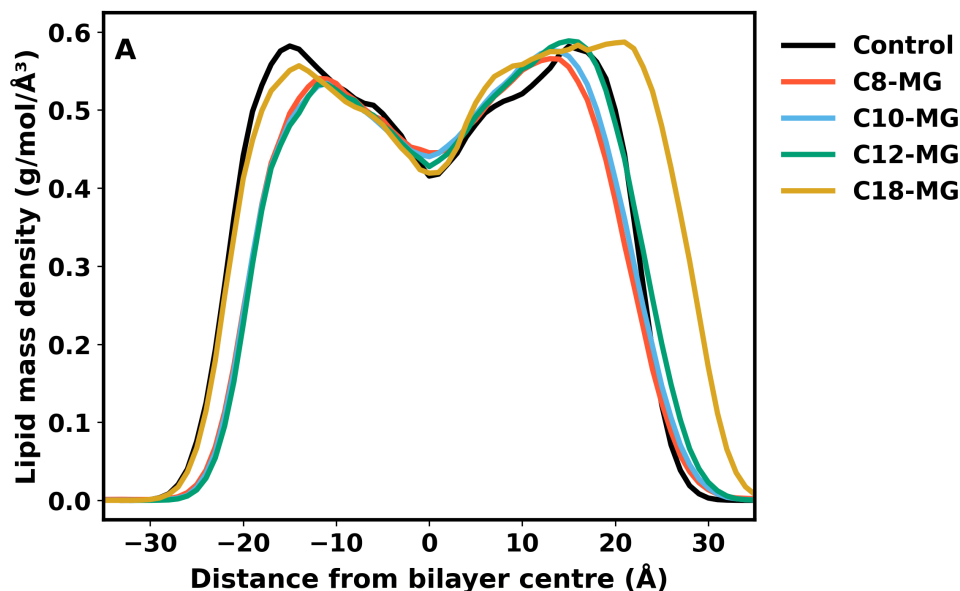


Figure 5.13: Average mass density profiles of the "MEMB" segment of POPC membranes with 100 molecules of C8-MG, C10-MG, C12-MG or C18-MG inserted into the upper leaflet. "MEMB" segment represents the total mass density of POPC and MG within the membrane.

Two trends that were apparent between the three MCMGs were that the impact on the upper leaflet decreased, and the impact on the lower leaflet decreased, as MG tail length increased^{5.7}. Interestingly, C12-MG caused a 1 Å movement away from the bilayer centre for upper leaflet headgroup positioning despite causing the greatest decrease of the three in the lower leaflet. This caused C12-MG to produce the lowest overall change from the control across the whole bilayer of the three at 4 Å (Figure 5.14). C10-MG displayed the greatest reduction in space between headgroups at 6 Å, and C8-MG in the middle at 5 Å. The 100GMS-A system was a significant increase in upper leaflet thickness and a smaller reduction in lower leaflet thickness, causing an overall increase by 5 Å.

The depth of water penetration did not follow these trends, however. The trend of

longer tail MCMGs causing greater disruption of the lower leaflet and lesser disruption of the upper leaflet within bilayer also aligned with the increased water permeation of these systems, as 100GML_A had the greatest increase in lower leaflet water permeation, reaching 2 Å closer to the core. In contrast, the greatest permeation into the upper leaflet was observed in 100GMCY_A, reaching +4.5 Å from the bilayer centre. Despite the significant increase in bilayer thickness in 100GMS_A water permeation remained higher than the control system, penetrating through to 7.5 Å. This was 2 Å closer to the core than in the control system, despite needing to penetrate a further 6 Å through the hydrophobic region of the bilayer to reach this point than in the upper leaflet of the control system. These differences in water permeation were also observed in the water density profiles for each system (5.15).

Furthermore, C8-MG and C10-MG loaded systems see a reduction in the depth of dip at the centre of the hydrophobic core, which would indicate the compaction of this region of the bilayer (Figure 5.14). This may be attributed to the disruption of lipid packing by MGs causing greater disorder at the hydrophobic core, causing a higher density of atoms to be present in the core of the bilayer, which in turn can be linked to the leaflet thinning observed across the bilayer in MCMG systems. This has been observed in coarse-grained MD simulations of antimicrobial peptides [269,270]

Table 5.7: Positions of membrane headgroup peaks and depth of water penetration (both given in Å) for the upper and lower leaflets of systems loaded with 100 MG molecules in relation to the centre of the membrane (0). Brackets represent change from control.

System	HG Position (U)	HG Position (L)	Water Depth (U)	Water Depth (L)
Control	14.5 (-)	-15.5 (-)	9.5 (-)	-9.5 (-)
100GMCY_A	12.5 (-2)	-12.5 (-3)	4.5 (+5)	-8.5 (+1)
100GMC_A	13.0 (-1.5)	-11.0 (-4.5)	6.0 (+3.5)	-8.0 (+1.5)
100GML_A	15.5 (+1)	-10.5 (-5)	8.5 (+1)	-7.5 (+2)
100GMS_A	20.5 (+6)	-14.5 (-1)	7.5 (+2)	9.5 (0)

The full density profile for C18-MG (Figure 5.14 C) did not follow the trends of MCMGs. While the lower leaflet POPC density distribution was comparable to MCMG systems, the upper leaflet did not form an obvious peak, rather a broader plateau-like shape from around +3 Å to +23 Å. C18-MG also did not have an obvious preferential

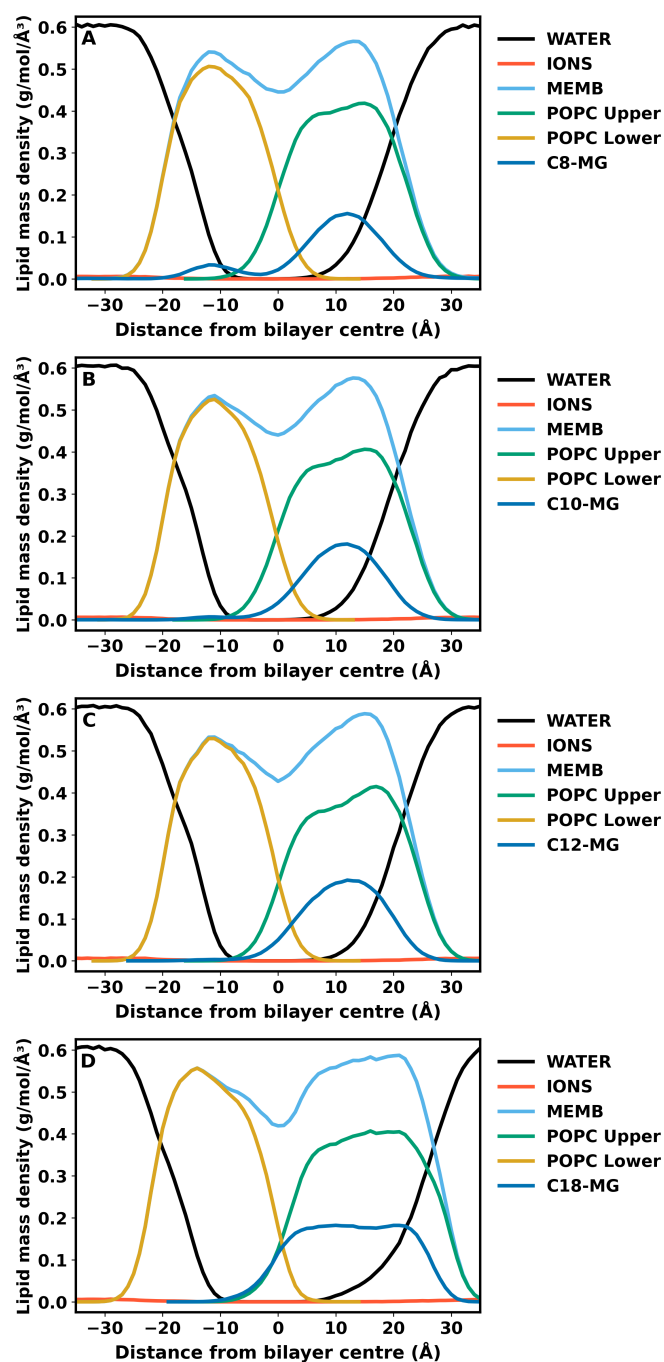


Figure 5.14: Average mass density profiles of each component of systems 100GMCY_A (A), 100GMC_A (B), 100GML_A (C), and 100GMS_A (D). "MEMB" represents the total mass density of POPC and MG within the membrane. "IONS" represents both Na^+ and Cl^- ions.

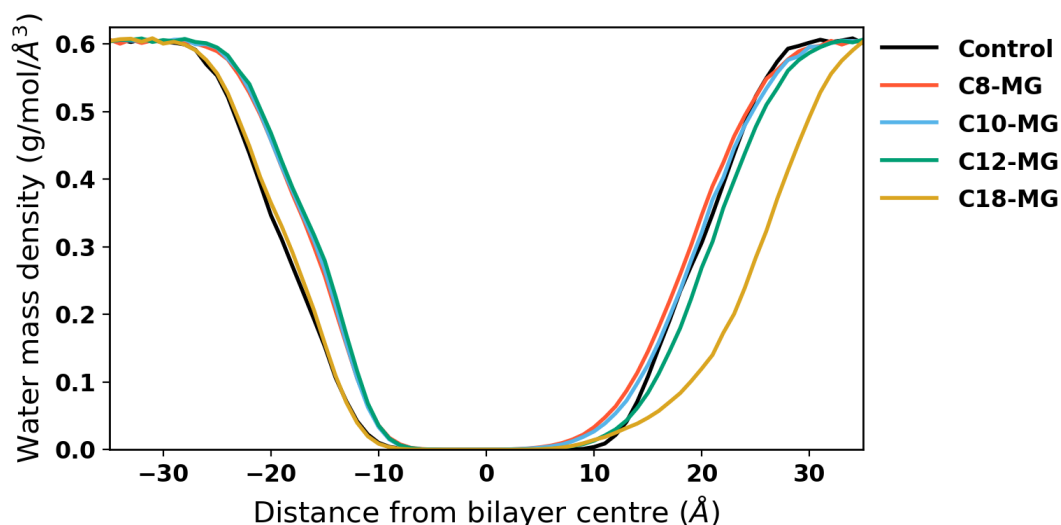


Figure 5.15: Average 1D mass density of water along the z-axis for systems Control (black), 100GMCY_A (orange), 100GMC_A (blue), and 100GMC_A (yellow), 100GML_A (green) and 100GMS_A (yellow).

position and is present throughout the upper leaflet at roughly an equal density. This was likely due to the tail length of C18-MG being similar to that of the POPC Sn1 tail and consequently these molecules occupy a larger area within the membrane than MCMGs.

The significantly different impact of C18-MG on the leaflet structure were also seen in the (S_{CD}) profiles shown in Figure 5.16. While insertion of all MGs caused an increase in upper leaflet order, the 100GMS_A system displayed a significantly greater increase than those containing MCMGs. Additionally all carbons of the POPC Sn1 tail were significantly affected in this system while in 100GMCY_A and 100GMC_A the effect on order in comparison to the control became negligible from C13 onwards. C12-MG also influenced order across the entire acyl chain, suggesting MG tail length to be a factor in how much order is affected across the bilayer. The inverse was seen in the lower leaflet (Figure 5.16 B), with all systems displaying reduced order compared to the control in the lower leaflet. The extent of changes to lipid ordering between MCMGs was proportional to tail length. Whole membrane S_{CD} profiles (Figure 5.16 C) indicate that C18-MG insertion caused an overall increase in chain order within the system, while C8-MG and C10-MG caused an overall decrease in order. C12-MG did

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not significantly alter order in comparison to the control. The high order of the upper leaflet in 100GMS_A could be observed visually in the trajectory, with very strong alignment of C18-MG tails with the bilayer normal (Figure 5.17).

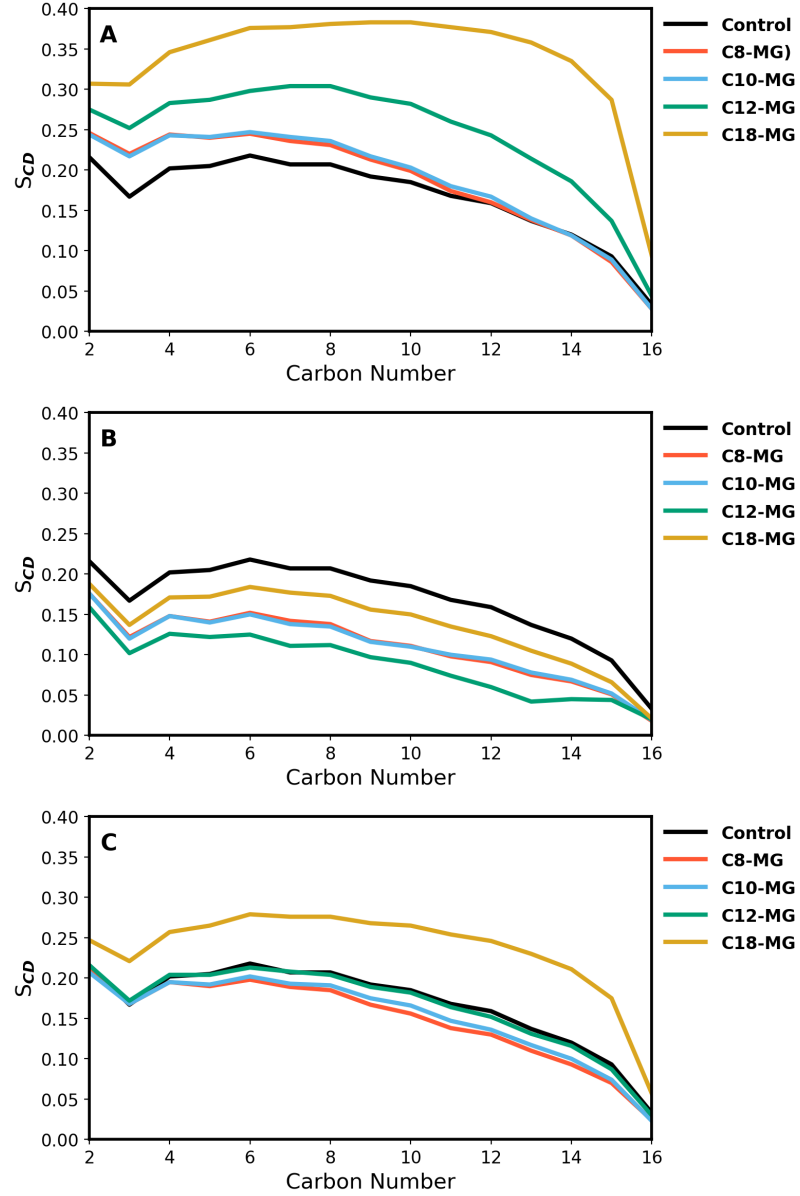


Figure 5.16: Average bilayer deuterium order parameters for 100GMCY_A, 100GMC_A, 100GML_A, and 100GMS_A systems for the Sn1 palmitoyl tail of POPC in the upper membrane leaflet (A), lower membrane leaflet (B) and whole membrane (C). High S_{CD} (0.5) represents strong alignment with bilayer normal. Carbon number represents the carbon position along the Sn1 palmitoyl acyl chain.

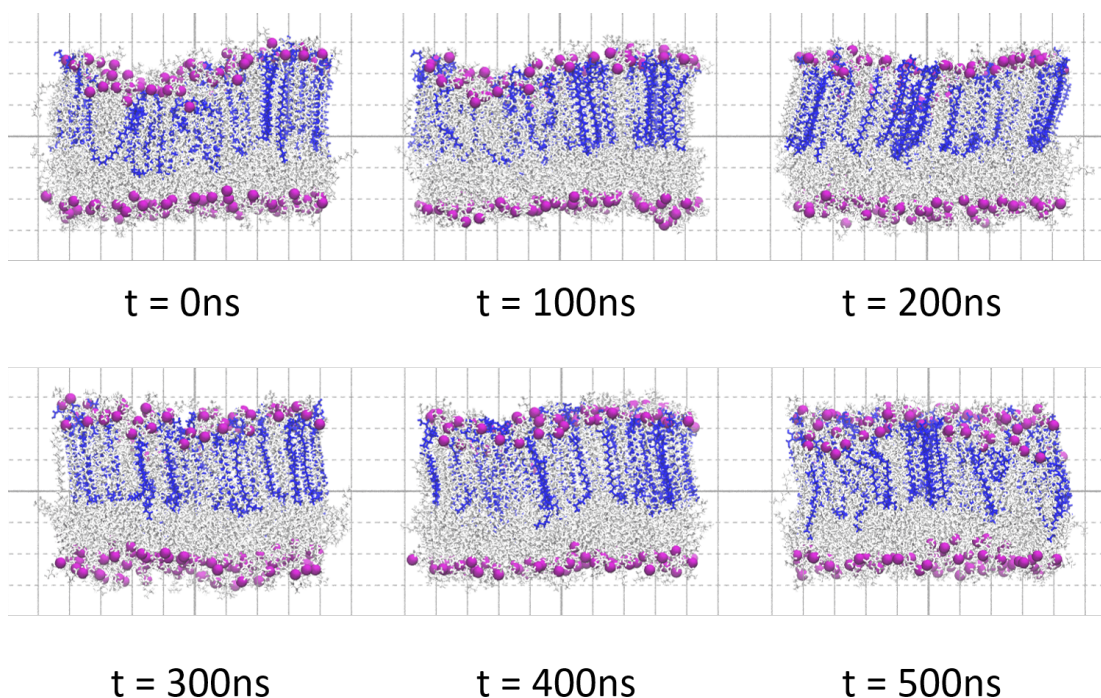


Figure 5.17: Organisation and tail alignment of a C18-MG loaded bilayer over time.

Influence of MG loading symmetry across leaflets

As observed with bilayer thickness in Figure 5.9, changes to other bilayer properties were also closer to control values than asymmetrical systems were. Full density profiles for symmetric systems in Figure 5.18 highlighted, as expected, a highly symmetrical distribution of system components across segments. Preferential positions of MG within the upper leaflet for MCMGs sit at around 12 Å in the upper leaflet, which was also observed in asymmetrically loaded systems (Figure 5.14 A-C). Unlike in Figure 5.14 D a more obvious density peak at 15 Å was observed in the symmetrical density profile for C18-MG. The peaks observed for both MG and POPC in the upper leaflet were also present in the lower leaflet at the same positions.

In comparison to control and asymmetric systems (Figure 5.19 A, C, E, G) the overall density profile of the bilayers were much closer to that of the control system than the asymmetric systems, although bilayer thickness was similar between symmetric and asymmetric systems. Symmetrical C8-MG and C10-MG systems

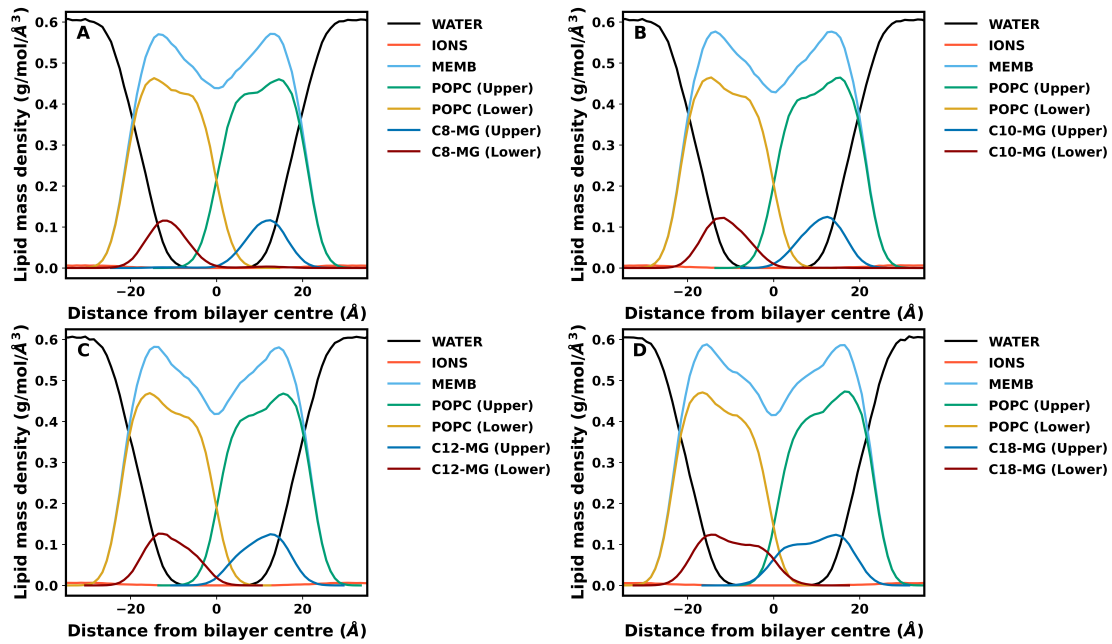


Figure 5.18: Average 1D mass density profiles along the z-axis of each component of systems 100GMCY_S (A), 100GMC_S (B), 100GML_S (C), and 100GMS_S (D). "MEMB" represents the total mass density of POPC and MG within the bilayer. "IONS" represents both Na^+ and Cl^- ions

maintained a reduction in the depth of the trough at the centre of the membrane, which suggests that lipid packing is disrupted by MCMGs regardless of symmetry. From Table 5.8 it can be seen that headgroup positioning differs significantly in some symmetrical systems. When comparing C18-MG systems, the significant increase in distance between headgroups and the membrane centre in the upper leaflet was significantly lower, reducing by 4 Å to a +2 Å increase in the upper leaflet, resulting in an overall distance 3 Å drop in distance between density peaks. Similarly, for C12-MG the significant lower leaflet thinning of the asymmetric system was not observed in the symmetrical system, having only a 1 Å reduction. For C8-MG and C10-MG, reductions in headgroup distance from the bilayer centre remained in symmetrical systems, though it was reduced by 0.5 Å in the upper leaflet and 2.5 Å in the lower leaflet of the C10-MG system, and by 1 Å in the lower leaflet only of the C8-MG systems. Determining the depth of water penetration also revealed that MCMG systems continued to display increased water penetration in comparison to control

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systems, though all were lesser than their asymmetric counterparts. Despite increased water penetration being observed in the 100GMS_A system, water penetration instead decreased in 100GMS_S by 2 Å. This aligned with the 2 Å increase in bilayer thickness observed in this system. These density profiles therefore indicate that more significant morphological changes to the bilayer across both leaflets when MG concentration is localised to a single leaflet.

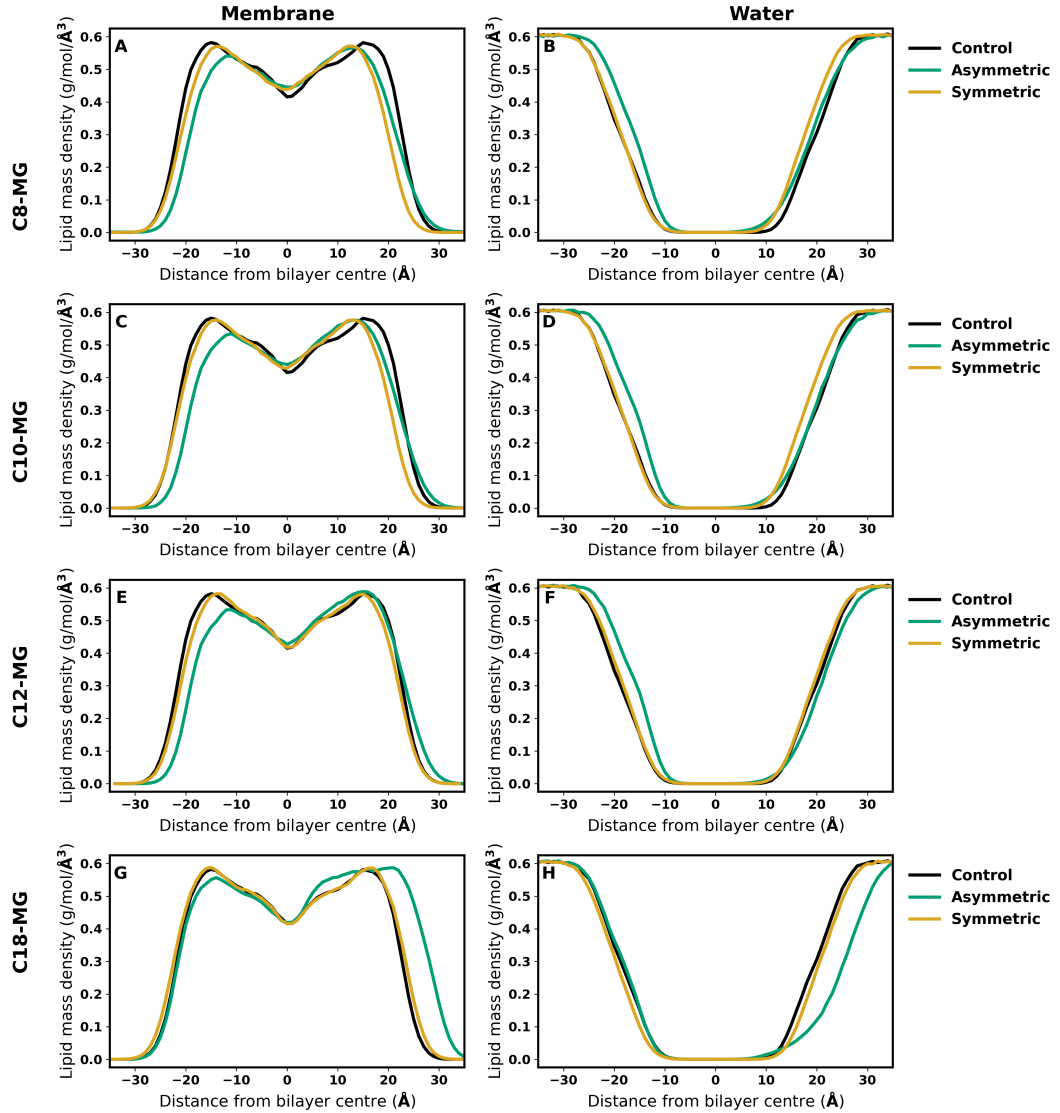


Figure 5.19: Comparison of changes to bilayer structure and water penetration in symmetric and asymmetrically loaded MG systems for (A-B) C8-MG, (C-D) C10-MG, (E-F) C12-MG, and (G-H) C18-MG.

Table 5.8: Positions of membrane headgroup peaks and depth of water penetration (both given in Å) for the upper and lower leaflets of systems loaded with 100 MG molecules in relation to the centre of the membrane (0). Brackets represent change from control.

System	HG Position (U)	HG Position (L)	Water Depth (U)	Water Depth (L)
Control	14.5 (-)	-15.5 (-)	9.5 (-)	-9.5 (-)
100GMCY_A	12.5 (-2)	-12.5 (-3)	4.5 (+5)	-8.5 (+1)
100GMCY_S	12.5 (-2)	-13.5 (-2)	8.5 (+1)	-8.5 (+1)
100GMC_A	13.0 (-1.5)	-11 (-4.5)	6.0 (+3.5)	-8.0 (+1.5)
100GMC_S	13.0 (-1)	13.5 (-2)	8.5 (+1)	-8.5 (+1)
100GML_A	15.5 (+1)	-10.5 (-5)	8.5 (+1)	-7.5 (+2)
100GML_S	14.5 (0)	14.5 (-1)	8.5 (+1)	-9.5 (0)
100GMS_A	20.5 (+6)	-14.5 (-1)	7.5 (+2)	9.5 (0)
100GMS_S	16.5 (+2)	-15.5 (0)	10.5 (-1)	-10.5 (-1)

Differences in phospholipid packing between MG symmetries can be observed in reported order parameters (Figure 5.20). Unlike asymmetric systems where the upper leaflet increased in order and the lower leaflet decreased, both leaflets displayed of symmetrically loaded bilayers displayed similar changes in all systems. Insertion of C18-MG still caused an overall increase in order across the bilayer, though it was lesser than in 100GMS_A. C12-MG produced a slight increase in order across both leaflets, where a slight decrease was observed in the asymmetric system. Both C8-MG and C10-MG continued to slightly reduce order across the bilayer in both leaflets, although again this was lesser than the asymmetrical equivalents. The extent of the reduction in order for symmetrical C8-MG and C10-MG systems was greater at the end of the Sn1 tail than the early carbons, which may explain the reduction in hydrophobic core depth for these systems' density profiles. The less drastic changes to order in these systems compared to asymmetric systems also aligns with disruption of phospholipid packaging.

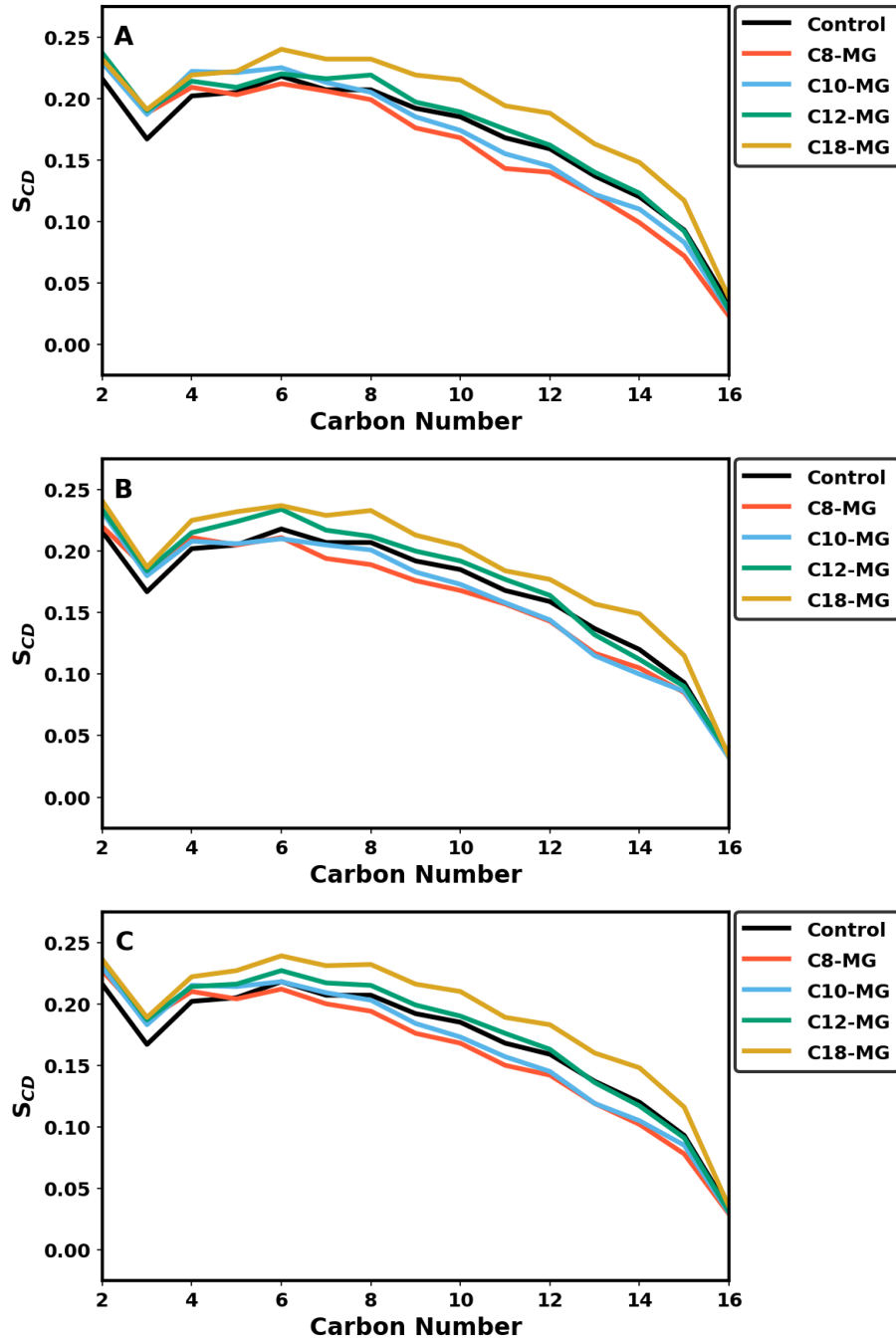


Figure 5.20: Average bilayer deuterium order parameter profiles for 100GMCY_S, 100GMC_S, 100GML_S, and 100GMS_S systems for the Sn1 palmitoyl tail of POPC in the upper membrane leaflet (A), lower membrane leaflet (B) and whole membrane (C). High S_{CD} represents strong alignment with bilayer normal. Carbon number represents the carbon position along the sn1 palmitoyl acyl chain.

5.4.4 Monoglyceride Behaviour Within Bilayers

It has been documented that amphiphilic molecules have the capability to move from one leaflet of a lipid bilayer to the opposite, reorienting in line with phospholipid head groups in that leaflet (also known as flip-flop events) [268]. This can be seen rarely in phospholipids, particularly at the timescale simulated, but is more common in smaller molecules such as MGs, which have also been observed to exit the bilayer into the water box (expulsion/exit events). (Figures 5.21, 5.22). Both processes are energetically unfavourable for MGs, and translocation of lipid molecules across a leaflet generally occurs on a timescale not normally capturable on an all-atom MD timescale [271–273]. Consequently, these events occurring on a 500 ns timescale can give greater understanding of bilayer disruption altering the ease of MG movement across the membrane. Specifically for MGs, potential energy barriers for MG flip-flop and exit were calculated for the POPC bilayer system and other systems in Chapter 7 of this thesis. These indicate that for these molecules the flip-flop or exit rate is at a sub-microsecond scale, and therefore more likely to be observed in the MD simulation, though documenting MG behaviour remains useful in understanding how they interact with the bilayer and external aqueous environment.

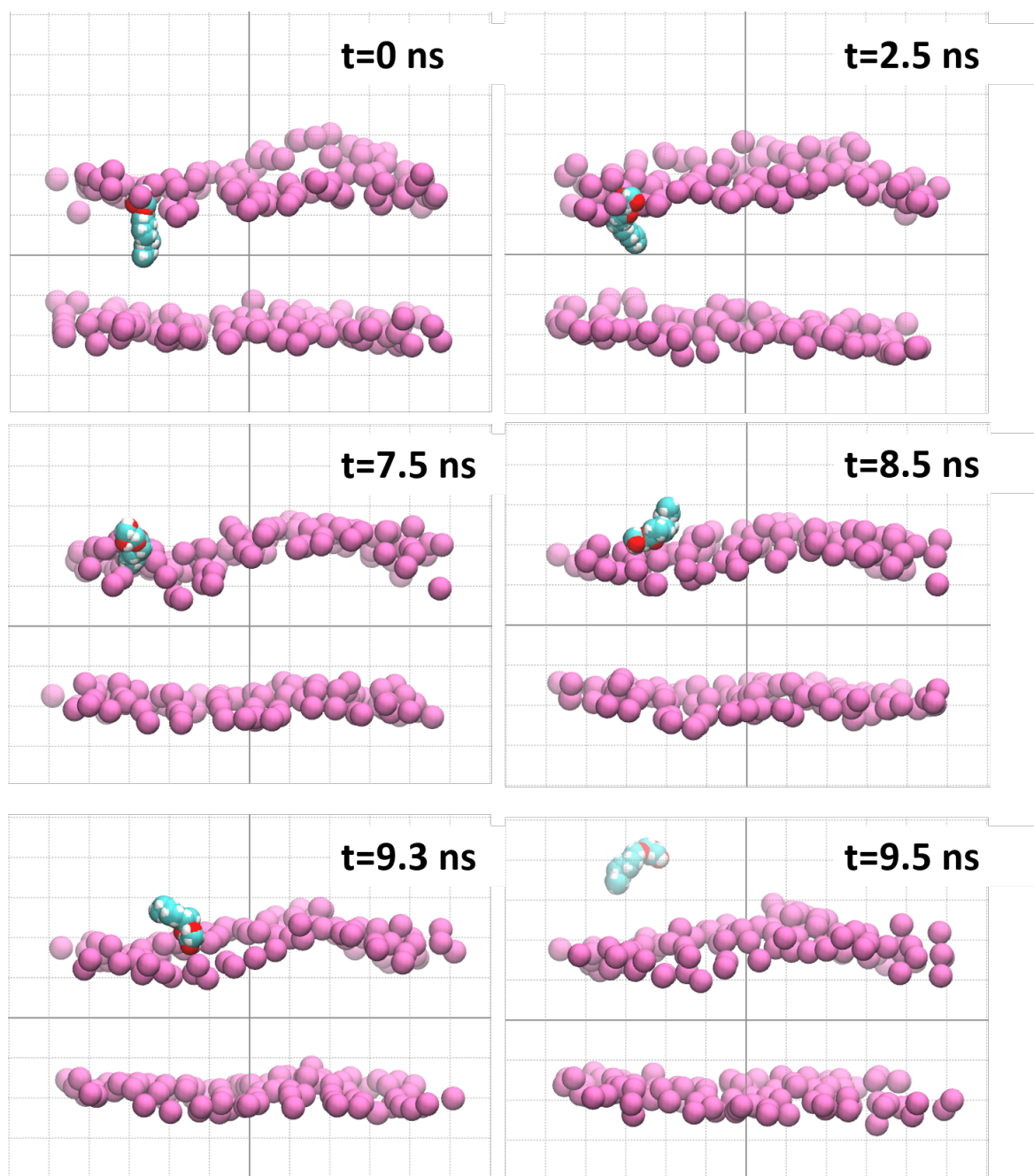


Figure 5.21: Stages of C8-MG exit from a lipid bilayer. Purple spheres represent phosphorus atoms at the headgroup region of each leaflet. Red indicated C8-MG headgroup Squares represent $10 \text{ \AA}^2$.

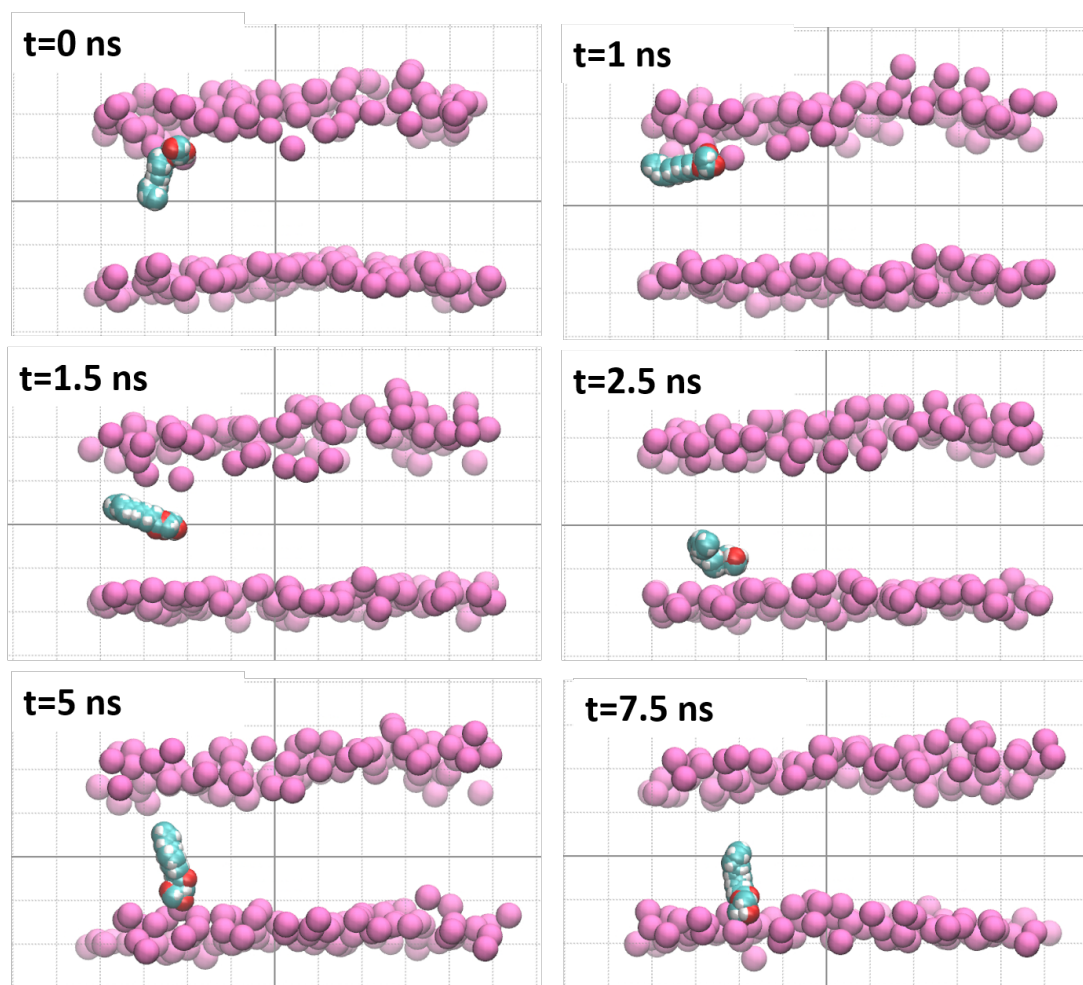


Figure 5.22: Stages of C8-MG flip-flop from the upper leaflet to the lower leaflet. Purple spheres represent phosphorus atoms at the headgroup region of each leaflet. Red indicates C8-MG headgroup. Squares represent $10 \text{ \AA}^2$.

Both processes of flip-flop and exit occurred over in under 10 ns. In MG exit the monomer initially moved towards the headgroup region, after which the tail oriented perpendicular to the bilayer normal while the headgroup remained in line with the headgroup region of the bilayer for 1-2 ns before detaching. This is likely because the headgroup of MGs have stronger intermolecular forces with other nearby atoms, as the hydroxyl groups present in the headgroup forms hydrogen bonds with other molecules. During lipid flip-flop the MG became disoriented with the bilayer normal as it passed through the hydrophobic core. As it passed to the lower leaflet, the molecule again began to reorient with the lower leaflet, with the headgroup orienting with the lower leaflet lipid-water interface and the tail aligned with the hydrophobic core.

Influence of MG Concentration Within the Bilayer

As highlighted by (Figure 5.10 C), despite C10-MG insertion only into the upper leaflet of the membrane, molecules of C10-MG displayed both flip-flop and exit events throughout the simulation. A summary of these events within the C10-MG loaded systems can be found in Table 5.9. This Table displayed a very slightly increased rate of MG translocation events occurring in systems that is heavily-loaded with MGs than crossing to the lower leaflet, which may suggest a correlation between MG concentration and reduced energy barriers for MG molecules to cross leaflets or exit from the membrane. The 100GMC_A system was also the only system of the five where expulsion was observed, which suggested both that this system is also the least stable, and that energy barriers to flip-flop may become lower as bilayers become more disrupted. This is investigated in Chapter 7 section 7.4.5 using US to calculate free energy barriers to MG movement from its preferred position within the leaflet to the bilayer centre.

The correlation between increasing MG concentration and the extent of amphiphile translocation may further indicate a reduction in membrane stability. [35] Expulsion of MG molecules from the membrane and flip-flop to the opposite leaflet of the membrane are ordinarily energetically unfavourable processes due to the amphiphilic nature causing a preference to remain associated with the hydrophilic exterior/hydrophobic

Table 5.9: Counts of flip-flop and expulsion events for C10-MG loaded systems. UL indicates the final count of MG in upper leaflet. LL indicates the final count of MG in lower leaflet. WB indicates final MG count in water box. Brackets indicate percentage of total MG.

System	Flip-flop	Exit	MG count (UL)	MG count (LL)	MG count (WB)
12GMC_A	0	0	12 (100)	0	0
28GMC_A	1 (3.6)	0	27 (96.4)	1 (3.6)	0
44GMC_A	2 (4.5)	0	42 (95.5)	2 (4.5)	0
100GMC_A	5 (5)	1 (1)	94 (94)	6 (6)	0

interior structure of lipid bilayers. This may suggest that in loaded membranes where the upper leaflet is under compression and the lower leaflet is under tension, these transport mechanisms become favourable as a means to relieve stress.

It should be noted that the transport of C10-MG from the upper leaflet to the lower via expulsion into the water box, followed by diffusion across the periodic boundaries observed in Table 5.9, is an artefact of the simulation procedure. Nevertheless, expulsion from the upper leaflet itself provides a viable route to reduce stress. Relief of membrane asymmetry has been reported experimentally to reduce the extent of membrane deformations caused by the addition of amphiphilic molecules. [191]

Influence of Inserted MG Structure

A consistent inverse correlation between MG chain length and lipid translocation events was noted in asymmetric 100MG systems. The 100GMCY_A system experienced significantly more translocation events than other MCMG systems while no changes to MG leaflet location could be observed across the entire trajectory (Table 5.10). Both processes for C8-MG are displayed in Figure 5.21. The differences in translocation probability between MGs may have contributed to the variance in antimicrobial efficacy of the tested MGs. For example, it may be difficult for longer chain MGs to enter the cell membrane and accumulate to a sufficient localised concentration to cause significant disruption. Conversely, shorter chain MGs may not remain in a leaflet long enough to produce their disruptive effects, which may explain reported differences in MG efficacy. [35,274,275] This is investigated further in Chapter 7.

Table 5.10: Counts of flip-flop and expulsion events for asymmetric MG loaded systems. UL indicates the final count of MG in upper leaflet. LL indicates the final count of MG in lower leaflet. WB indicates final MG count in water box.

System	Flip-flop	Exit	MG count (UL)	MG count (LL)	MG count (WB)
100GMCY_A	7	26	64	26	0
100GMC_A	1	5	94	6	0
100GML_A	2	0	99	1	0
100GMS_A	0	0	0	0	0

Influence of MG Loading Symmetry Across Leaflets

The increased stability of symmetrically loaded bilayers was further reflected in the reduced occurrence of MG translocation events during the simulated trajectories (Table 5.11). In 100GMCY_A, the system which experienced the most translocation events, the number of MG flip-flop events were 2 times more frequent and exit events were 8.6 times more frequent than the symmetrically loaded equivalent. Similarly, 100GMC_A had 3 times more flip-flop events than its symmetrical counterpart and exhibited numerous exit events while the symmetrical system only had one. Due to the energetically unfavourable nature of translocation events within a membrane, this provides further evidence of systems stability and suggests a bilayer tension reduction occurring from the return to a stable state. Additionally, in the 100GML_A system, flip-flop of one molecule was detected, which was not seen in the symmetrically loaded equivalent. C12-MG has been reported to have a flip-flop rate of seconds, so the detection of the process happening at a 500 ns timescale further suggests that asymmetric stress induced by MG insertion into a leaflet promotes the translocation of MGs to the inner leaflet in an attempt to relieve stress. [276]

Table 5.11: Counts of flip-flop and expulsion events for symmetrically loaded systems. UL indicates the final count of MG in upper leaflet. LL indicates the final count of MG in lower leaflet. WB indicates final MG count in water box. Brackets indicate percentage of total MG. “U<L” and “L<U” represent flip-flop direction of MG from upper to lower leaflet and lower to upper leaflet respectively. Brackets for final MG count represent the number of molecules originating from the opposite leaflet which are included in the final count.

System	Flip-flop	Exit	MG (UL)	MG (LL)	MG (WB)
100GMCY_S	3 (1 $U \rightarrow L$, 2 $L \rightarrow U$)	2 (1 U, 2 L)	51	48	1
100GMC_S	1 (L $\rightarrow$ U)	0	51	29	0
100GML_S	0	0	50	50	0
100GMS_S	0	0	0	0	0

5.4.5 Very High Monoglyceride Concentrations

In addition to the previously stated work carried out in this chapter, another set of simulations were performed where 200 molecules of each MG were loaded into the membrane - double the concentration of the previous highest concentration in this chapter. This was done with the aim of trying to push the bilayer towards significant destabilisation to attempt to understand potential mechanisms of antimicrobial activity further. In particular, this allowed further exploration of MG flip-flop and exit in the context of asymmetric stress relief.

Asymmetric Loading of Monoglycerides

Unlike lower concentrations, where systems displayed at most induced curvature as the only significant deformation of the bilayer structure, more severe deformations of the bilayer structure in several different systems was observed early on in the simulations.

In all three replicates systems loaded with 200 molecules of C8-MG destabilisation of the bilayer structure occurred (Figure 5.23). This began with the induction of significant curvature in the upper leaflet of the membrane, which merged into a bud-like structure and sat on top of the upper leaflet, stretching the z box dimension. This structure was composed of both C8-MG and POPC. Between 100 and 200ns, after the bud had formed, this structure proceeded to detach from the upper leaflet and began to float in the water box, where it remained for the rest of the production run. The structure had MG and POPC headgroups facing the water box, with no partitioning

between MG and POPC, making it analogous to a micelle.

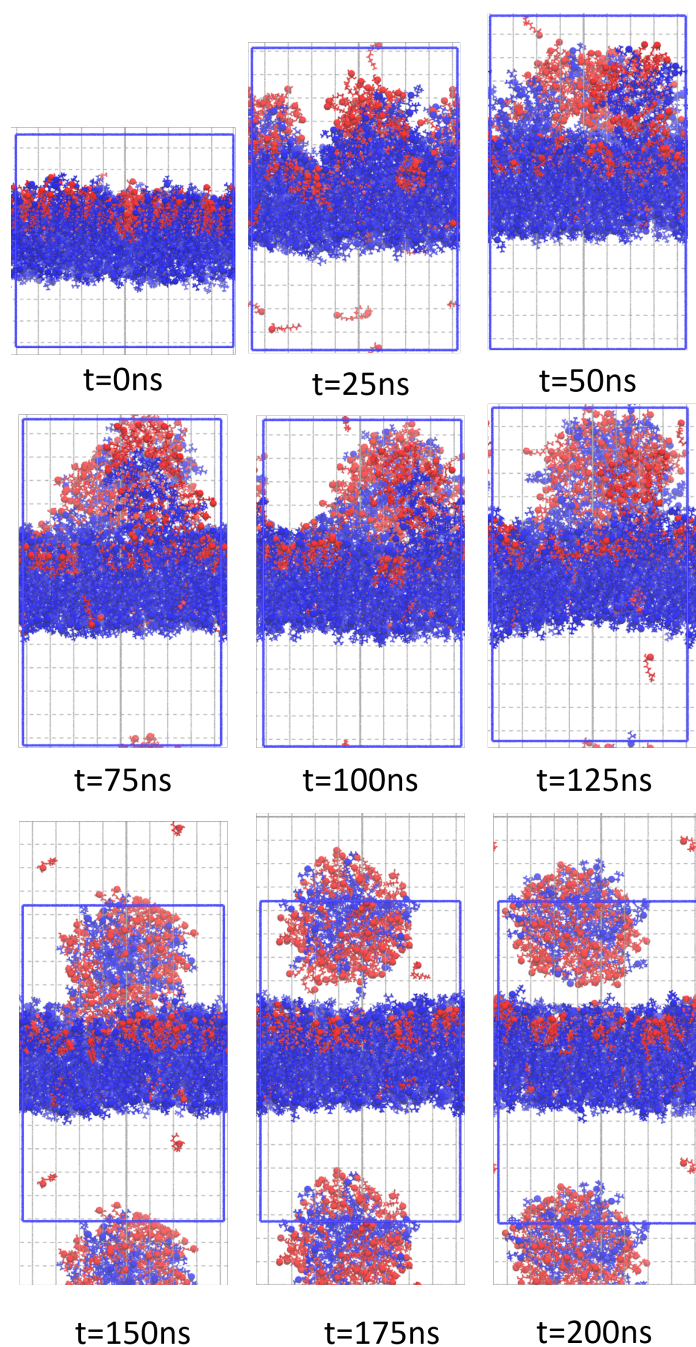


Figure 5.23: Stages of leaflet destabilisation, structure formation, and subsequent budding from the upper leaflet of a system loaded with 200 molecules of C8-MG over a 200ns timespan. Blue represents POPC. Red represents C8-MG. The blue box represents the simulation box; $\pm z$ axis PBC were employed for the purpose of clarity.

The detachment of the "micelle" was a distinguishing feature of the C8-MG loaded system. In both C10-MG and C12-MG loaded systems, although a bud developed in the upper leaflet, as in the previously discussed system, no detachment from the leaflet occurred. Instead, the protrusion grew to a length of around 50 Å over the remaining course of the trajectory. This protrusion had a similar structure to the micelle-like nature of the detached bud in the C8-MG system, with headgroups facing towards the surrounding water. Failure of these structures to detach from the membrane may suggest stronger non-bonded interactions stabilising the membrane in systems loaded with C10-MG and C12-MG due to their longer acyl tails, though with only 500 ns of simulation time it is unclear whether these structures simply require additional time to detach in comparison to systems disrupted by C8-MG.

Notably, while this behaviour was observed in all three repeats of 200GMCY_A loaded systems, formation of the bud did not occur in in one C10-MG replicate and two C12-MG replicates. Examination of these trajectories revealed that the likely cause for this was due to the rapid flip-flop of MGs to the lower leaflet of the bilayer over a short period of time (Figure 5.24). In the C10-MG system which failed to form a bud, significant curvature was induced in the upper leaflet, similar to Figure 5.23 $t=25$ ns, leading to a small hemispheric structure forming on the leaflet at approximately 8 ns into the simulation. This structure remained for approximately 20 ns before collapsing. This collapse occurred immediately following the movement of 41 molecules of C10-MG to the lower leaflet. This event appeared to stabilise the bilayer significantly, as for the remainder of the trajectory only 7 more C10-MG molecules flipped to the lower leaflet and one molecule exited into the water box, leaving a final C10-MG count of 151 in the upper leaflet and 48 in the lower. This is a significantly higher percentage of molecules which experienced flip-flop events than in the 100 C10-MG systems. This same behaviour was also observed in systems loaded with C12-MG. The overall process was slower to occur, with significant curvature beginning around 40 ns into the production run and persisting until 80 ns. Slightly fewer molecules of C12-MG flipped to the lower leaflet during the relief of this curvature, with 41 molecules flipping during the process, and a total of 44 in the lower leaflet by the end of the

simulation. Flip-flop of some POPC molecules was also observed in this system, which is significantly more energetically unfavourable than MCMG flip-flop, usually requiring days to move across leaflets without the assistance of proteins [277]. This occurring at a ns scale therefore indicates severe disruption of the bilayer system. Additionally, no exit events were experienced by C12-MG molecules in this particular simulation.

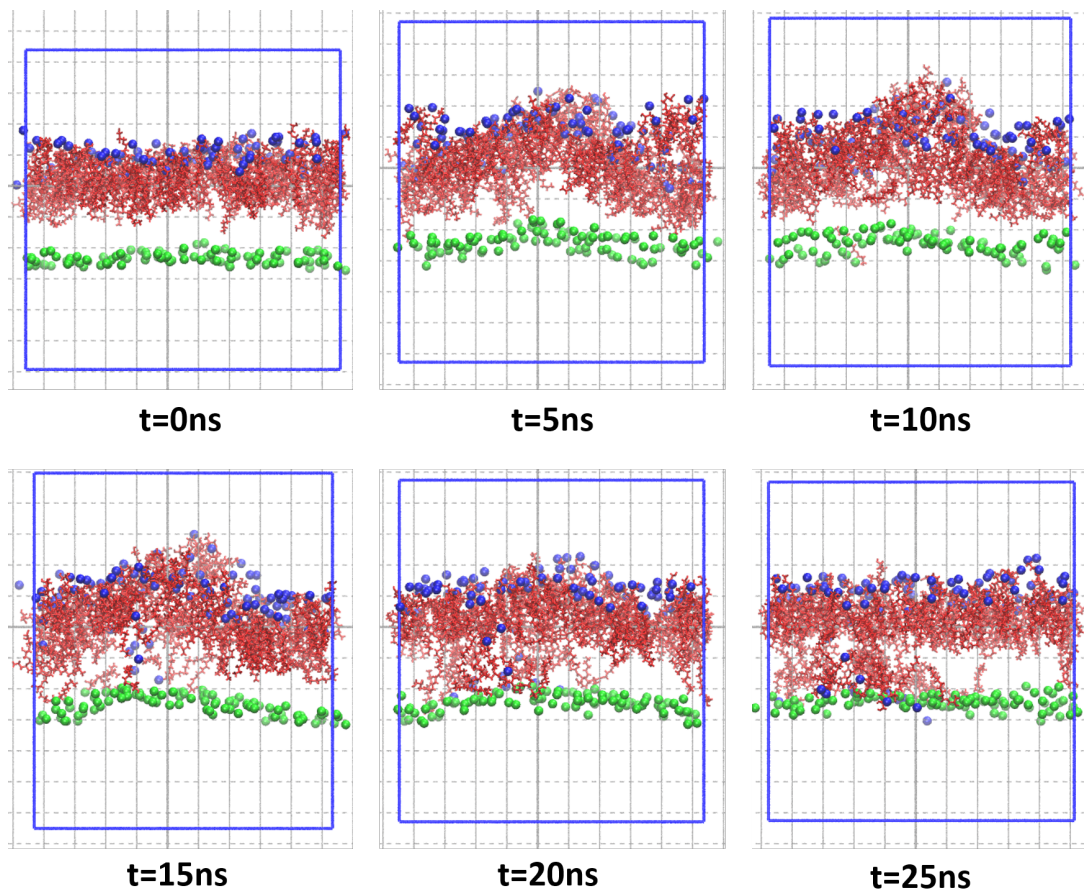


Figure 5.24: Stages of bilayer planarity returning to a heavily disrupted POPC bilayer via mass flip-flop in a system loaded with 200 molecules of C10-MG over a 25 ns timespan. Blue represents the head groups of POPC which started in the upper leaflet. Green represents the head groups of POPC which started in the lower leaflet. Red represent C10-MG. The blue box represents the simulation box.

While no bud formed on the upper leaflet of the 200GMS_A bilayer system, the changes observed in the 100GMS_A system discussed in section 5.4.3 were amplified in this bilayer. The bilayer density profile revealed a more extreme expansion of the upper leaflet and compaction of the lower leaflet (Figure 5.25). This was agreed upon by the

presented order parameters for the system, which showed extremely high alignment with the membrane normal for the upper leaflet and system as a whole, and very low alignment with the lower leaflet. The highly ordered upper leaflet experienced the formation of an indent, where high tail interdigitation between the two leaflets occurred. This indent moved in position across the trajectory, due to the lateral diffusion of the molecules within the membrane, however the specific molecules in this interdigitated region did not change (Figure 5.26). Water penetration was asymmetrically distributed with minima of 7.5 and -12.5 Å, losing 2 Å in depth on each side from the 100GMS_A system, suggesting reduced fluidity within this system in comparison.

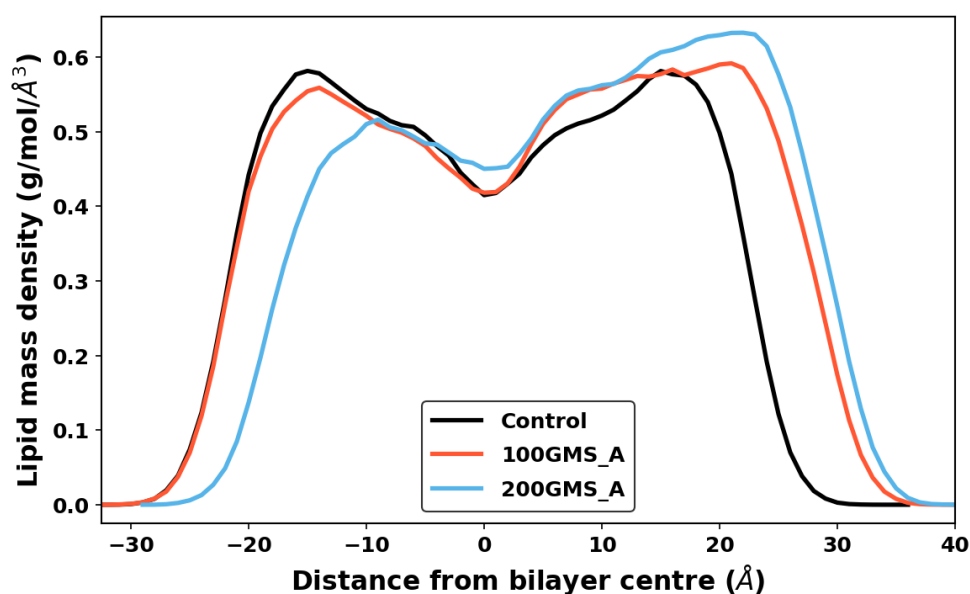


Figure 5.25: 1D density profile along the z-axis for MEMB segment (POPC+C18-MG) for systems Control (black), 100GMS_A (red), and 200GMS_A (blue). "MEMB" represents both POPC and C18-MG.

Symmetric Loading of Monoglycerides

As it was observed in previous Sections that systems with symmetrised MG loading saw significant lower deviation of bilayer structure and properties in comparison to the control than systems loaded asymmetrically at a count of 100 MGs in the system, it was decided to also perform a set of symmetrised systems with 100 MGs in each leaflet.

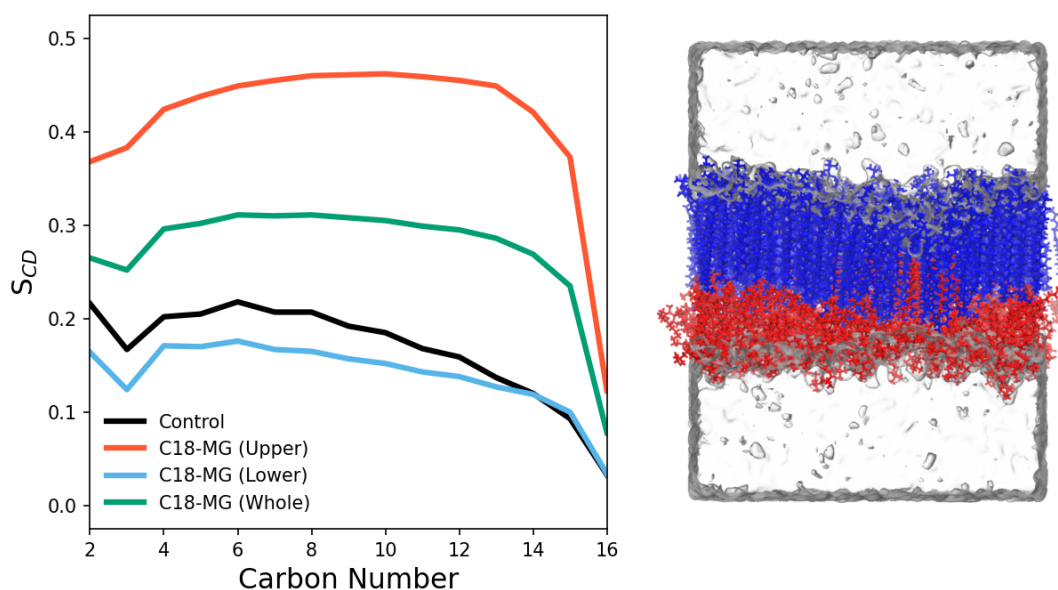


Figure 5.26: POPC Sn1 tail deuterium order parameters for 200GMS_A and control system (left). Snapshot of the high interdigitated region of bilayer (right). Blue represents the upper leaflet of the membrane, red represents the lower leaflet of the membrane.

In symmetric systems, no outright loss of bilayer integrity was observed, though trends within the system follow those discussed in Section 5.4.3, but to an amplified degree.

The average thickness of each system were 35.2, 36.6, 38.2, and 45.0 Å for C8-MG, C10-MG, C12-MG, and C18 respectively. For MCMG systems this was consistently lower than the average bilayer thickness of asymmetrically loaded 100MG systems, instead being around the same thickness of the asymmetric system for C12-MG, and lower than either for C10-MG and especially C8-MG. C18-MG displayed the opposite effect, as expected, displaying an increased average thickness than either system containing 100 molecules of C18-MG. xy thickness distribution maps revealed regions of slightly higher and slightly lower thickness across MCMG systems with a maximum of 2 Å in difference between region, indicating relative stability in the bilayer structure. On the contrary, C18-MG displayed a significant dip in thickness at the centre of the xy plane matched with large regions of high thickness on each side, with a difference of up to 8 Å between these regions (Figure 5.27). This was observed in the trajectory of a "pinch" between the two leaflets, which formed around 300 ns into the production run.

(Figure 5.28). Around this pinch lipids were visibly disordered with tail interdigitation between leaflets, but showed strong alignment with the bilayer normal away from it, which aligned with the observed thickness distribution of the xy thickness map. Compared to the 200GMS_A system, the overall changes to thickness were greater in the symmetrised system which, combined with dent formation, suggests that reaching a steady state of equilibrium between the two leaflets does not always correlated with a significant reduction in the extent of property deformation in MG loaded bilayers, given a sufficiently disruptive count of MG is present across the system.

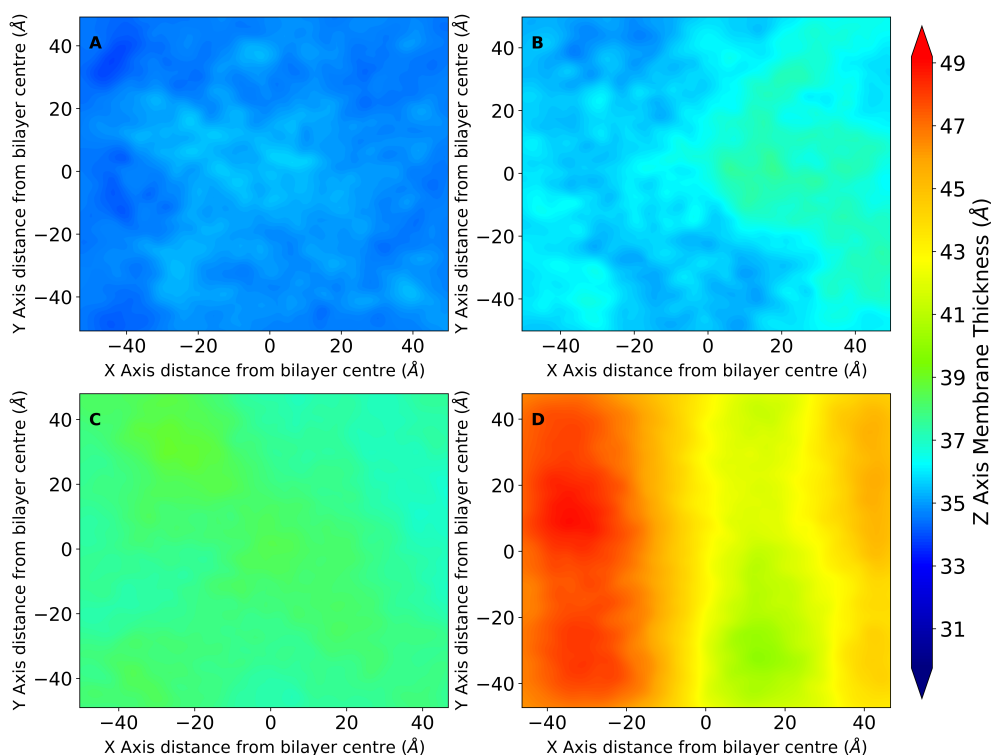


Figure 5.27: 2D average membrane thickness maps across the xy plane for the production runs of symmetrical C8-MG (A), C10-MG (B), C12-MG (C), and C18-MG (D) systems with 100 MGs in each leaflet.

As was observed in symmetrically loaded systems with 50 MG present in each leaflet, mass density profiles displayed a fairly even distribution of density across both leaflets of the bilayer, with similarly placed headgroups 5.29. The overall width of the bilayer was reduced slightly in all MCMG systems in comparison to the symmetrical 100MG systems (consisting of 50 MG in both leaflets of the bilayer),

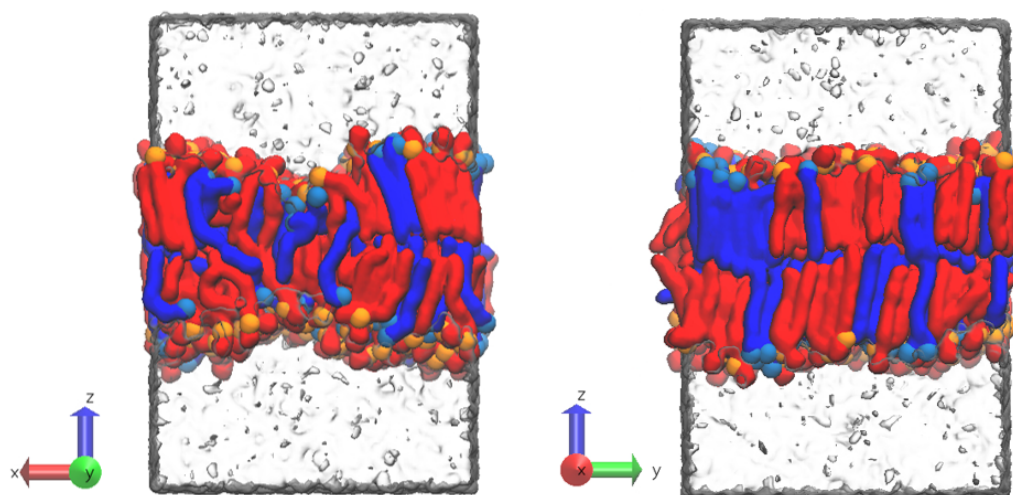


Figure 5.28: View of pinched lipid bilayer containing 100 molecules of C18-MG in each leaflet. Red represents POPC, blue represents C18-MG. Orange and blue spheres represent the headgroups of POPC and C18-MG respectively. Images taken from final frame of trajectory.

again indicating that bilayer properties can still be impacted by MG insertion even when symmetrical. In comparison to asymmetrical 100MG systems the upper leaflet displayed a greater reduction in thickness and the lower leaflet a lesser reduction in thickness though headgroup positioning did not significantly change. Unlike MCMG systems, the 200GMS_S system displayed significant expansion of the lower leaflet, which aligns with the very high deuterium order parameters observed in this system. For MCMG systems water penetration remained roughly in line with Z-axis membrane thickness, however penetration was observed significantly deeper into the upper leaflet in 200GMS_S (5.29 H). This was likely caused by the deformation of the bilayer structure from the pinched bilayer in this system disrupting lipid packing in a region of the bilayer.

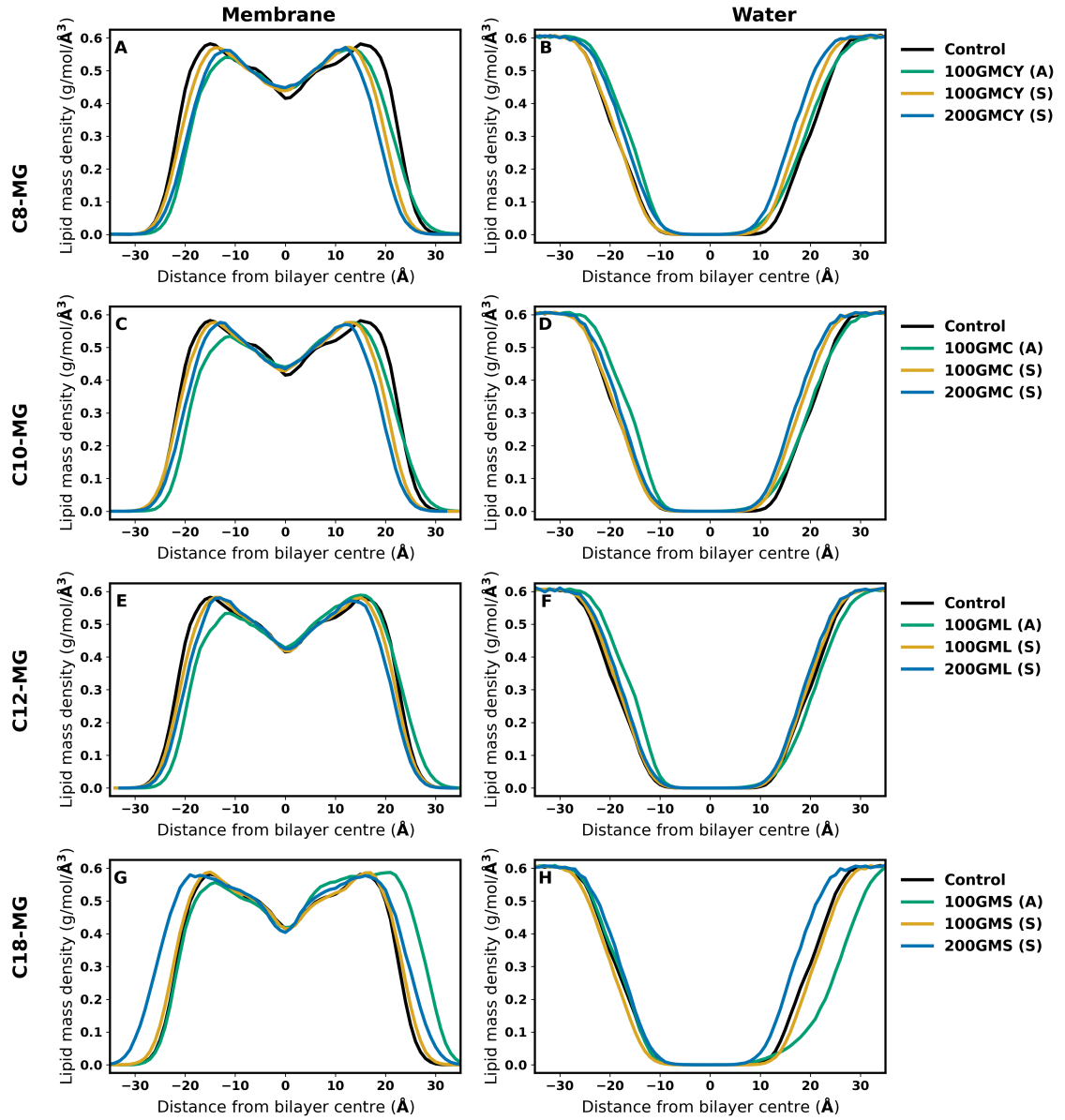


Figure 5.29: Comparison of changes to bilayer and water 1D density distribution in 100MG symmetric and asymmetrically loaded systems and 200MG symmetrically loaded systems for (A-B) C8-MG, (C-D) C10-MG, (E-F) C12-MG, and (G-H) C18-MG.

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Deuterium order parameters revealed that order across the two 100MG and symmetrical 200MG system were very similar across the whole bilayer, and both symmetrical MCMG systems showed similar order within leaflets^{5.30}. For C18-MG, however, the system 200GMS_S displayed significantly higher order between leaflets than 100GMS_S, which was closer to the control. This again suggested that significant disruption continued to occur in this system despite having no MG-driven asymmetric stress between leaflets.

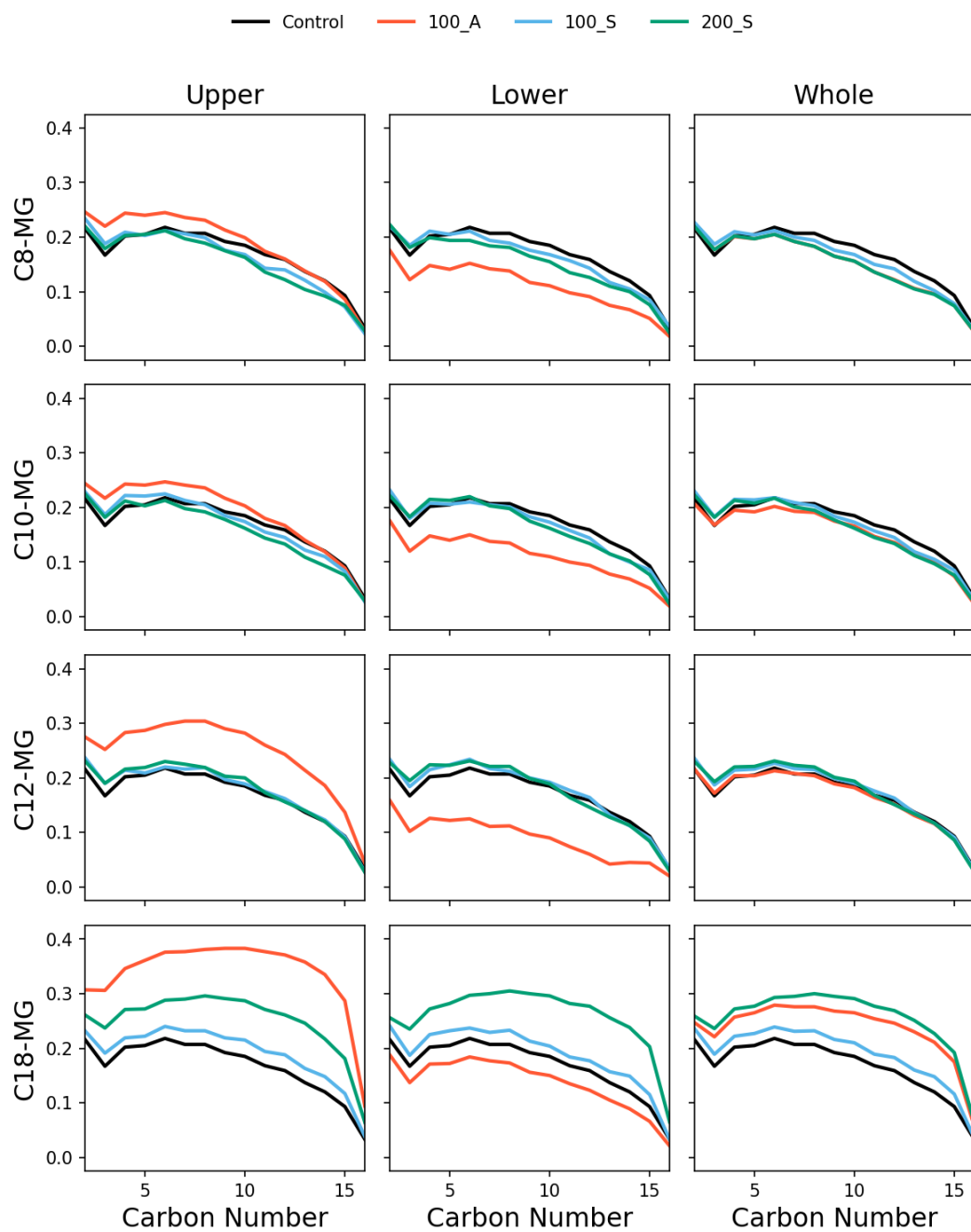


Figure 5.30: Deuterium order parameters across the upper leaflet, lower leaflet, and whole bilayer for symmetrical and asymmetrical 100MG systems and symmetrical 200MG systems.

Overall trends in stability of MCMG systems were also reflected in the rates of MG exit and flip-flop, which was comparable to the other set of symmetric systems. All molecules of C12-MG across the two leaflets were entirely stable, experiencing no flip-flop events. Additionally, only 3 flip-flop events, all moving from the lower leaflet to the upper leaflet, were observed in 200GMC_S with no exit events, which was consistent with the 100GMC_S system. System 200GMCY_S experienced flip-flop events in both leaflets and exit events at roughly the same extent in both leaflets, with a final C8-MG concentration of 103/97 in the upper and lower leaflet respectively. Flip-flop of MGs in the C18-MG system also occurred by the end point of the simulation, with the head groups of three molecules moving to the opposite leaflet, which was not observed in any other C18-MG system. This occurred around the thinner, "pinched" region of the bilayer.

When specifically comparing these three MG loading dynamics (50 in each leaflet, 100 in each leaflet, 100 in the upper leaflet only) a key aspect of the mechanism is that the insertion of MGs into the bilayer structure produces a baseline stress on the bilayer separate from the asymmetric stresses addressed in the previous section, which is correlated with overall MG count throughout the bilayer system. When comparing 100_S systems (50 MG per leaflet) and 200_S systems (100 MG per leaflet) systems it was consistent that for all MG tail lengths systems containing 100 MG per leaflet had more pronounced changes to bilayer properties in comparison to the control system, as shown in Figures 5.29 and 5.30. Overall water penetration across both leaflets was increased in all systems, and a greater reduction in bilayer thickness was observed in MCMG systems. The thickness profile for 200GMS_S was different to 100GMS_S, seeing greater expansion of the lower leaflet than the upper leaflet, though both did see expansion. In reference to deuterium order parameters, alignment of POPC tails with the bilayer normal was generally similar across all carbons for the MCMGs systems, with overall order displaying particularly high similarity between systems. For C18-MG order across all leaflets was more significantly increased, which aligned with the more significant changes to thickness comparative to the other bilayer models. These factors suggest that increasing the MG count across the bilayer continues to increase the extent

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to which the bilayer properties are disrupted, which aligns with discussion regarding the 100_S systems, where even when the system was in mechanical equilibrium changes to bilayer properties still occurred. Mechanically, when considering the discussed forces related to MGs that likely drive bilayer disruption, this aligns with the concept that higher MG counts present within an individual leaflet increase the stress placed on the bilayer due to greater geometric mismatch with the undisrupted bilayer structure driving more significant perturbation of properties.

A more interesting comparison can be made between the 200_S systems and the 100_A bilayer systems at the same chain length. It has been shown in data across sections 5.4.1 through 5.4.4 that even when MG are present in only one leaflet of the bilayer properties across both leaflets are affected. When comparing these two systems, many parameters were similar, or higher for 200_S systems in comparison to the 100_A systems. For tail order, order was very similar for both distributions across all three MCMGs for overall order despite the significant differences in order observed between the upper and lower leaflet in asymmetric and symmetric systems. The asymmetric loading producing a slightly greater decrease in fluidity at tail carbons near the hydrophilic headgroup region. Overall bilayer thickness, from the density profiles of the membrane, were similar despite symmetrical systems seeing roughly even thinning of both leaflets while the lower leaflet was more thinned by the presence of MG in the upper leaflet, as the distance between headgroups was similar. Likewise, water penetration was slightly greater in the lower leaflet of the asymmetric system and greater in the upper leaflet of the symmetrical system. This suggests that the overall disruption profile of a symmetrical system with 100 MG in each leaflet is very similar to that of one with 100 MG in a single leaflet. This is strong evidence to support that asymmetry in MG loading is likely an amplifier of the extent of disruption when considering overall MG monomer count within the bilayer, and possibly indicates the importance of MG count within an individual leaflet in driving bilayer deformation. As has been repeatedly discussed across this chapter, the accumulation of MGs within a leaflet is likely to exacerbate the stress MGs place on the bilayer. For asymmetric systems this also causes a significant imbalance in stress profiles across

the two leaflets, which can lead to more significant structural disruption. As some structural perturbation was observed in the symmetrical systems with 100 MG in each leaflet, this suggests that both a high MG count in each leaflet of a symmetrical system causing hydrophobic mismatch within the leaflet and an imbalance in stress profiles between leaflets can lead to curvature induction in the bilayer structure.

5.4.6 Discussion

Summary of Findings

The most obvious insight to be gained from the results presented is the importance of MG concentration in disrupting membrane properties. The extent of all disruption observed within systems containing 12/28/44/100 MG molecules was exacerbated by the addition of more MG into the leaflet, and at a count of 200 significant destabilisation of bilayer planarity began to be observed. The high concentration of MG used in these simulations is analogous to that immediately following an interaction of a MG-based LNP with a lipid bilayer. LNPs incorporating other known antimicrobial molecules have been found to cause the destruction of cell membranes, which may suggest that a high localised concentration of MG within the bilayer induces changes to membrane morphology [278]. This may provide possible explanation of LNPs having increased antimicrobial potency than free MGs.

The concentration of C10-MG in these systems was strongly correlated with disruption of the lipid bilayer system. This might explain the experimentally reported minimum antibacterial concentrations. Even in systems where the bilayer structure was not compromised, there were notable changes to bilayer fluidity and permeability, which is another property of the cell membrane reported to be disrupted experimentally by MGs [39]. Though a simplified model system such as the POPC bilayer featured in this chapter cannot highlight this in full, increased bilayer permeability can cause increased passive transport across the bilayer, which can in turn lead to the inhibition of nutrient and waste exchange pathways as the diffusion gradient between the cell and external environment is altered, or inhibit transport protein function [263, 279]. The opposite is true for increased bilayer thickness, which can increase energy barriers

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to nutrient transport and can lead to the accumulation of waste products in the cell which can lead to toxic effects.

Clear trends emerged in how bilayers become destabilised by MGs, dependent on the length of the acyl tail structure. The easiest to observe of these was how classes of MG produce different effects on the bilayer. With two classes utilised in this study - medium chain MGs and long chain MGs - MCMGs generally decreased bilayer stability, while long chain MGs appeared increase bilayer stability, primarily through a reduction in bilayer fluidity. This is agreed upon experimentally, as these molecules have uses as permeation enhancers and stabilisers respectively [280,281]. As the antimicrobial potential for MCMGs has been linked to altering the cell membrane permeability, causing the leakage of cellular components, this observed behaviour would also make sense in the context of lipid bilayer destabilisation mechanisms [35,282]. Within the MCMG group, C8-MG displayed the most significant disruption of the bilayer, while C12-MG produced the least disruption, and C10-MG was in between the two in terms of disruption. This indicates a clear trend of shorter tail length being correlated with increased bilayer property disruption. This is likely due to the structure of MGs. As discussed throughout this chapter, hydrophobic mismatch is likely an important driving factor in disrupting bilayer phospholipid packing in particular. C8-MG has the shortest tail of the three MCMGs, and therefore would produce the most significant hydrophobic mismatch with the bilayer. Additionally, geometrically C8-MG is the most conical of the three MCMGs, so it is the most geometrically mismatched with POPC geometry and therefore the most likely to induce curvature in the bilayer.

It appears that a factor which influences bilayer characteristics in these systems is changes to ordering of POPC molecules within the leaflet due to MG packing. This appears to be directly related to the structure of MGs and where they sit within the bilayer leaflets, which aligns with literature as MGs are known to disrupt phospholipid packing [283]. The reduction in lipid packing density observed in MCMG systems is likely due to shorter structure of these molecules in relation to the membrane, as the shorter chain MGs have a reduced length in comparison to POPC. For C18-MG, the acyl tail length of C18-MG is comparable to the tails of POPC phospholipids, which

would force greater alignment of POPC with the membrane normal following C18-MG insertion, resulting in an increase in leaflet thickness.

The planar structure of the lipid bilayer itself was also found to experience significant disruption at high concentrations. At relatively lower concentrations, this manifested as the induction of increased curvature in the bilayer, primarily associated with the leaflet of the membrane the MGs are inserted into. This was also likely due to changes to lipid packing in the bilayer induced by MG insertion, as molecules with differing ratios of headgroups to tail lengths have less dense packing, which can induce curvature in the membrane structure, disrupting bilayer integrity [284]. At its most severe, this induced curvature could result in the formation of budding-like behaviour on the surface of the membrane in MCMG systems, which would either remain or bud off from the membrane, as observed in asymmetric 200 MG systems. This is likely due to the accumulation of stresses within the bilayer which are generated by the incorporation of MGs into the leaflet, as has been observed with other amphiphilic molecules in experimental studies [191,285]. Antimicrobial MGs have been reported to induce budding in lipid bilayer models, which aligns with this observed behaviour [27].

Furthermore, findings presented in this chapter presents evidence that asymmetric stresses in particular are a driving force for bilayer instability, as has been hypothesised in previous literature [27, 39]. Asymmetric MCMG systems all experienced the flipping of MG to the lower leaflet, at rates inversely related to MG tail length. This flip-flop to the lower leaflet increased the mechanical equilibrium between the two leaflets, therefore reducing the asymmetry of the stress exhibited on the upper leaflet of the bilayer. This also aligns with observed differences between asymmetric and symmetrically loaded MG systems, where properties were less disrupted in systems where asymmetry between leaflets was completely relieved, therefore indicating that asymmetric stress relief may reduce the extent of destabilisation across the bilayer system. Additionally, the movement of MGs to the lower leaflet would reduce the number of molecules with mismatched geometry to POPC in the upper leaflet, therefore reducing the extent of curvature induction the leaflet experienced. In replicates of 200 MG-containing bilayer systems where disruption of bilayer planarity

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was not persistent, mass flip-flop of MG to the opposite leaflet of the membrane was observed, occurring roughly at the same time as significant induced curvature in the bilayer. Both this process and budding of a micelle-like aggregate structure from the membrane could be classified as asymmetric stress relief mechanisms, as the bilayer became more stable following these events occurring.

C8-MG was found to be much more likely to exit the bilayer than flip to the lower leaflet, which may explain why the 200GMC_A system saw budding off from the upper leaflet, while other MCMG buds instead either remained tethered to the leaflet, or were relieved by flip-flop towards the lower leaflet. By simulating a state of equilibrium between the two leaflets in terms of MG count it was determined that this state is associated with a reduction in disruption of membrane properties, tending towards control values. While this does indicate that bilayers have the ability to relieve the damage caused to their properties by MGs, at high concentrations it was still apparent that lasting differences to all membrane properties remained in comparison to the control, indicating changes caused by MG insertion cannot be fully reversed while MGs remain in the bilayer structure, as MGs still disrupt phospholipid packing within the bilayer. Due to the shorter tail of C8-MG, monomers have weaker hydrophobic interactions with the bilayer than C10-MG and C12-MG, and therefore these molecules likely have lower energy barriers for leaving the bilayer as they are less anchored into the structure. This may also influence why C8-MG was more likely to exit the bilayer than cross through the hydrophobic core of the bilayer to flip-flop to the lower leaflet.

Notably, while it appears that the disruptive effects of MGs is inversely correlated with MG tail length, experimentally it has been reported that C10-MG has greater antimicrobial efficacy than C8-MG. There are several possible reasons why this is not reflected within these simulations. Firstly, the methodology employed for this chapter utilises an equal number of MGs for each species, which may not be accurate experimentally, as C8-MG has a higher CMC threshold and higher minimum antimicrobial concentration than longer tail MGs, indicating micelles containing C8-MG are less stable, and therefore micelle interactions with a lipid bilayer may not deliver as many molecules of C8-MG as longer MCMGs threshold. [34] Similarly,

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C8-MG was the least stable MG within the bilayer, experiencing by far the most exit events, and displayed a preference for exiting the bilayer over flip-flopping to the other leaflet. Given the short timescale of these simulations, this may suggest that bilayers C8-MG induced disruption can be relieved quicker than larger MGs, preventing longer term destabilisation of the bilayer. Geometrically, due C8-MG having the most extreme hydrophobic mismatch with the lipid bilayer for the three MCMGs it makes sense physically that these molecules produced the greatest disruption in the bilayer.

In the context of the experimental hypotheses that were set out to be evaluated using MD in this chapter, the following aspects of the mechanism can be further elucidated upon by the findings of this chapter. Antimicrobial MGs only being reported to cause disruption above CMC thresholds aligns with the disproportionate increase in bilayer disruption at higher MG concentrations observed in these simulations. As these MGs were located within a small modelled section of the bilayer, this may also suggest that a high localised concentration of MG within a region of the bilayer can be linked to a high degree of disruption. As discussed above, C8-MG appeared to cause more bilayer disruption than C10-MG and C12-MG at equal concentrations over the timescale simulated despite having low experimentally reported antimicrobial efficacy, which aligns with C8-MG having the most extreme conical geometry of the three MCMGs. This could suggest that lower concentrations of C8-MG are delivered to bilayers via LNPs than C10-MG or C12-MG experimentally, or that due to having lower stability within the bilayer, C8-MG molecules do not remain in the bilayer long enough to cause significant damage to the membrane.

Strong evidence of the hypothesised importance of asymmetric stress generated by MG insertion in driving the deformation of lipid bilayers can be seen through the data presented in this chapter. Simulation of equal MG concentrations at differing distributions between the two bilayer leaflets revealed differences in the behaviour between these systems, with symmetrical systems displaying greater stability and properties closer to the control system than asymmetrical MG systems did in comparison to the control. Additionally, strong curvature and budding was not observed in asymmetrically loaded systems, which suggests that reducing the

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MG concentration within a specific leaflet likely reduces the extent of disruption exhibited in the bilayer. Furthermore, two potential relief mechanisms to the stresses that accumulate across a leaflet due to MG insertion were also identified at high concentration. These were the expulsion of MGs, the formation of buds on the upper leaflet of the bilayer, and mass flip-flop of MG to the lower leaflet of the bilayer. This therefore gives insight into the methods bilayer can use to reach states similar to the created equilibrium systems in response to MG-associated destabilisation, and how specific mechanisms may be preferred in response to specific MG geometries.

Limitations of Study

Finally, there are some caveats regarding this presented work. As discussed, the models used in this chapter specifically focus on the effect of MG concentration within the membrane, rather than concentration within solutions which is more commonly used when referring to MG concentrations experimentally. Therefore, it is difficult to directly compare findings with experimental data on the subject as it does not account for stages involving bilayer interaction with lipid micelles prior to incorporation of MG into the structure. It also does not account for potential differences in localised concentration between different MGs, and could not simulate a large lipid bilayer with high concentration of MG in only one section of the bilayer, as the entire bilayer would not have a uniformly high MG concentration across it in experimental studies.

While the presented approach is useful in elucidating the fundamentals of MG interactions with membranes at a very fine level of detail, larger scale simulation methods such as coarse-graining would allow the study of larger bilayer regions. Coarse grained simulations would facilitate the observation of longer time scale mechanisms, such as membrane budding studied experimentally by Hua *et al.* (2023). In this study, budded vesicles displayed a diameter of 20-40 nm, 2-4 times the xy dimensions of systems investigated in this study. [285] Larger scale simulations such as CG could be used to allow such interactions, and potentially earlier phases of micelle interaction with the membrane, to be observed. However this comes at a cost of a loss of detail in the behaviour investigated in here. The information provided in this chapter may

therefore be used as a baseline to allow future longer scale simulations to be conducted.

Some questions could be raised regarding how MGs are inserted into the bilayer and the MG counts utilised in this study. As discussed in the previous chapter, MGs were inserted directly into the bilayer prior to the simulation in order to allow better control of initial MG count and location within the bilayer. While this was done to create a system analogous to a high localised MG concentration in a region of the bilayer as might be found following interaction with a micelle, this strategy does not necessarily correlate to MG insertion dynamics into real cell membranes, as MG monomers insert into the bilayer individually rather than all at once, which is not fully comparable to conditions present in these systems, as during equilibration the initial bilayer model was forced to respond to the stress of all inserted molecules at once. Additionally, very high MG counts are present in later systems in this chapter. Micelles are normally composed of 50 - 100 surfactant molecules, so 100 molecules in the bilayer would represent a very high MG count, and 200 MG within a region of the bilayer of this size is likely unrealistically unachievable experimentally without prior deformation of the bilayer occurring. Despite this to an extent casting doubt on whether these exact behaviours could be observed outside of *in silico* environments, high MG counts allowed for significant disruption in the systems to be captured on simulation timescales, in particular details such as the micelle-like NP structure formations observed in 200 MG containing systems which would likely not appear at a sub-microsecond timescale otherwise.

There are some inherent limitations with the model used for the lipid bilayer in these simulations. Being exclusively composed of POPC, an argument can be made that these findings do not give any direct insight into the antibacterial applications of MGs as this bilayer model is most comparable to mammalian cell membranes. In particular, it does not account for the negatively charged lipids in bacterial lipid membranes. This is directly addressed in the models utilised in the next chapter. Additionally, the lack of membrane proteins - which could not be included due to system size and were not considered vital due to MGs being reported experimentally to be primarily associated with lipid bilayer destabilisation - meant that no knowledge regarding how MGs may

affect protein mediated processes in the cell membrane could be gained. Despite the absence of specific details in the model, these results still ultimately have generated novel insight into fundamental aspects of MG behaviour within bilayer models, and can be utilised as a foundation for more complex systems going forwards.

5.5 Conclusions

The results presented from this series of all-atom MD membrane simulations provide novel insight into the dynamics of antimicrobial MG interactions with membranes and improve our understanding of mechanisms previously identified experimentally. Summarising, there are several key factors crucial for understanding MG interactions with lipid membranes.

- Membrane disruption by MGs increases alongside their concentration within the leaflet. The extent of disruption detected in these systems does not appear to be a linear progression correlated with MG:lipid ratio, which may indicate a minimum threshold of local MG concentration within a region of the bilayer above which the stress placed on the bilayer by MG insertion exceeds the system's capacity to tolerate, leading to significant perturbation, such as the curvature induction and budding observed in some systems.
- The length of the MG chain plays an important role in membrane disruption. Shorter chain MCMGs have greater antimicrobial efficacy, which aligns with these molecules displaying the most conical geometry in comparison to C18-MG, suggesting the hydrophobic mismatch within the bilayer system generated by these molecules likely plays an important role in their antimicrobial efficacy. No outright membrane fission was observed, which in part may be an artefact related to the size of the simulation box or too short a simulated timescale.
- C8-MG addition resulted in the most significant disruption of bilayer properties of the three MCMGs. This aligns with C8-MG having the most conical geometry and highlights that differences in antimicrobial potency observed between MGs of

different tail lengths is not directly correlated with the extent of bilayer disruption produced when present at equal concentrations within the bilayer.

- Changes to membrane thickness and fluidity appear to be factors influencing the destabilisation of bilayer properties. In particular, MGs with known antimicrobial potency consistently influence bilayer behaviour to tend towards liquid-disordered phase-like properties. These are variables which have been observed to change in similar experimental work in response to MG exposure.
- Bilayer stress generated by MG-induced asymmetry seems to be a driving force in bilayer destruction. This is in line with previous hypotheses that significant membrane deformations caused by amphiphile insertion are reduced by flip-flop of amphiphiles to the inner leaflet of the membrane. [29, 191] The simulations presented here additionally indicate that while an ideal equilibrium state is much more stable, disruption of membrane properties in comparison to an untreated control remains, therefore suggesting that while the asymmetric insertion of MGs amplifies bilayer deformation, there remains a baseline stress from MG insertion that alters bilayer properties even if asymmetric stress in the bilayer is entirely absent.

While these findings provide interesting insight into the actions of MGs, further investigation using various computational and experimental methods may allow better understanding of MG induced bilayer disruption to further understand its mechanisms.

Chapter 6

Destabilising Interactions Between Monoglycerides and Bacterial Cell Membrane Models

6.1 Rationale

Although the POPC bilayer systems studied in the previous chapter provide excellent mechanical insight into the ways that monoglycerides influence the destabilisation of a lipid bilayer, and allow deeper understanding of existing experimental hypotheses, these cannot be directly treated as evidence towards understanding the antibacterial mechanisms of MGs. This is primarily because the POPC bilayer model used for the study is not strongly reflective of the lipid composition of bacterial cell membranes.

This chapter presents a second study, which aims to better replicate the cell membrane component of bacterial cell envelopes by employing the use of lipid bilayers with compositions which more accurately reflect bacterial membranes in order to better understand how lipid bilayer destabilisation can be linked to the antimicrobial mechanisms of MGs. The interactions between medium chain MGs C8-MG, C10-MG, and C12-MG with models representing the Gram-positive plasma membrane and the Gram-negative inner membrane respectively were simulated and analysed in order to understand the similarities and differences of how these models may respond to MG

insertion.

6.2 Chapter Aims

1. Accurately simulate lipid bilayer models for the Gram-negative inner membrane, and the Gram-positive plasma membrane.
2. Identify key differences and similarities between monoglyceride-induced disruption of the three membrane models used in this chapter and the previous.

6.3 Methodology

6.3.1 System Setup

The initial lipid bilayer structure for each system in this chapter was generated using the CHARMM-GUI membrane builder. [239]. Two different phospholipid ratios were used to represent the G+ bacterial plasma membrane (G+PM) and G- bacterial inner membrane (G-IM) respectively. Lipid species used to construct these model systems are listed in Tables 6.1 and 6.2. Lipids selected for this study were chosen based on models constructed and validated as accurate representations of the lipid bilayer component of the G+ and G- bacterial cell membrane by Pogozheva *et al.* (2022). Moreover, all lipid species were available for bilayer construction within the CHARMM-GUI Membrane Builder [1].

Control systems containing no MG were created for both models. MG was inserted into bilayers during system construction in CHARMM-GUI as C18-MG asymmetrically inserted into the upper leaflet. MGs were then shortened to the required length using an in-house Tcl script to C8-MG, C10-MG, and C12-MG. MG were inserted into the upper leaflet at a count of 100, 150, and 200, and 50, 100, and 150 for the G-IM. Additionally, a further 6 systems were created using POPC to allow direct comparisons at these MG concentrations. These systems followed the same protocol and had 50 or 150 molecules of each MG inserted solely into the upper leaflet. Data collected in the previous chapter for asymmetric POPC/MG systems with 100 and 200 molecules in

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Table 6.1: Bilayer composition of the Gram-positive bacterial plasma membrane model system (G+PM). Lipid counts were identical between the outer and inner leaflets. X(Y/Z) represents the structure of the head group (X) and the Sn1 (Y) and Sn2 (Z) lipid tails, respectively. PAICL2 has two acyl tails at the 1' and 3' positions. PG = phosphoglycerol, PE = phosphatidylethanolamine. CL = cardiolipin.

Lipid Name	Lipid Head/Tails	Count Per Leaflet	Charge
MAIPG	PG(14:0/a15:0)	22	-1
AIPG	PG(a15:0/i15:0)	27	-1
PAIPG	PG(16:0/a15:0)	9	-1
DPPG	PG(16:0/16:0)	7	-1
MAIPE	PE(144:0/a15:0)	9	0
AIPE	PE(a15:0/i15:0)	11	0
PAIPE	PE(16:0/a15:0)	4	0
DPPE	PE(16:0/16:0)	3	0
PAICL2	CL(1'-[16:0/a15:0],3'-[16:0/a15:0])	8	-2
Leaflet Charge			-81 e

Table 6.2: Bilayer composition of the Gram-negative inner membrane model system (G-IM). Lipid counts were identical between the outer and inner leaflets. X(Y/Z) represents the structure of the head group (X) and the Sn1 (Y) and Sn2 (Z) lipid tails, respectively. N:1(XZ) represents a double bond present in the acyl chain at the carbon X along position. cy indicates the presence of a cycloacyl group in the acyl chain. PVCL2 has two acyl tails at the 1' and 3' positions. PG = phosphoglycerol, PE = phosphatidylethanolamine. CL = cardiolipin.

Lipid Name	Lipid Head/Tails	Count Per Leaflet	Charge
PMPE	PE(16:0/cy17:0)	46	0
POPE	PE(16:0/18:1(9Z))	13	0
QMPE	PE(15:0/cy17:0)	12	0
OYPE	PE(18:1(9Z)/16:1(9Z))	8	0
PMPG	PG(16:0/cy17:0)	10	-1
PYPG	PE(16:0/16:1(9Z))	9	-2
PVCL2	CL([16:0/18:1(11Z)], [16:0/18:1(11Z)])	2	-2
Leaflet Charge			-32 e

the bilayer were also utilised in this chapter to draw specific comparisons. Table 6.3 reports the models and MG counts used in each system.

The CHARMM36m all-atom forcefield was used to conduct all simulations. [208] Additional parameters for C8-MG, C10-MG, and C12-MG were used due to their absence in the CHARMM36m FF. The creation of these parameters was detailed in the previous Chapter, and these parameters are available in the SI (Appendix B).

All systems were solvated by adding 40 Å of TIP3P water either side of the membrane along the z axis while NaCl ions were added to set the solution concentration

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Table 6.3: Relevant System information. Bilayer model indicates the composition of the lipid bilayer. G+PM and G-IM indicates the use of the Gram-positive plasma (Table 4.2) and Gram-negative inner (Table 6.2) bacterial membrane model lipid compositions respectively. POPC indicates 100 molecules of POPC per leaflet. MG Count represents starting MG count in the upper leaflet.

System Name	Bilayer Model	MG Species	MG Count
CTRL_GPPM	G+PM	None	0
GTRL_GNIM	G-IM	None	0
GPPM_100_GMCY	G+PM	C8-MG	100
GPPM_100_GMC	G+PM	C10-MG	100
GPPM_100_GML	G+PM	C12-MG	100
GPPM_150_GMCY	G+PM	C8-MG	150
GPPM_150_GMC	G+PM	C10-MG	150
GPPM_150_GML	G+PM	C12-MG	150
GPPM_200_GMCY	G+PM	C8-MG	200
GPPM_200_GMC	G+PM	C10-MG	200
GPPM_200_GML	G+PM	C12-MG	200
GNIM_50_GMCY	G-IM	C8-MG	50
GNIM_50_GMC	G-IM	C10-MG	50
GNIM_50_GML	G-IM	C12-MG	50
GNIM_100_GMCY	G-IM	C8-MG	100
GNIM_100_GMC	G-IM	C10-MG	100
GNIM_100_GML	G-IM	C12-MG	100
GNIM_150_GMCY	G-IM	C8-MG	150
GNIM_150_GMC	G-IM	C10-MG	150
GNIM_150_GML	G-IM	C12-MG	100
POPC_50_GMCY	POPC	C8-MG	50
POPC_50_GMC	POPC	C10-MG	50
POPC_50_GML	POPC	C12-MG	50
POPC_100_GMCY	POPC	C8-MG	100
POPC_100_GMC	POPC	C10-MG	100
POPC_100_GML	POPC	C12-MG	100
POPC_150_GMCY	POPC	C8-MG	150
POPC_150_GMC	POPC	C10-MG	150
POPC_150_GML	POPC	C12-MG	150

to 0.15 mol/L. As negatively charged lipids were present in the bilayer, an additional 162 Na^+ ions were added to G+PM model systems, and 64 Na^+ ions were added to the G-IM model to neutralise the net negative charge of the bilayer. All systems underwent an initial NVT simulation for 10 ns to undergo energy minimisation, using colvar restraints which were reduced in strength as the simulation continued. The CHARMM-GUI equilibration protocol was followed, conducting the equilibration in 6 steps with progressively reducing restraint strength in each step. Following this, the NPT ensemble was used for 100 ns of equilibration and 500 ns of production runs. The

production trajectories were ran at laboratory conditions with constant temperature (298.15 K) and pressure (1.01325 bar) with a 2 fs time step. A 12Å vdW cut-off and PME were employed.

All models were simulated for 600 ns each with the first 100 ns used for equilibration and to allow relaxation of the membrane system. This 100 ns period was initially excluded from data analysis presented below, and further explanation of why this cut-off was selected is described in *Equilibration of systems*, though it was necessary for some analysis to be performed within this 100 ns threshold due to notable behavioural changes occurring to bilayers at this time. All simulations were performed using NAMD3 CUDA version. [211]

6.3.2 Analysis

Pre-preparation of trajectories for analysis was performed using an in-house Tcl script (Appendix C) which centred the bilayer in the middle of the simulation box. Area per lipid (APL) was calculated using VMD membrane analysis tool MEMBPLUGIN version 1.1.32. [242, 259] using the atoms C2, C1 and C3 for each membrane phospholipid and C3 for each MG. To calculate average bilayer thickness the difference in average z-coordinate of the phosphorous atoms in the upper and lower leaflets respectively was used. The 2D thickness across the xy plane was calculated every 20 steps of the trajectory using the membrane thickness map function of MEMBPLUGIN version 1.1.32. Lipid tail order parameters (S_{CD}) were calculated for the Sn1 chain of DPPG for the G+PM model and POPE for the G-IM model every 50 steps of the trajectory. These lipids were selected due to being the most abundant lipids in each models with a 1-palmitoyl tail, to maintain consistency with POPC, which were compatible with MEMBPLUGIN. Partial density profiles for each system were calculated using the Density Tool plugin in VMD. [258] Partial density profiles were calculated for the water, ions, phospholipids, and MG for the upper and lower leaflets individually for each system along the z-axis with 1 Å spacing between bins and were calculated every 50 steps of the trajectory. All data was processed and visualised using Python.

6.4 Results

6.4.1 Model Validation

As both bacterial phospholipid bilayer models used for this chapter had been previously published in literature, it was essential that the constructed models produced for using in this series of simulations accurately reflected the characteristics reported by Pogozheva *et al.* (2022). Comparisons between these systems are displayed in Table 6.4.

Table 6.4: Bilayer parameters for control systems. Pogozheva indicates values taken from Pogozheva *et al.* (2022) [1] Thickness represents the average distance between P atoms in the upper and lower leaflet of the bilayer. APL represents the average area taken up by each lipid across the xy plane. S_{CD} is the alignment of C16:0 acyl tails of phospholipids with the membrane normal. Water penetration indicates the closest position to the membrane centre $z=0$) of water molecules present along the z axis.

System	Source	Thickness (Å)	APL (Å ²)	S_{CD}	Water Penetration (Å)
G+PM	Pogozheva	37.0	61.5	0.23	-9.2/9.2
G+PM	This chapter	37.9	64.3	0.19	-9.5/9.5
G-IM	Pogozheva	38.3	64.0	0.22	-10.3/10.3
G-IM	This chapter	39.0	62.4	0.18	-10.5/10.5

Metrics for these systems were generally in good agreement with reported literature values, indicating that the control systems used for this study were accurately simulated. There was some variance from values reported by Pogozheva *et al.* for all metrics, however this could be explained with differences in simulation setup, as reported literature values were collected from a simulation conducted with a constant temperature of 310.15 K while simulations in this chapter were ran with a constant temperature of 298.15 K. Simulations were also produced using OpenMM rather than NAMD, which may have caused some differences in calculations.

6.4.2 Gram-Positive Plasma Membrane

Impact of Monoglyceride Structure

As displayed in the previous chapter, differences the tail length of saturated MCMGs can produce changes in the degree of property disruption produced in a POPC lipid

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bilayer at high concentrations. Therefore, identifying whether these effects could also be observed in bilayer models that more accurately represent bacterial membranes is important in providing evidence that the hypotheses investigated in model bilayer systems in the previous chapter regarding biological membrane disruption could also be applied to antimicrobial contexts. The three MCMGs used in the previous chapter (C8-MG, C10-MG, and C12-MG) were pre-inserted into the upper leaflet of the membrane of both the Gram-positive bacterial plasma membrane and Gram-negative inner membrane models to investigate this. As 100 MG was the count used in the previous chapter that showed the greatest changes to bilayer properties without inducing deformation of the upper leaflet, this was chosen as the starting MG count for these systems in order to analyse the influence of MGs on bacterial bilayer models. The thickness values of the membrane systems are listed in Table 6.5. Reported thickness values indicate a trend consistent with those reported in the previous chapter - that C8-MG produces more significant thinning of the bilayer than C10-MG or C12-MG. The difference in thickness was not significantly different between MGs at this concentration, though it was significantly different in comparison to the control for all three.

Table 6.5: Average bilayer thickness for G+PM systems loaded with 100 MG. Change from control expressed in Å and percentage change are also reported. All values were rounded to 1 decimal point. Brackets represent standard deviation.

	GPPM_100_GMCY	GPPM_100_GMC	GPPM_100_GML	CTRL_GPPM
Thickness (Å)	35.2 (0.5)	35.6 (0.5)	36.3 (0.5)	37.9 (0.5)
Change (Å)	-2.7	-2.3	-1.6	0
% change	-7.1%	-6.1%	-4.2%	0%

The lack of significant difference between the three MCMG systems in disruption of the G+PM model was also reflected in their density profiles (Figure 6.1), as no significant differences between membrane lipid headgroup positioning was observed, although the extent of the headgroup peak differed between the three MCMGs, with the C12-MG containing system displaying the most pronounced upper leaflet peak of the three. In each system the density profiles showed disruption of membrane lipid positioning across both leaflets, but particularly in the upper leaflet, which indicated

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that the phospholipid packing disruption observed in the previous chapter remains present in the G+PM model. All lipid species were shown to be affected by this disruption, therefore indicating that bilayer packing disruption by MGs is not biased towards specific lipid species within these systems. As is discussed later in this chapter, Na^+ ions accumulated in high concentration close to the lipid-water interface due to the negatively charged headgroups of membrane lipids, which was not observed in the POPC density profiles due to POPC being a zwitterionic phospholipid. Therefore, it can be observed from these density profiles that similar disruption to membrane lipid packing within the bilayer caused by MGs discussed in the previous chapter was also observed within the more complex, G+PM analogous model.

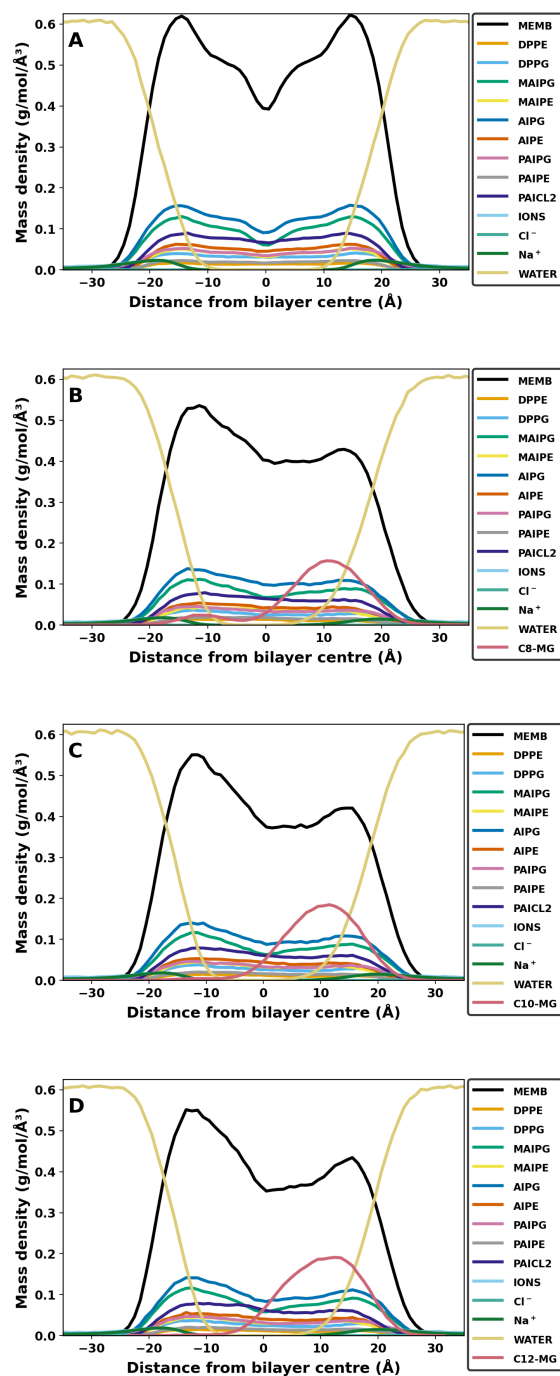


Figure 6.1: 1D average density profiles along the z-axis for control (A), C8-MG (B), C10-MG (C), and C12-MG (D) containing G+PM systems. "MEMB" represents the density of the phospholipid bilayer excluding added MG.

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Deuterium order parameters for systems at a given concentration identify differences to the fluidity for each leaflet (Figure 6.2). Upper leaflet C12-MG caused the most significant increase in order, and therefore most significant reduction in fluidity at a 50% increase across most of the chain. Likely this is due to the longer tail of C12-MG causing more rigidity towards the hydrophilic heads of the chains. The lower leaflet in comparison was very disordered, at a greater than 50% decrease at carbons 6-8 and all sitting around 0.1, where 0 is completely disordered and 0.5 is perfectly ordered along the bilayer normal. Overall, all three systems displayed reduced order at these concentrations, which is in line with what was observed in the previous chapter.

This overall decrease in fluidity can be observed in the depth of water penetration for the systems, where water was present in the bilayer up to 10 Å from the centre of the bilayer, compared to the -14 Å seen in the control (Figure 6.3, Table 6.6). These systems also had an observable increase in water penetration in the upper leaflet compared to the control, though this slope was much more gradual below a mass density of 0.2 than in the lower leaflet, which followed the same slope as in the control.

Table 6.6: Depth of water penetration values for 100 C8-MG, C10-MG, and C12-MGs containing G+PM systems. All values were rounded to 1 decimal point.

System	Water Penetration (UL) (Å)	Water Penetration (LL) (Å)
Control	9.2	9.2
GPPM_100_GMCY	4.5	7.5
GPPM_100_GMC	5.5	8.5
GPPM_100_GML	6.5	8.5

The ion density profiles identify no significant changes in Cl^- ions between MG-loaded systems (Figure 6.4). A much higher number of Na^+ ions were present in the G+PM and G-IM to neutralise the density of negatively charged lipid species, comparatively. In the control system, Na^+ ions were most highly concentrated at the head group region of the membrane at approx +/- 20 Å and peaks were had an equal size across the upper and lower leaflet headgroup regions. In systems where MG was present, the height of these peaks were lower on both ends, but especially the upper leaflet where MG was present, suggesting that the presence of MGs alters the accumulation of Na^+ ions at both leaflets. Additionally, the peak on the lower leaflet

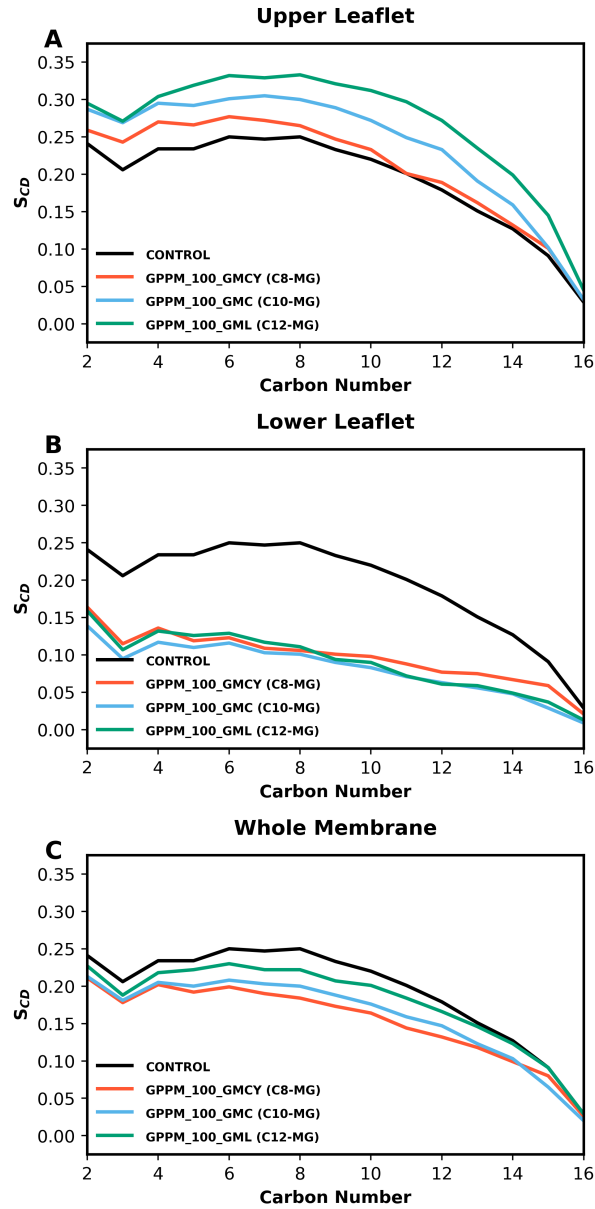


Figure 6.2: Deuterium order parameters for 16:0 acyl tail of phospholipid DPPG in G+PM systems with added C8-MG, C10-MG, and C12-MGs for the upper leaflet (A), lower leaflet (B), and both leaflets (C).

was about 1 Å closer to the centre, aligning with the compression of membrane leaflets being more weighted towards a reduction in thickness in the lower leaflet. Of note, the outer leaflet peak was reduced from 0.023 g/mol/Å³ to 0.015g/mol/Å³ in MG loaded systems. This suggests Na⁺ ion accumulation at the upper leaflet of leaflet perturbed

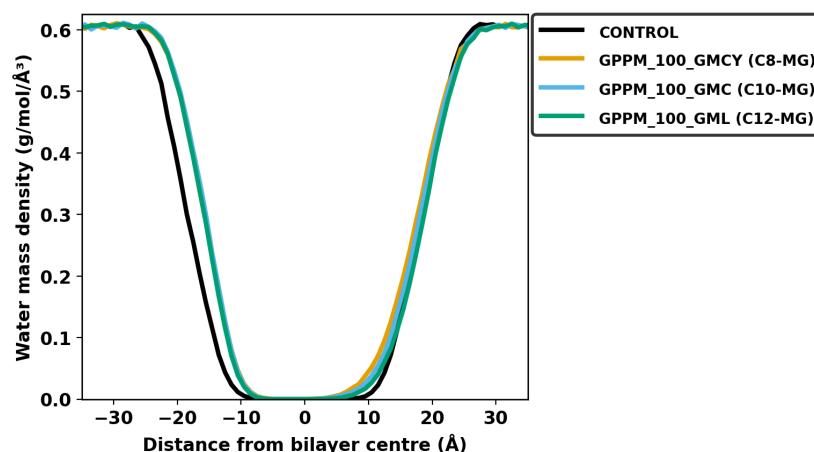


Figure 6.3: 1D average water mass density profile along the z-axis of water molecules for 100 C8-MG, C10-MG, and C12-MGs containing G+PM systems.

by MGs had a less uniform accumulation at headgroups, which was reflected in the greater penetration of Na^+ ions towards the membrane centre in the upper leaflet. The reduction in peaks may also be due to the lateral expansion of both bilayer leaflets while the number of Na^+ ions stays the same, so density is lower. This did not affect the density of Cl^- ions due to these ions in bulk solution not interacting with the bilayer, while Na^+ ions were attracted to the negative phospholipid headgroups of the bilayer.

From this data it can be gathered that all MCMGs trialled in the previous chapter also have the capability to produce disruption of bilayer characteristics in models that more accurately represent the G+ plasma membrane. Observed trends also indicate that, as in the previously investigated POPC model in the previous Chapter, C8-MG loaded systems displayed the most significant disruption of bilayer characteristics, while systems containing C12-MG displayed the least disruption of the three MGs. This suggests that the evidence presented in the previous chapter regarding the importance of MG structure on disruption of bilayers can likely be applied to understanding MCMG mechanisms of antimicrobial activity.

Impact of Monoglyceride Concentration

Following investigation of whether trends in the influence of MG structure efficacy could also be observed in lipid bilayer models more closely representative of bacterial

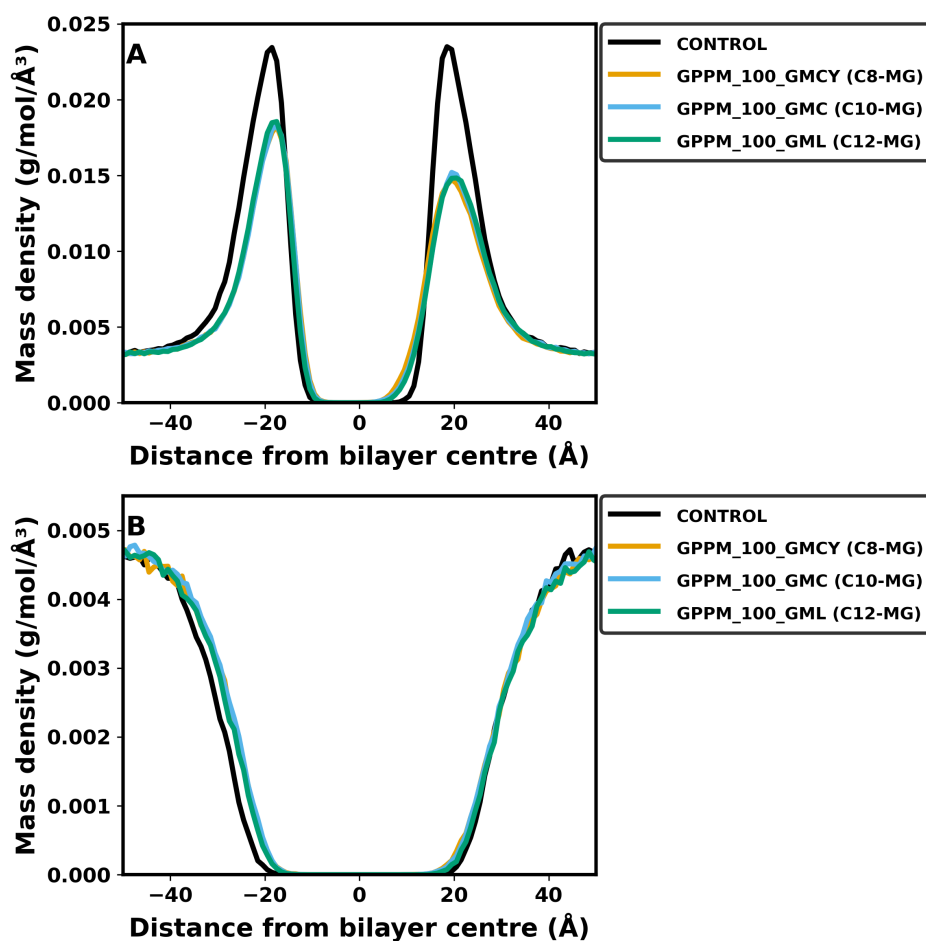


Figure 6.4: 1D average mass density profile of Na⁺ (A) and Cl⁻ (B) ions for 100 C8-MG, C10-MG, and C12-MGs containing G+PM systems.

compositions, how the concentration of MG may influence the extent of disruption was also investigated. As noted in the Methodology section, 150 and 200 of each MG added asymmetrically was explored for the G+PM. As a whole, G+ membrane systems displayed high planar stability across all concentrations tested, with the exception of GPPM.200.GMCY. The loss of planar bilayer integrity was observed only in the system GPPM.200.GMCY, as shown in Table 6.7.

The beginning stages of bilayer planarity disruption could be observed almost immediately following the initialisation of the unrestrained production run for GPPM.200.GMCY, characterised by an initial buckling of the bilayers at the lipid centre and formation of a globular structure composed of both C8-MG and membrane

Table 6.7: Integrity of bilayer planarity in G+PM systems. "Yes" indicates a system where planarity was disrupted. "No" indicates a system where planarity remained throughout the simulation.

MG Concentration	C8-MG Disruption	C10-MG Disruption	C12-MG Disruption
100	No	No	No
150	No	No	No
200	Yes	No	No

phospholipids on the upper leaflet (Figure 6.5). The membrane structure continued to increase in size until around 50 ns, when the buckling of the membrane was relieved and the rest of the bilayer, other than the aggregate structure resting on top, returned to a planar state with low induced curvature. The structure was loosely tethered to the upper leaflet throughout the remainder of the simulation, taking on a spherical morphology which could be considered similar to a mature bilayer bud prior to scission. Some expulsion of MGs into the water box was also observed in this system, with the majority of molecules that left the bilayer doing so from the bud, which suggested that MGs are less stable within the bud than in the membrane. This structure could be interpreted as a small-scale analogue to formation of membrane budding, or tubule formation on the cell membranes which has been observed experimentally [27].

Reported thickness values for each system are listed in Table 6.8, while system percentage change is listed in Table 6.9. It was consistent for each MG that remained planar that a reduction in thickness in comparison to the control was observed when MGs were inserted into the structure. For each system there was a decrease in thickness between the concentrations of 100 and 150 molecules in the upper leaflet. The reduction was not identical between the three MGs, with C8-MG causing a 1 Å reduction, compared to 0.6 Å for C10-MG and 0.8 Å for C12-MG. Additionally, at 200 MG, when significant disruption was observed in C8 MG, no thickness values calculated continued the expected further decrease in thickness, instead staying the same for the system containing C10-MG and increasing for the systems containing C8-MG and C12-MG respectively. For C8-MG, this value was likely due to the method that the MEMBPLUGIN membrane thickness tool uses to calculate the average thickness of bilayers. Because bilayer thickness is calculated as the average difference in space along

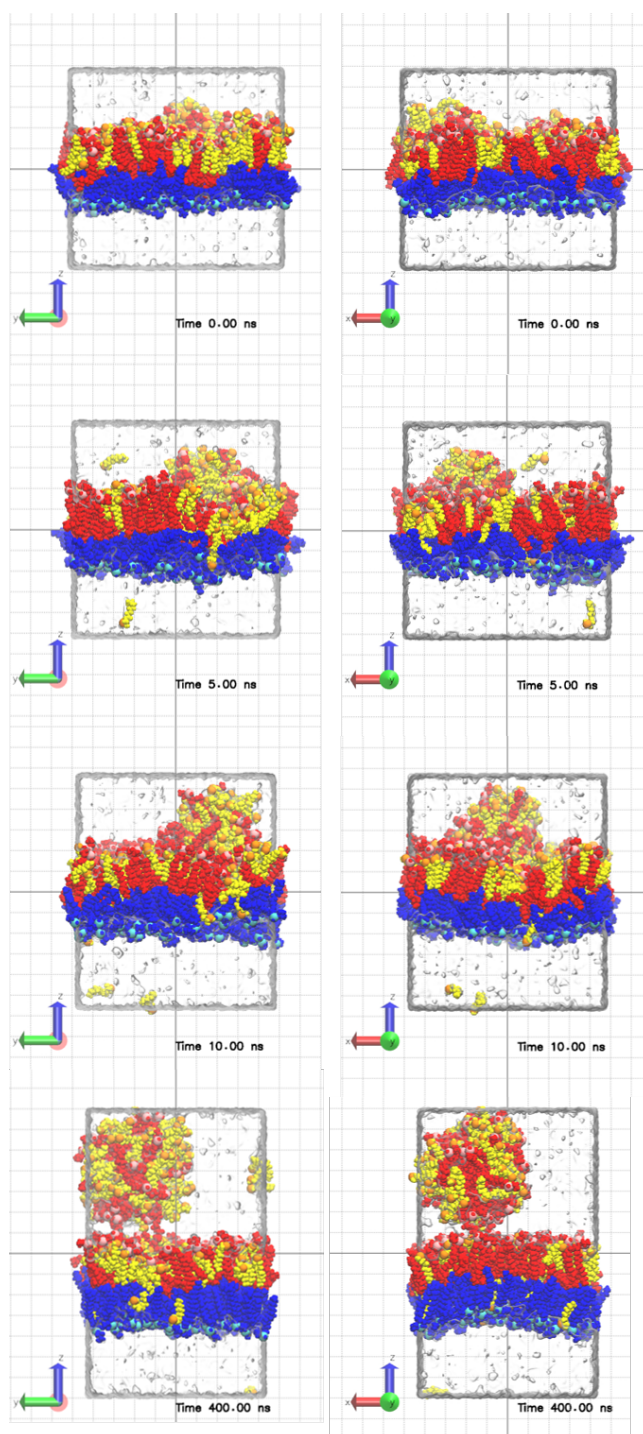


Figure 6.5: Bud formation formation on G+PM model lipid bilayer containing 200 molecules of C8-MG inserted into the upper leaflet from two different side on perspectives. Each square represents 1 nm^2 .

the z-axis between phosphorus atoms in the upper leaflet and phosphorus atoms in the lower leaflet, the breaking of lipid bilayer planarity causes the thickness measurements to rapidly increase, inflated by the position of phosphorus atoms in the bud, rather than solely that of the upper leaflet (Fig 6.6).

Table 6.8: Average bilayer thickness of G+PM systems with varying concentrations of C8-MG, C10-MG, or C12-MG added. All values were rounded to 1 decimal point. Brackets represent standard deviation. * marks a system where budding occurred.

MG Concentration	C8-MG	C10-MG	C12-MG	Control
0	-	-	-	37.9 (0.5)
100	35.2 (0.5)	35.6 (0.5)	36.3 (0.5)	-
150	34.2 (0.6)	35 (0.7)	35.5 (0.5)	-
200	49.9 (2.0)*	35 (0.6)	36.1 (0.6)	-

Table 6.9: Percentage change in bilayer thickness in comparison to control for G+PM systems with varying concentrations of C8-MG, C10-MG, or C12-MG added. All values were rounded to 1 decimal point. * marks a system where budding occurred.

MG Concentration	% Change C8-MG	% Change C10-MG	% Change C12-MG
100	-7.1	-6.1	-4.2
150	-9.8	-7.7	-6.3
200	+31.7	-7.7	-4.8

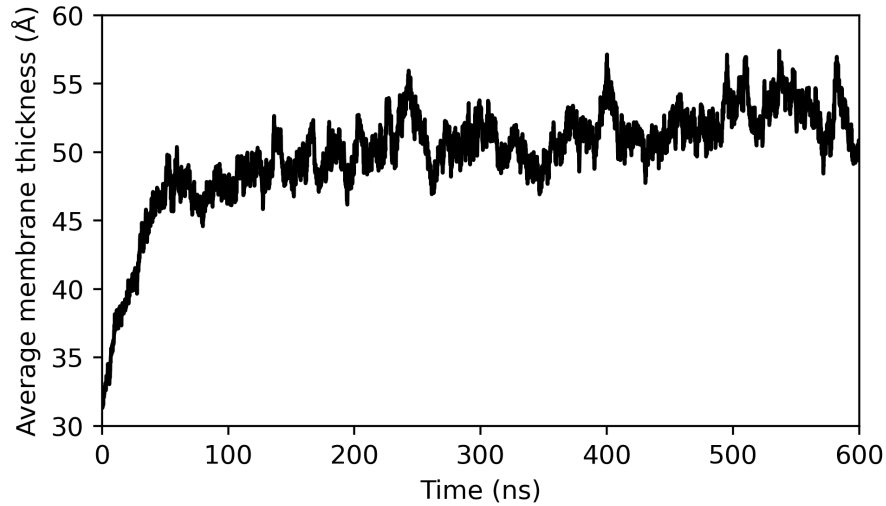


Figure 6.6: Time vs average bilayer thickness along the membrane normal for system GPMM_200_GMCY.

For the observed increase in thickness in the system containing C12-MG, the

mapped thickness over time values reveal a rise in bilayer thickness to around 36.75 Å at approximately 150 ns, which plateaued around this value for the remaining 450 ns of the simulation (Figure 6.7 A). Prior to this point, bilayer thickness was calculated to be around 35 Å. The time point of this leap coincided with significant movement of C12-MG molecules to the lower leaflet of the bilayer (Figure 6.7 B). As discussed in the previous chapter, systems with high MCMG concentration in both leaflets showed lower deviation from control systems across all parameters than systems with MCMG only present in one leaflet. The observed increase in thickness being correlated with the significant movement of C12-MG to the lower, reducing the degree of leaflet asymmetry, this would align with the idea that the relief of asymmetric stresses can reduce the impact of MG mediated disruption.

The behaviour of MGs within the bilayer also differed between concentrations. C8-MG was more likely to exit the bilayer than flip-flop, which was consistent with observations in POPC systems. At a count of 100 MG in the system, 24 of the 100 C8-MG molecules left the membrane at one point during the simulation, while 3 flopped to the lower leaflet. The system GPPM_150_GMCY had a proportionately lower number of C8-MG molecules leave the bilayer at 26 molecules (13%), 8 of which reinserted into the lower leaflet and 18 returned to the upper. Unlike in GPPM_100_GMCY, 32 molecules flipped to the lower leaflet during the simulation, leaving the lower leaflet with a MG count of 40 at the end of the simulation. Outside the aggregate structure which formed in the system GPPM_200_GMCY, movement both into the water box and the lower leaflet was observed. Movement of C8-MG to the lower leaflet occurred simultaneously with the formation of the bud, though more C8-MG molecules exited into the bud than crossed to the lower leaflet. No membrane phospholipids moved to the lower leaflet in this system, but some did get taken into the upper leaflet bud structure.

The C10-MG system displayed more flip-flop events than expulsion events. The system GPPM_100_GMC displayed very little movement in general, with a final MG concentration of 4 molecules in the lower leaflet, two of which flip-flopped, while two exited into the water box then crossed PBCs, re-inserting into the lower leaflet. At

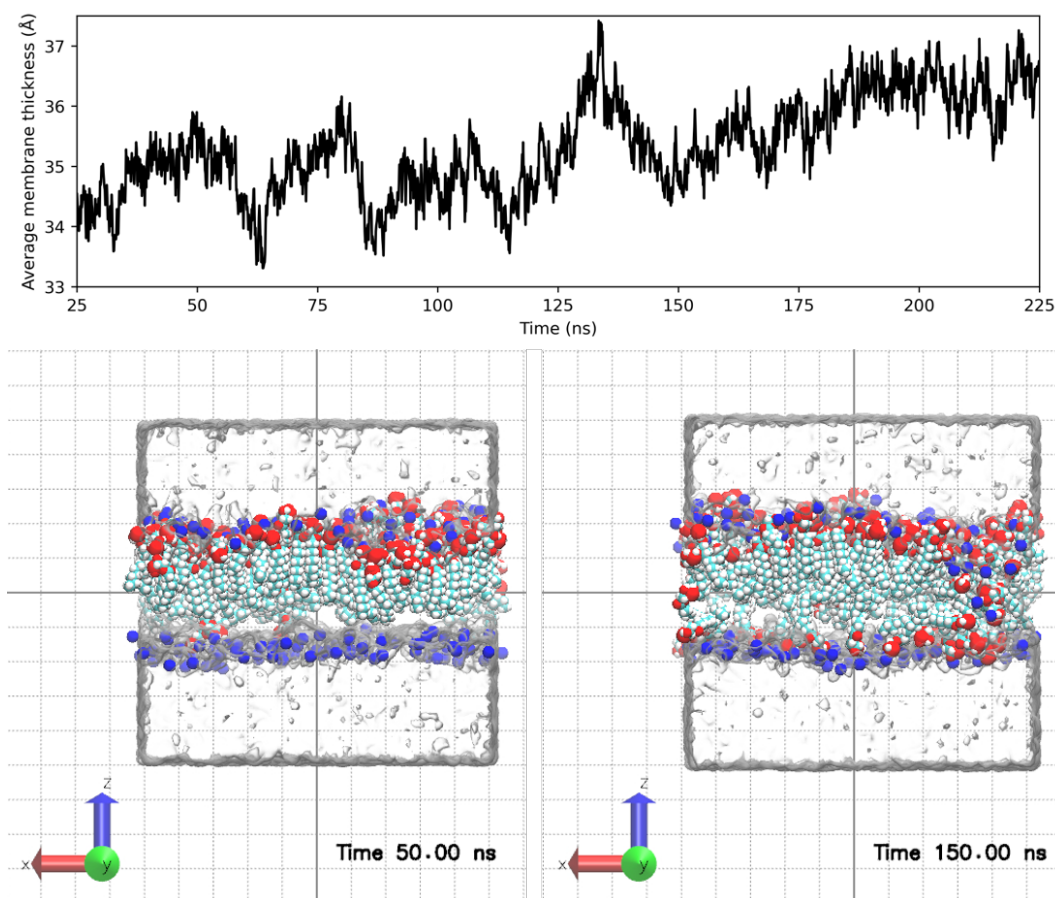


Figure 6.7: A) Time vs average bilayer thickness along the membrane normal for system GPPM.200_GML. B) C12-MG positioning in the lipid bilayer before and after the increase in bilayer thickness shown on bilayer thickness plot, demonstrating significant flip-flop of C12-MG to the lower leaflet.

GPPM.150_GMC, again only a couple molecules exited the bilayer. The first movement of a C8-MG to the lower leaflet was observed at 32 ns in the production run, after which only a few other C10-MG molecules moved to the lower leaflet until approx 350 ns, where 9 molecules flip-flopped to the lower leaflet in rapid succession, occurring around the same time temporary spontaneous curvature formation in the bilayer appeared. Mass translocation to the lower leaflet of C10-MG immediately following curvature induction was also observed in the GPPM.200_GMC system, occurring at 20 ns and 370 ns. Only 4 molecules moved from the lipid bilayer into the water box over the course of the simulation. Three re-inserted into the bilayer structure in the lower leaflet, and

two back into the upper leaflet. Coinciding with negative curvature induction at 10 ns, C10-MG count in the lower leaflet spiked from 8 to 35 molecules, and the curvature at 370 ns did not significantly impact MG count in the lower leaflet.

In the system GPPM_100_GML, no movement of C12-MG into the water box and only one molecule move to the lower leaflet occurred. Movement began at 320 ns and took 30 ns to reorient in the lower leaflet. At a count of 150 C12-MG, one molecule left the upper leaflet into the water box and later reinserted back into the upper leaflet. 13 molecules flip-flopped to the lower leaflet during the simulation. In GPPM_200_GML no molecules left the membrane, however a significant flip-flop to the lower leaflet occurred around 120 ns, following formation of transient spontaneous curvature, where lower leaflet C12-MG count jumped from 5 molecules to 43 over the course of 50 ns.

A consistent behavioural trend for all MCMGs in these systems was that at the highest concentration, 200, all systems had a final lower leaflet concentration in the 35-40 range, though this does not account for the MG content of the bud present in GPPM_200_GMCY. Most movement of MGs across the bilayer occurred in large bursts associated with the formation of curvature in the bilayer, and expulsion of MGs from the bilayer into the water box did not appear strongly correlated with any changes to membrane morphology occurring.

Interestingly, in the GPPM_150_GMCY system production run, at approximately 160 ns, it was observed that a small, transient pore spontaneously formed in the membrane, remaining for 40ns before closing again (Figure 6.8). The maximum diameter of this pore was 20 Å. The movement of sodium ions through the membrane to the opposite side could be seen in the simulation, facilitated by movement through the pore, which stopped as the pore closed. Although the pore had closed from 200 ns onwards, a highly disordered region between leaflets remained in the bilayer structure for a further 100 ns. This led to the formation of a second pore at 250ns, which fluctuated in size until it also closed around 300 ns. After this, no further pores appeared in the bilayer. Several MG molecules moved to the lower leaflet around the time that this was resolved, though it is unclear whether MG movement was associated with the closing of the pore. When pore formation began at 160 ns, 21

Chapter 6. Destabilising Interactions Between Monoglycerides and Bacterial Cell Membrane Models

MGs were present in the lower leaflet, 127 in the upper leaflet, and 2 in the water box. Both pores had a high concentration of MG surrounding the structure, and the time period where the pore was present was associated with high movement of MGs from the upper leaflet to the lower leaflet. Additionally, when a pore formed headgroups of both membrane phospholipids and MG reoriented to face the hydrophilic water channel across both leaflets.

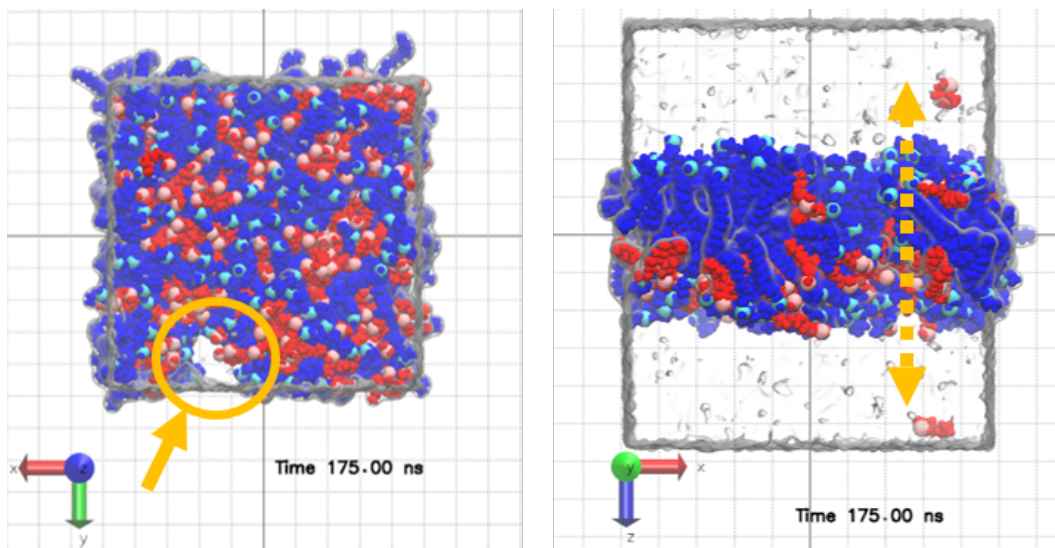


Figure 6.8: Pore formed in a G+PM bilayer model loaded with 150 molecules of C8-MG from two angles. Cyan and pink spheres represent headgroups of membrane phospholipids and C8-MG respectively. Location of Pore indicated in orange.

Deuterium order parameters highlighted that there were not large differences in overall order between MG concentrations, all having similar S_{CD} values across their 16:0 (stearyl) acyl chains (Sn1 tails, Figure 6.9). It was consistent that the lower leaflet displayed a significant decrease in order across all carbons, while the increase in order in the upper leaflet was heavily influenced by the tail length of respective MGs, with C8-MG having little impact on order of carbons towards the end of the Sn1 phospholipid tail. This was also observed in S_{CD} plots in POPC bilayers in the previous chapter. The sudden increase in bilayer thickness towards control values observed in GPPM_200_GML aligned with these order parameters, as the degree of order in the membrane differs less in comparison to the control than the GPPM_150_GML system, which reported lower bilayer thickness.

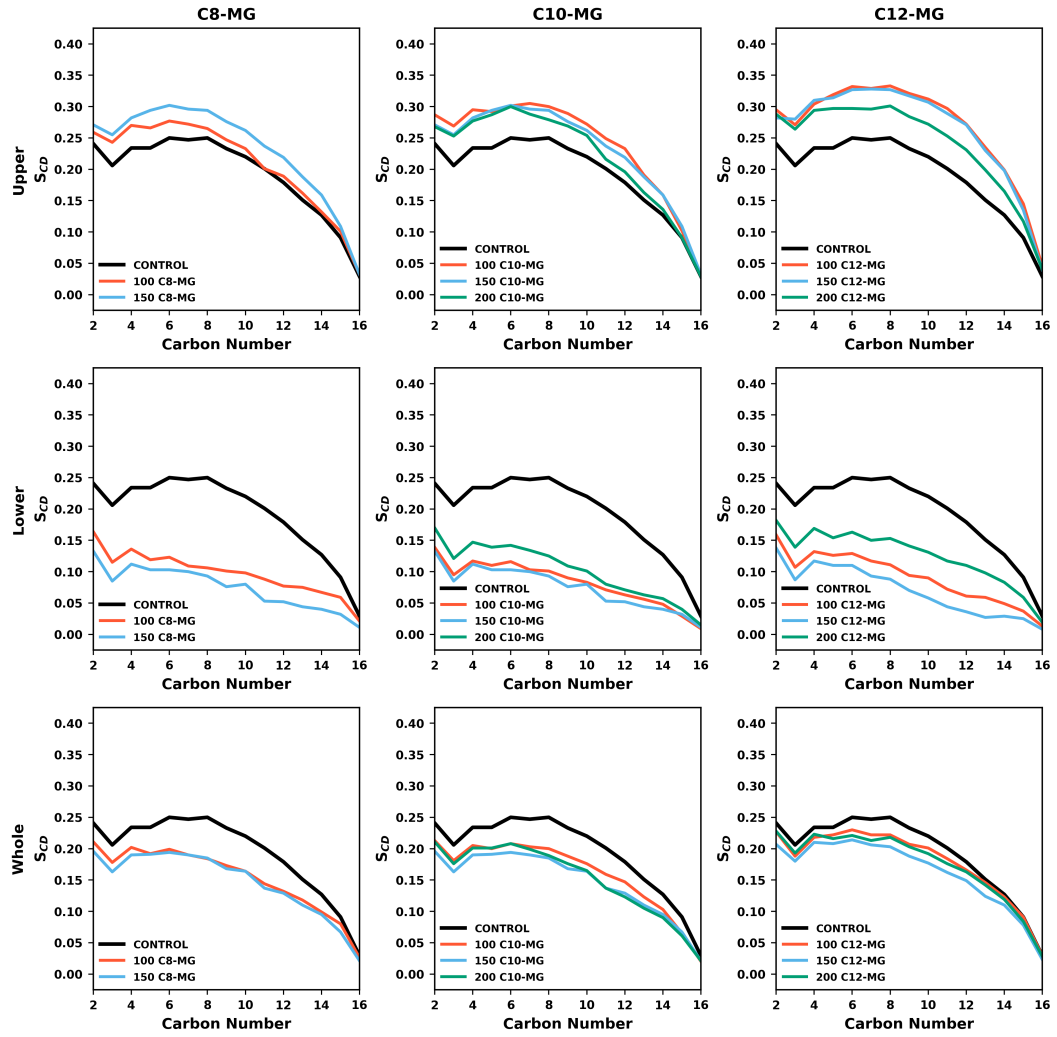


Figure 6.9: Deuterium order parameters for the 16:0 acyl tail of PMPG across the upper leaflet, lower leaflet, and whole bilayer for G+PM systems with varying concentrations of C8-MG, C10-MG, or C12-MG added.

It is evident from the presented results that more significant disruption of G+PM bilayers tended to occur at greater MG concentrations in the upper leaflet, as was observed in POPC systems. At very high disruption of 200 MG in the systems budding was also observed in one system, which may suggest slightly more resistance of the G+PM bilayer to MGs than the POPC bilayer had. Additionally, evidence of mass movement of MGs to the lower leaflet of a bilayer reducing the extent of property perturbation aligned with hypotheses regarding the importance of asymmetric stress in

driving the deformation of lipid bilayers and subsequent methods of asymmetric stress relief. Therefore, observations of the G+PM system display strong similarity to the outcomes of evaluating hypotheses regarding bilayer destabilisation in the equivalent POPC systems.

6.4.3 Gram-Negative Inner Bacterial Membrane

Impact of Monoglyceride Structure

As with the G+PM, the G-IM was initially modelled containing 100 MG in the upper leaflet. At this concentration, however, significant disruption of the upper leaflet was observed in two of the systems - GNIM_100_GMCY and GNIM_100_GMC. The extent of this disruption prevented the tools used to calculate some system properties from producing accurate values, due to the tools requiring a planar membrane structure. Because of this, systems discussed in this section will instead be at the concentration of 50 MG, as each of these systems showed structural stability throughout the simulations. 100 MG systems for the G-IM are discussed in greater detail later within this section of results.

Reported thickness values for the 50 MG systems (Table 6.10) again reflect that the presence of C8-MG was associated the most disrupted lipid bilayer properties of the three tested MGs. The decrease in thickness was fairly linear between MGs, with a 0.2 Å difference between C10-MG and C12-MG, and a 0.2 Å difference between C8-MG and C10-MG, though there was no significant difference between MGs based off error calculations. All thickness values for MG loaded systems showed significant difference to the control.

Table 6.10: Average bilayer thickness for G-IM systems loaded with 50 MG. Change from control expressed in Å and percentage change are also reported. All values were rounded to 1 decimal point. Brackets represent standard deviation.

	GNIM_50_GMCY	GNIM_50_GMC	GNIM_50_GML	CTRL_GNIM
Thickness (Å)	37.3 (0.5)	37.5 (0.4)	37.8 (0.5)	39.0 (0.5)
Change (Å)	-1.7	-1.5	-1.2	0
% change	-4.4%	-3.8%	-3.1%	0%

Density profiles (Figure 6.10) also showed no significant differences between the

three MGs at this concentration. The overall density at the upper leaflet rose slightly with acyl chain length, particularly the peak at the headgroup region of the upper leaflet which is likely due to the physical size difference between the three MGs. Water penetration was generally similar between all three systems (Table 6.11). Water did not penetrate significantly past the headgroup region of the membrane, and the "dry" size of the hydrophobic core was 18-19 Å in all systems. Water permeated closer to the bilayer centre in comparison to the control dry thickness (21 Å) for all systems, even accounting for the overall decrease in bilayer thickness (-1.7 to -1.2 Å). Water penetrated slightly deeper into the upper leaflet of the bilayer which aligns with the greater disruption of headgroup packing of leaflets loaded with MG (Figure 6.10). A small, shallow peak in density of 0.013 g/mol/Å³ at -13 Å was observed in the GNIM_50_GMCY system that was not observed in GNIM_50_GMC or GNIM_50_GML. This suggests that at lower concentrations C8-MG was more likely to move from its position in the upper leaflet, either into the water box, or across the bilayer, than C10-MG and C12-MG at low concentrations.

Table 6.11: Depth of water penetration values for 100 C8-MG, C10-MG, and C12-MGs containing G+PM systems. +/- represent maximum penetration depth along the z-axis for each leaflet.

System	Water Penetration (UL) (Å)	Water Penetration (LL) (Å)
GNIM_50_GMCY	+8.5	-9.5
GNIM_50_GMC	+9.5	-9.5
GNIM_50_GML	+8.5	-10.5

GNIM_50_GMCY had a final lower leaflet count of 8 C8-MG molecules. None of these molecules flip-flopped from the upper leaflet to the lower leaflet of the membrane, therefore all MG molecules present in the lower leaflet were artefacts of PBCs being crossed. GNIM_50_GMC and GNIM_50_GML both had a final MG count in the lower leaflet of 1. In GNIM_50_GMC only 1 C10-MG molecules exited the bilayer, and no molecules flipped to the lower leaflet, whereas GNIM_50_GML had one molecule move to the lower leaflet and none exit the bilayer. The lack of MG movement within the bilayer indicated relatively strong bilayer stability at a count of 50 MG for all three systems.

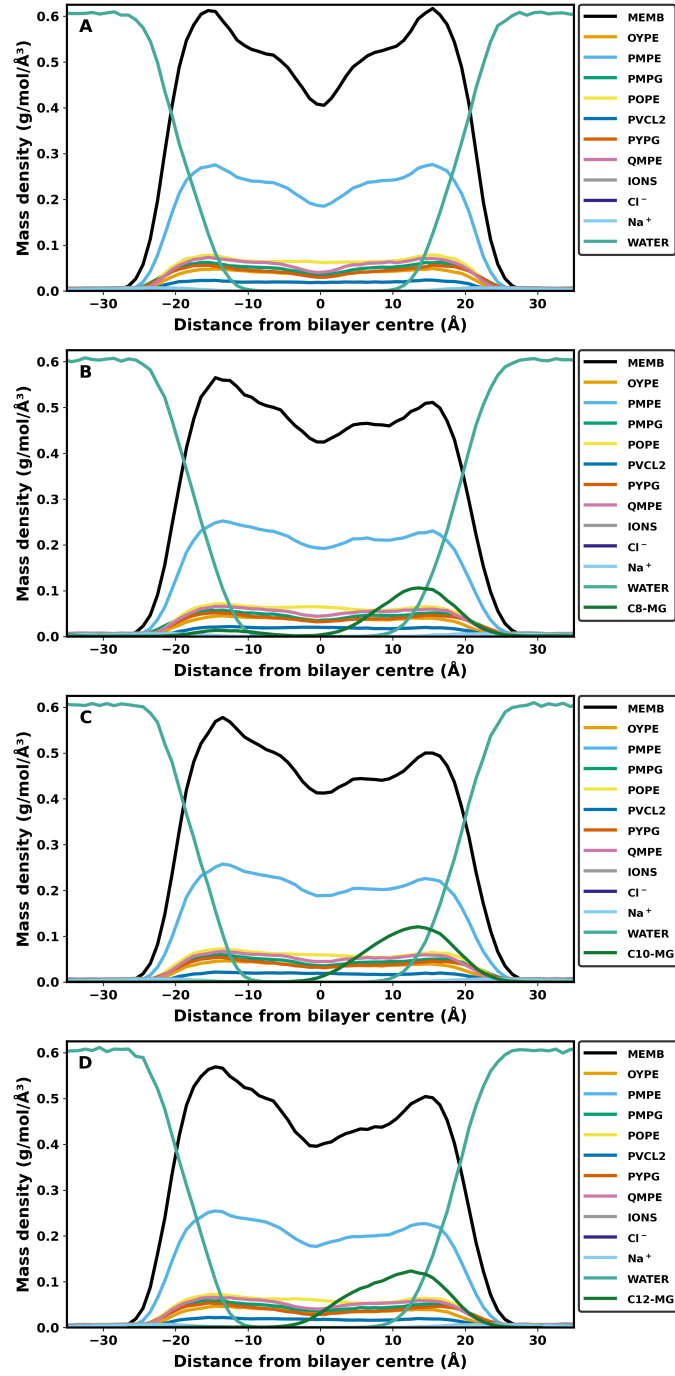


Figure 6.10: Average 1D mass density profiles along the z-axis for C8-MG, C10-MG, and C12-MGs containing G-IM systems Control (A), GNIM_50_GMCY (B), GNIM_50_GMC (C), and GNIM_50_GML (D). MEMB represents the density of the phospholipid bilayer excluding added MG.

A small increase in overall bilayer fluidity was observed in the systems based on calculated deuterium order parameters (Figure 6.11). Around a 50% decrease in order was observed in the lower leaflet across all carbons, while increases to order in the upper leaflet were mainly associated with the early carbons (C2-C10) of the acyl tail. The whole bilayer deuterium order parameter profile (Figure 6.11 C) indicated a minor drop in overall order some carbon atoms in the acyl chain. There was no significant difference between MGs for the whole membrane or lower leaflet, but MG tail length did have some influence on order in the upper leaflet, as has been noted in previous upper leaflet S_{CD} profiles, with C8-MG having lesser influence on the phospholipid acyl tail than C10-MG and C12-MG.

Overall, it is more difficult to identify key differences between MGs at this concentration compared to the 100 in the G+PM, however it does appear that the trend of the shortest MCMGs causing the most significant disruption of the bilayer remains consistent with the other membrane models simulated. This consistency therefore can be linked to the general bilayer disruptive properties of MGs to the antimicrobial capabilities of these molecules simultaneously, providing further evidence that significant disruption of bilayer properties is primarily associated with very high localised MG concentrations.

Impact of Monoglyceride Concentration

To complement the 50 MG concentration studied in the G-IM systems, 100 and 150 of each MG were also added asymmetrically to further understand bilayer disruption. At these high concentrations of MG it was very consistent that the loss of bilayer planarity through the initiation of budding occurs. The speed of budding initiation in each system is described in Table 6.12.

At a count of 100 all systems except GNIM_100_GML experienced budding to some degree, and all displayed bud formation at 150 molecules. Bud formation in GNIM_100_GMCY and GNIM_100_GMCY was slower than in their respective 150 MG systems, and the size of the buds were lower (Figure 6.12).

Average bilayer thickness values are reported in Table 6.13, with percentage change

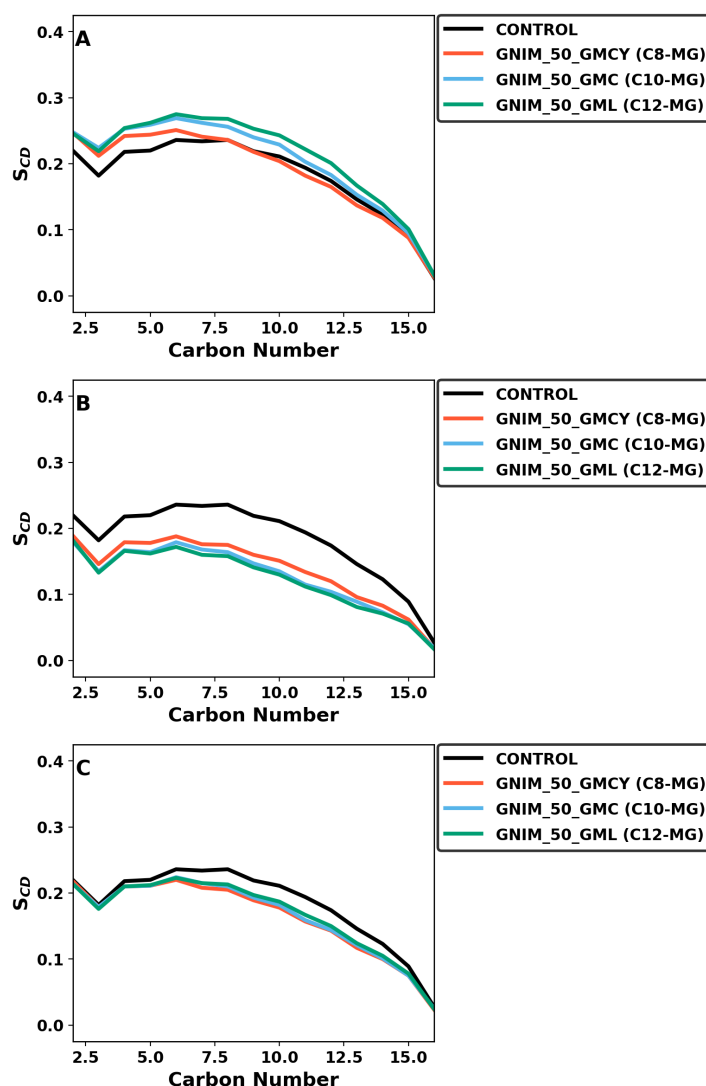


Figure 6.11: Deuterium order parameters for the 16:0 acyl tail of POPE in G-IM systems with added C8-MG, C10-MG, and C12-MGs for the upper leaflet (A), lower leaflet (B), and both leaflets (C).

Table 6.12: Simulation time from initiation of production run until the beginning of upper leaflet budding for G-IM systems loaded with C8-MG, C10-MG, or C12-MG. TTD represents time from the start of the simulation to the beginning of bilayer planarity disruption in ns.

MG Concentration	TTD C8-MG	TTD C10-MG	TTD C12-MG
50	-	-	-
100	70	300	-
150	10	25	20

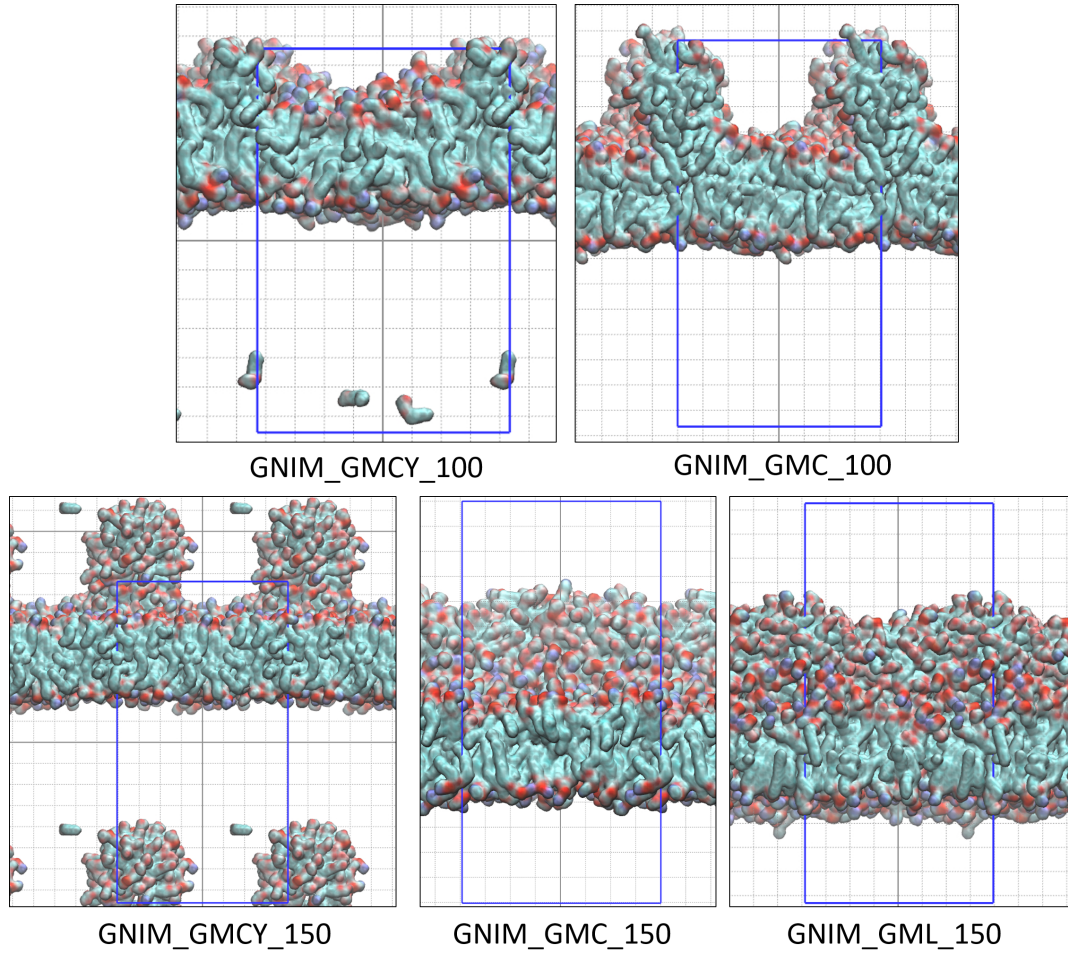


Figure 6.12: Final frame of each G-IM simulation where budding occurred. Blue box represents the periodic boundaries of the simulation box. Periodic images were added, and water and ions hidden for clarity.

in Table 6.14. Due to limitations of the tools elaborated upon later in this chapter, all these values include bud structures, and therefore do not accurately represent thickness in the planar section of the bilayer. Despite the budding that occurred in GNIM_100_GMCY and GNIM_100_GMC, a decrease in overall membrane thickness was observed in these systems in comparison to the control. For C8-MG this was likely due to the bud not maturing over the simulation, so it remained contained to a smaller portion of the leaflet and at a height of 20 Å. Both GNIM_50_GML and GNIM_100_GML displayed a significant reduction in thickness was observed in comparison to the control, with GNIM_100_GML being 0.5 Å greater than GNIM_50_GML.

Table 6.13: Average bilayer thickness of G-IM systems with varying counts of C8-MG, C10-MG, or C12-MG added. All values were rounded to 1 decimal point. Brackets represent standard deviation. * marks a system where budding occurred.

MG Count	C8-MG Disruption	C10-MG Disruption	C12-MG Disruption	Control
0	-	-	-	39.0 (0.5)
50	37.3 (0.5)	37.5 (0.4)	37.8 (0.5)	-
100	38.3 (1.1)*	39.6 (3.6)*	36.3 (0.5)	-
150	49.5 (4.3)*	46.5 (1.2)*	48.0 (1.5)*	-

Table 6.14: Percentage change in bilayer thickness in comparison to control for G-IM systems with varying counts of C8-MG, C10-MG, or C12-MG added. All values were rounded to 1 decimal point. * marks a system where budding occurred.

MG Count	% change C8-MG	% change C10-MG	% change C12-MG
50	-4.4	-3.8	-3.1
100	-1.8*	+1.5*	-6.9
150	+26.9*	+19.2*	+23.1*

Density profiles for GNIM_50_GML and GNIM_100_GML showed alterations to the positioning of membrane lipids in both leaflets but especially the upper leaflet where C12-MG is present (Figure 6.13). The partial density distribution of the lower leaflets were less disturbed than the upper leaflet where C12-MG was present. C12-MG remained contained to the upper leaflet of the bilayer at both concentrations. GNIM_50_GML had one molecule move to the lower leaflet and no movement occurred in GNIM_100_GML. Water penetration depth in the lower leaflet did not alter between systems, remaining at -10.5 Å, but at 100 C12-MG water penetration in the upper leaflet was significantly increased, being present through to +5.5 Å.

Deuterium order parameters for each system indicated an overall drop in order of around 0.05 (a 20% decrease), and therefore indicates increased overall fluidity in the membrane (Figure 6.14). GNIM_100_GML saw significantly greater changes to order in each direction than in the 50 MG equivalent system.

Due to limitations in tools it was difficult to produce direct, data driven analysis of how MG concentration affected destabilisation of G-IM bilayer models. However, the bilayer budding observed in systems indicates significant disruption of bilayer properties, as this behaviour is hypothesised to be associated with high asymmetric stresses in a membrane caused by amphiphile insertion [27,285]. Given this, it appears

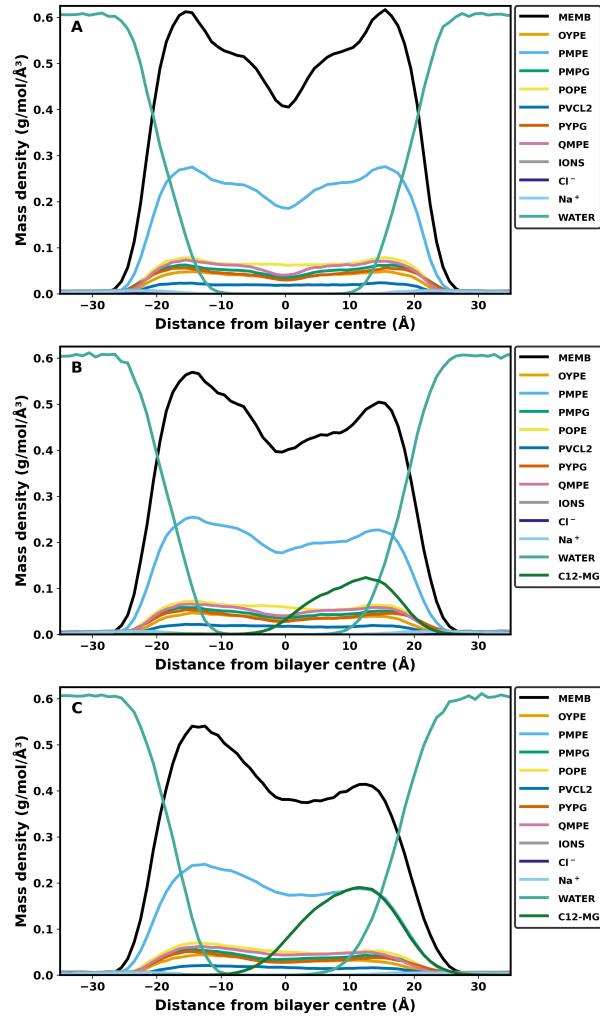


Figure 6.13: 1D average mass density profiles along the z-axis for C12-MG containing G-IM systems Control (A), GNI.50_GML (B), and GNIM.100_GML (C). "MEMB" represents the density of the phospholipid bilayer including added MG.

that hypotheses regarding membrane destabilisation investigated in POPC and G+PM systems can also be applied the the G- inner membrane.

6.4.4 Comparisons Between Bilayer Systems

Although trends generally matched up with those observed in the previous chapter, it is useful to directly compare systems where possible in order to understand how the behaviour of different bilayer systems may differ upon exposure to MGs. This section compares the bilayer models to the POPC model used in the previous section.

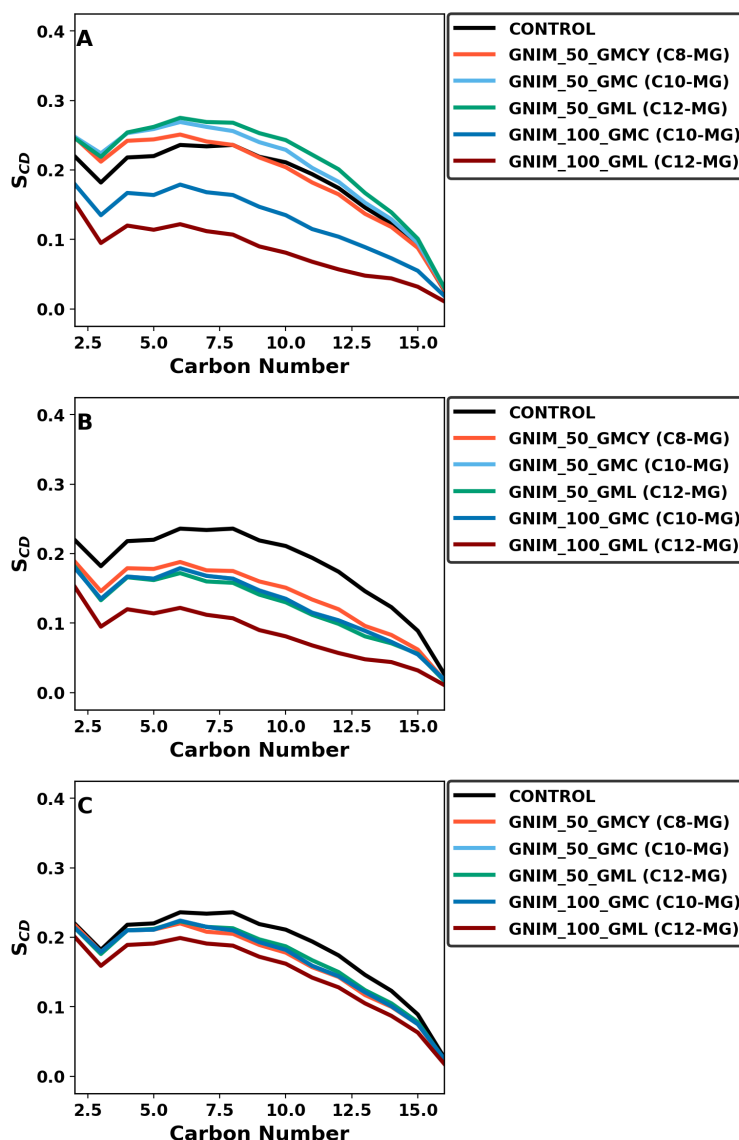


Figure 6.14: Deuterium order parameters for the 16:0 acyl tail of POPE across the upper leaflet, lower leaflet, and whole bilayer for G-IM systems with varying concentrations of C8-MG, C10-MG, or C12-MG added.

All values were compared at all concentrations possible, though as discussed in the previous section some of these parameters were unable to be calculated in systems where budding occurred.

Bilayer Thickness

Percentage change in bilayer thickness across all systems is reported in Table 6.15. Excluding systems where budding occurred, a consistent trend was noticeable, when comparing specifically systems where budding did not occur across all three models, that the most disruption to bilayer thickness occurred in systems containing C8-MG, then C10-MG, then C12-MG in that order. Both bacterial membrane models showed greater disruption MGs than the POPC bilayer for each MG. The only concentration where all three systems were simulated and no budding occurred were in systems with 100 C12-MG added. At this concentration the G-IM model was more disrupted than the G+PM.

Table 6.15: Percentage change in average bilayer thickness in comparison to control for G+PM, G-IM, and POPC systems with varying concentrations of C8-MG, C10-MG, or C12-MG added. All values were rounded to 1 decimal point. * represents a system where budding occurred

MG	Concentration	G+PM	G-IM	POPC
C8-MG	50	-	-4.4	-3.6
	100	-7.1	-1.8*	-6.1
	150	-9.8	+26.9*	+17.1
	200	+31.7*	-	+31.6*
C10-MG	50	-	-3.8	-2.5
	100	-6.1	+1.5*	-4.8
	150	-7.7	+19.2*	-6.4
	200	-7.7	-	-7.9
C12-MG	50	-	-3.1	-0.8
	100	-4.2	-6.9	-2.8
	150	-6.3	+23.1*	-4.8
	200	-4.8	-	+39.2*

Density Profiles

Bilayer thinning was also observed in the mass density profiles for each system (Figure 6.15). In all systems, the extent of bilayer thinning was not symmetrical, with thinning being biased towards the lower leaflet. The peak in the headgroup regions of the bilayer was lower in the upper leaflet of all non-control systems, which indicated more variance in phospholipid positioning in this region of the bilayer.

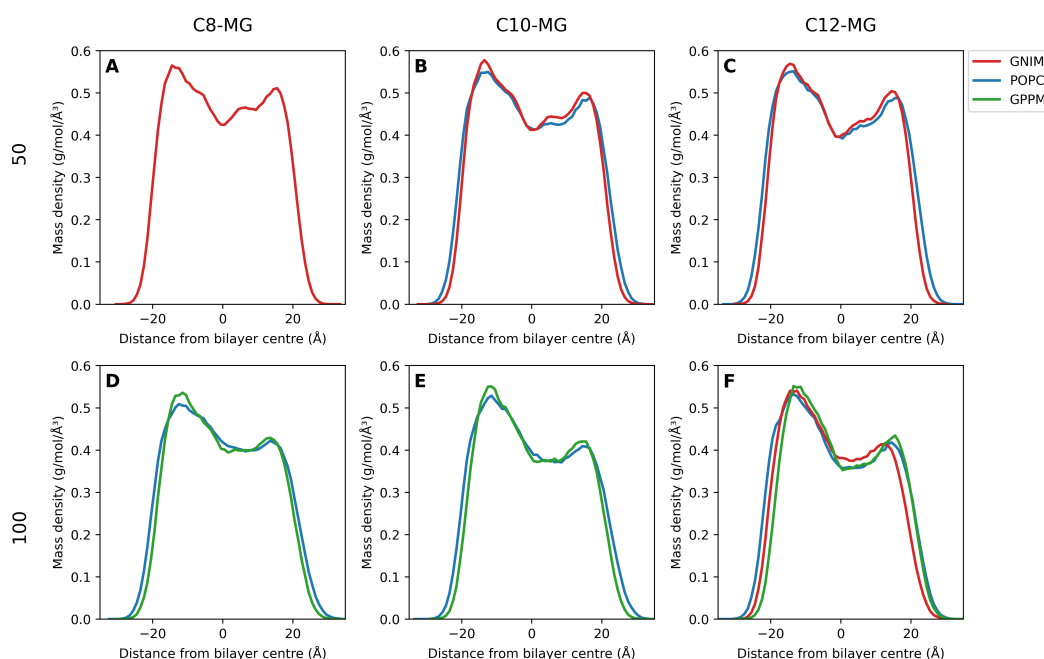


Figure 6.15: 1D average mass density profiles along the z-axis of bilayer phospholipids for G+PM, G-IM, and POPC bilayer systems at a count of 50 and 100 MH molecules in the bilayer. Density profiles for only systems where budding did not occur shown.

Partial density profiles for MG placement revealed that all three MCMGs tended to form a peak at around +12 Å, which indicates a preferred position for MC placement in the bilayer (Figure 6.16). This did not shift as MG concentration grew, however for C8-MG and C12-MG a small secondary peak was displayed around -11 Å, indicating the movement of MGs and their subsequent reorientation with the lower leaflet headgroups.

Water penetration depth through the membrane is highlighted in Figure 6.17 and Table 6.16. The depth of water penetration was increased across all MG containing systems. At 50 MG differences were not very pronounced between G-IM and POPC systems, both experiencing a 1-2 Å reduction to their respective controls. At 100 MG differences, a 4-5 Å increase in permeation across leaflets was observed in systems. At 150 MG the penetration increase was 6-10 Å, and 6-9 Å at 200 MG. It was consistent in all systems that C8-MG caused the greatest increases in water penetration, though the range of values was within 2 Å of the other two MGs. Increases in water penetration were typically more pronounced in the upper leaflet, where MGs began, than the lower

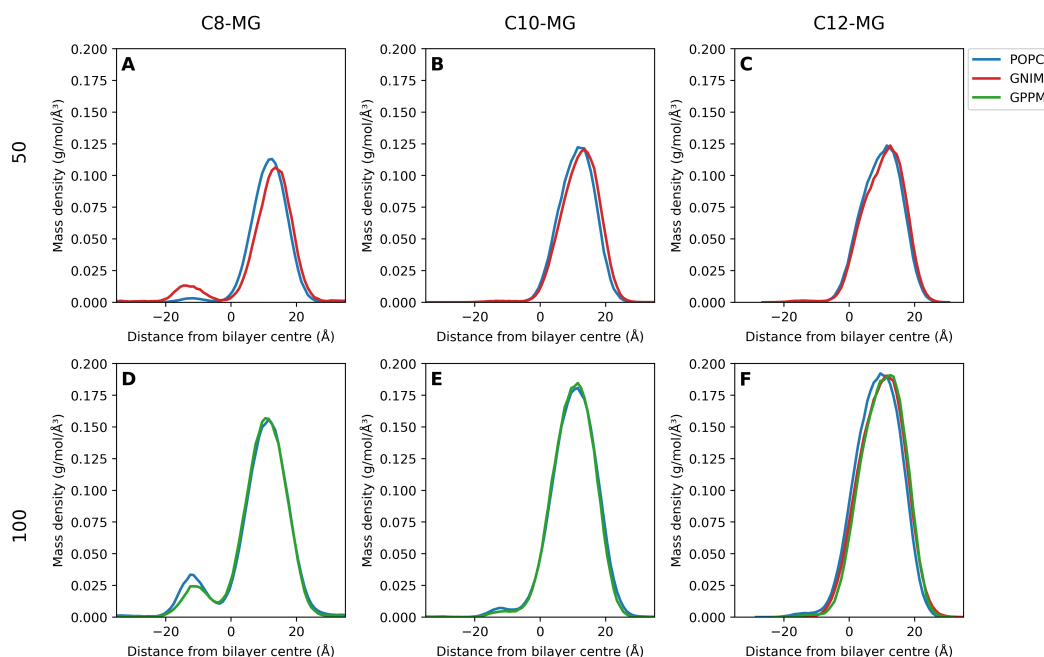


Figure 6.16: 1D average mass density profiles along the z-axis of C8-MG, C10-MG, and C12-MG molecules for G+PM, G-IM, and POPC bilayer systems at a count of 50 and 100 MH molecules in the bilayer. Density profiles for only systems where budding did not occur shown.

leaflet of the membrane. The depth of water penetration was not significantly different in increase to the observed increase in thickness at a MG count of 50 MG, so this may account for increased penetration at comparatively low concentrations, however water permeation depth outpaced the decrease in thickness observed in systems containing 100, 150, or 200 molecules.

Deuterium Order Parameters

Deuterium order parameters are displayed in Figure 6.18. Overall, though all bilayer systems containing MG showed a reduction in order, the POPC bilayer systems showed less significant changes to order than the other systems (Figure 6.18 A, D, G). This may be because baseline order in the POPC control was slightly lower, as order between bilayer models at high concentrations was similar. Additionally, POPC has a different headgroup to the DPPG and POPE lipids analysed for S_{CD} which would influence membrane order, as PE headgroups are known to have tighter packing

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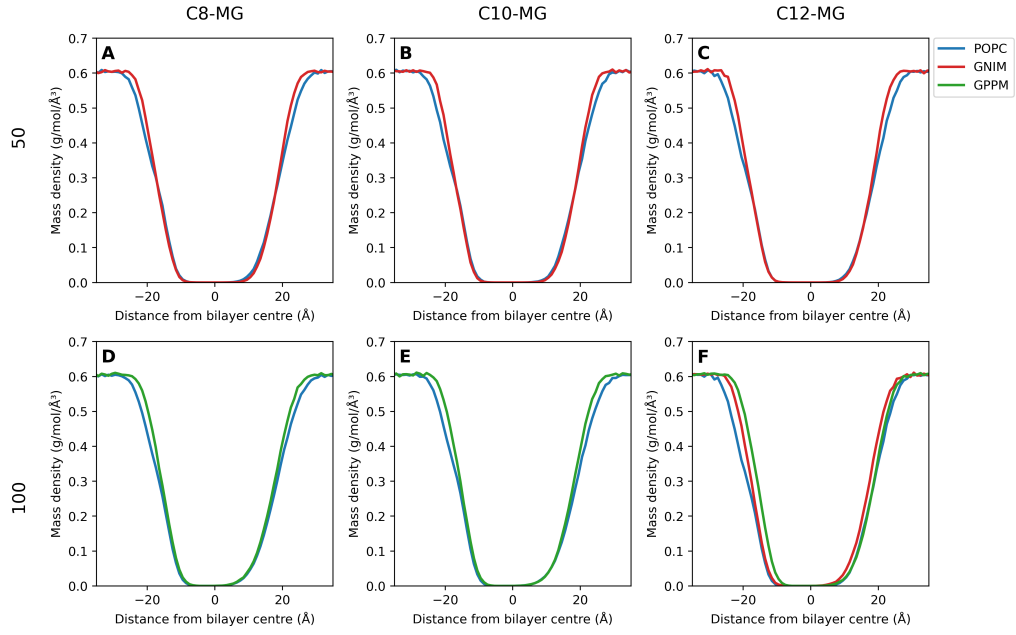


Figure 6.17: 1D average mass density profiles along the z-axis of water molecules for G+PM, G-IM, and POPC bilayer systems at a count of 50 and 100 MH molecules in the bilayer. Density profiles for only systems where budding did not occur shown.

Table 6.16: Depth of water penetration into bilayer core (Å) for G+PM, G-IM, and POPC systems with varying concentrations of C8-MG, C10-MG, or C12-MG added. -/+ represents depth in the lower and upper leaflets respectively. All values were rounded to 1 decimal point. NC indicates that water penetration could not be calculated due to budding occurring.

MG	Concentration	G+PM	G-IM	POPC
Control	0	-9.5/+9.5	-10.5/+10.5	-9.5/+9.5
C8-MG	50	-	-9.5/+8.5	-8.5/+7.5
	100	-7.5/+4.5	NC	-8.5/+4.5
	150	-4.5/+4.5	NC	NC
	200	NC	-	NC
C10-MG	50	-	-9.5/+9.5	-9.5/+8.5
	100	-8.5/+5.5	NC	-8.5/+5.5
	150	-6.5/+2.5	NC	-7.5/+4.5
	200	-6.5/+5.5	-	-4.5/+5.5
C12-MG	50	-	-10.5/+8.5	-10.5/+7.5
	100	-8.5/+6.5	-10.5/+5.5	-10.5/+5.5
	150	-7.5/+5.5	NC	-9.5/+2.5
	200	-6.5/+6.5	-	NC

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between molecules. [286] There was no significant differences in overall order at the same concentration in MG loaded systems between the three models.

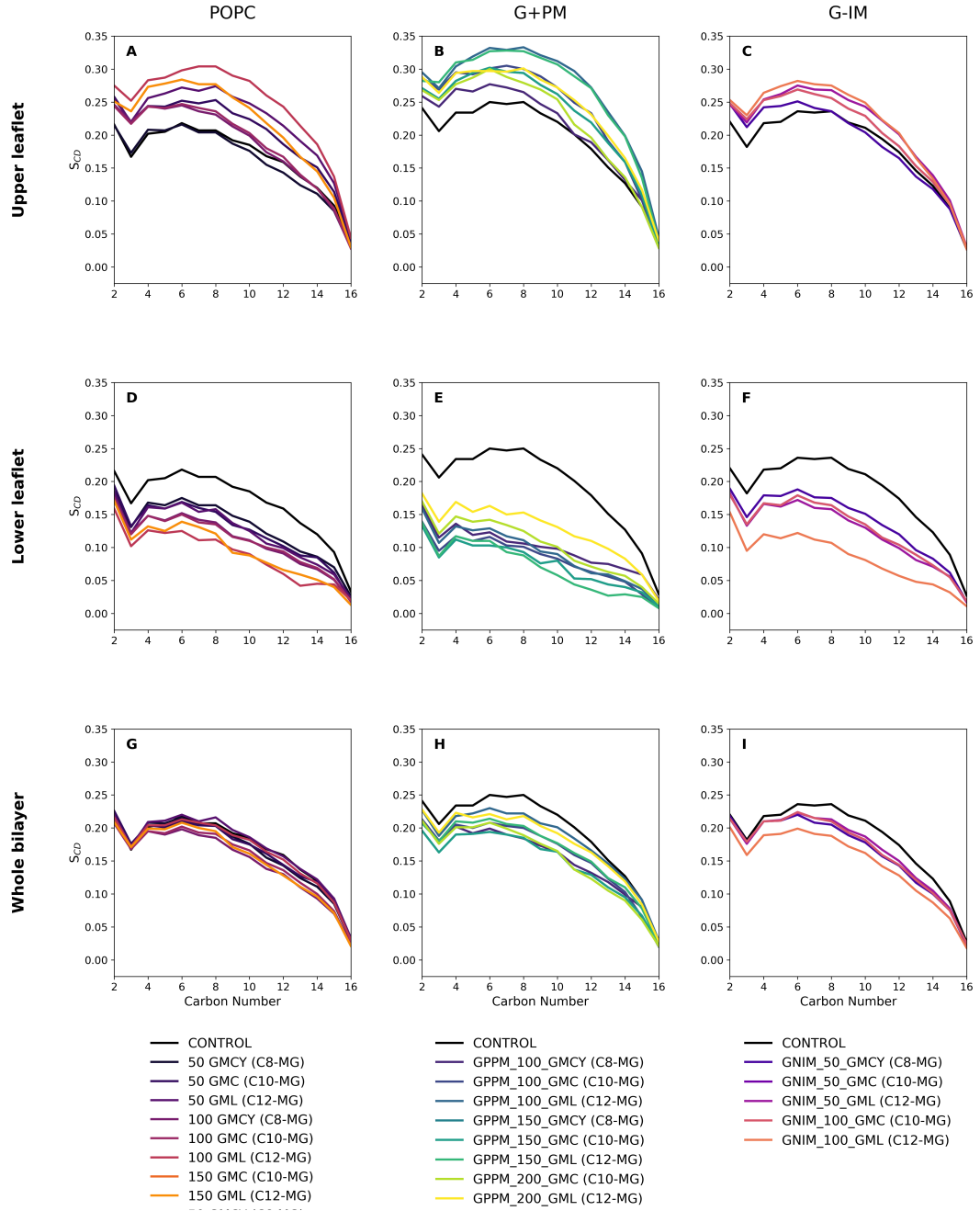


Figure 6.18: Deuterium order parameters for the 16:0 acyl tail of POPC, DPPG (G+PM), and POPE (G-IM) phospholipids across the upper leaflet, lower leaflet, and whole bilayer for G+PM, G-IM, and POPC bilayer systems respectively with varying concentrations of C8-MG, C10-MG, or C12-MG added.

From these parameters a general trend can be observed regarding the extent of disruption the three bilayer models investigated experience in response to MG concentrations. G-IM systems tended to show the greatest susceptibility to MGs of the three systems, even at concentrations which didn't induce budding. In systems where budding did occur, the G-IM tended to experience it at lower concentrations than the other two systems. G+PM showed the most resistance to the effects of MGs, suggesting the greatest stability of the three. POPC was more resistant than G-IM models but did display budding more often than G+PM systems. All systems were most susceptible to C8-MG, and C12-MG containing systems had higher thresholds to obvious stress-relieving behaviours such as budding and mass MG flip-flop occurring despite similar levels of destabilisation.

6.5 Discussion

6.5.1 Monoglyceride Bilayer Disruption Mechanisms

Observed mechanisms of bilayer destabilisation by MGs were very consistent with those observed in the previous chapter. This provides significant evidence that the findings of the first chapter regarding how the POPC lipid bilayer becomes disrupted by the presence of MGs can be translated to more realistic bacterial models, and therefore can be considered characteristics of the antimicrobial activity MGs exhibit against bacteria. From the additional simulations conducted in this chapter, a more thorough understanding of the behaviours observed in MD simulations, and how they can be contextualised within our existing knowledge of MG mechanisms of antimicrobial activity, can be formed.

An obvious aspect of the disruption mechanism of MGs is caused by the physical presence of the molecules within a lipid bilayer. The presence of MGs in the bilayer is known to disrupt structural characteristics of the membrane. MGs are known to disrupt phospholipid packing upon their insertion by physically wedging between phospholipid molecules. [283] This disrupts headgroup interactions, for example hydrogen bonding between phosphatidylethanolamine (PE) headgroups, and causes an increase in space

occupied by each headgroup as was observed in APL calculations when discounting MGs in the calculation. [283, 287]. Disruption of bilayer headgroup packing in turn alters the pressure profile of the bilayer, which causes the mechanical stress normally localised to the head groups to be transferred throughout the bilayer, driving structural changes to the core of the membrane, such as the thinning observed in all systems where MCMGs were present without budding (Table 6.15) and the reduction in the depth of the trough which appears at the centre of bilayer density profiles across this Chapter and the previous [288]. The physical wedging effect of MGs would be amplified by the presence of more molecules in the bilayer, which would align with low disruption of properties at low MG concentrations, and disproportionately extensive damage to the bilayer observed in systems with very high MG concentrations. This also aligns with the importance of MG positioning in driving property disruption, as assessed in Chapter 5, where systems that had an asymmetrical distribution of MGs were significantly more perturbed than those with a symmetrical distribution.

Geometrically, MGs can be described as conical, due to their single acyl tail and glycerol headgroup. Such geometry promotes positive curvature in planar bilayer systems [28]. The effects of this positive curvature was observed in systems within the last two chapters, especially at high concentrations. Bilayer behaviours observed in simulations that align with known effects of positive curvature induction include visible membrane bending or buckling of the lipid bilayer. Mechanically, this behaviour occurs when expansion of upper leaflet area per lipid due to MG addition causes expansion greater than the lower leaflet, causing the bilayer to form a bend to reduce free energy [289].

A greater acyl tail volume relative to the headgroup of MGs causes MG geometry to become less conical, and therefore C12-MG is the least conical of the three MGs looked at in this study. A more conical MG geometry should theoretically cause greater disruption of bilayer properties by inducing greater curvature, which would explain why at equal concentrations within the bilayer C8-MG displayed the most significant disruption of bilayer properties as it produces the greatest hydrophobic mismatch with the membrane lipids. Experimentally it is consistently reported that C10-MG or C12-

MG were the most potent antimicrobial MGs of these three [33,35,40,164]. This could be attributed to C8-MG CMC thresholds. C8-MG monomers have weaker hydrophobic interactions driving micellisation than longer tail molecules as the hydrophobic region of the molecule is smaller, so the CMC threshold for C8-MG is higher. As potency is broadly associated with MIC, meaning that a lower MIC is linked to higher potency, and antimicrobial activity in MGs is reported commonly above CMC thresholds, the comparatively high CMC threshold of C8-MG may therefore be associated with the molecule's lower potency experimentally, as a higher monomer concentration is required in solution to display antimicrobial activity. Once this MIC threshold has been reached, it is likely, and agreed upon from the data shown in this thesis, that C8-MG produces greater bilayer disruption due to the structural difference discussed above. Additionally, C8-MG has weaker hydrophobic interactions with other molecules than C10-MG and C12-MG, meaning monomers are less strongly associated with the micelle structure. This may mean that due to faster exchange of monomers between solution and the micelle more free MG monomers may be available to partition into the bilayer above CMC thresholds. This may not necessarily increase C8-MG insertion into the bilayer however, as the longer acyl tails of C10-MG and C12-MG cause these molecules to more favourably insert into the bilayer from solution [290]. Conversely, C8-MG may have a lower energy barrier to leaving the membrane than longer MGs, which would make it more difficult for critical thresholds in the bilayer for significant membrane disruption to occur to be reached. This would align with the observation that C8-MG exited the bilayer much more readily than C10-MG and especially C12-MG in all simulations at equal concentrations between the three MCMGs. Investigation in MG energy barriers for entry into and exit from lipid bilayers was performed in the following chapter.

At extremely high bilayer concentrations more extreme behaviour than curvature induction in the bilayer was observed. As discussed in Chapter 5 and earlier in this chapter, this behaviour was the formation of a structure on the upper leaflet of the membrane that contained both MG and phospholipid molecules. This behaviour can be considered membrane budding, which is a process driven by a high localised curvature in a region of the membrane and causes remodelling of the membrane to create a budded

deformation to reduce bilayer stress in extreme circumstances where bending does not provide enough relief [29,276,283]. As buds only formed in systems with very high MG counts within a bilayer this displayed behaviour would be in line with budding as a relief mechanism for the high asymmetric stress accumulation in these systems. Although there was evidence of budding forming from exposure to all three MGs in this chapter, C12-MG consistently required higher concentrations for it to occur in comparison to C8-MG and C10-MG in bacterial model systems. This aligns with the lesser disruption of planar bilayer characteristics observed in C12-MG systems compared to the others. In POPC bilayer there was one instance of budding occurring in a C12-MG system and not a C10-MG system, however this could be attributed to being due to the mass movement of C10-MG molecules to the lower leaflet following spontaneous curvature forming, and budding was observed in C8-MG at a count of 150 MG where it was not in the respective C10-MG and C12-MG systems.

Regarding the mechanisms of bilayer relief from asymmetry, it appears from these systems that MG flip-flop to the lower leaflet is very important to maintaining bilayer integrity in response to MG-induced asymmetric stress. In systems with very high concentrations, all initially showed the induction of curvature in the bilayer, which then collapsed in some systems into a more planar bilayer or destabilised further into budding in others. In systems where budding did not occur, there was consistently a very high lower leaflet MG concentration, which was primarily due to the mass movement of MG molecules to the lower leaflet over a short period of time. This behaviour was not surprising, as it has been reported in literature that movement of small amphiphilic molecules in the bilayer across leaflets can be induced in response to mechanical stress between leaflets in order to reduce asymmetric stresses across the bilayer. [291,292] Additionally, mass flip-flop of MGs reducing the extent of bilayer property perturbation was also observed in some systems analysed in Chapter 5. Flip-flop relief of asymmetric stress was most strongly associated with C12-MG of the three MCMGs, while the others appeared to relieve stress mainly through budding. This suggests that the energy barriers for movement out of the bilayer between C8-MG, C10-MG and C12-MG may differ enough that movement across the bilayer is more energetically favourable for

C12-MG than exiting the bilayer. This suggestion is investigated further in the next Chapter.

6.5.2 Implications of Bilayer Structure on Monoglyceride Susceptibility

As has been observed in the results presented within this chapter, it appears clear that different lipid bilayers display varying levels of susceptibility to MGs.

Between the bacterial models, the G-IM model consistently displayed greater disruption by MGs in comparison to the G+PM at equal concentrations. Structurally, these bilayers have two key properties which are influenced by the lipid composition of the leaflets - the charge of phospholipids in the bilayer, and the geometry of the membrane phospholipids.

In terms of charge, the G+PM model is significantly more negatively charged than the G-IM model. G-IM has an overall charge density of $-32 e$ per 100 lipids in a leaflet (Table 6.2), while the G+PM has a charge density of $-81 e$ per 100 lipids in a leaflet (Table 4.2). Experimentally, it has been observed that a high negative charge in a membrane results in increased steric exclusion of amphiphilic molecules, due to the accumulation of Na^+ ions at the headgroups, which could result in greater difficulty in MGs interacting with the bilayer [293]. This is a mechanism that primarily affects the insertion of MGs into the bilayer, so it is unlikely that differences in disruption observed within these simulations is attributable to the charge of the systems, as an equal concentration was placed into the bilayer prior to the start of the simulation, therefore bypassing any potential repulsion within the context of this study.

The cardiolipin content of the G+PM and G-IM models also may influence bilayer susceptibility. Cardiolipin is a dimeric phospholipid with 4 acyl tails. The increased area of cardiolipin in comparison to two-tailed phospholipids causes tighter local packaging in the bilayer, which in turn increases bilayer stability [294]. Additionally, cardiolipin has been shown to be associated with regions of negative curvature in lipid bilayers due to its inverted cone structure. [295] This negative curvature has been shown in some cases to counteract the positive curvature induced by some amphiphiles. [296] Due to this, there is potential that the negative curvature and

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overall stability induced by cardiolipin inhibits the degree of deformation that high MG concentrations induces. G+PM has a significantly higher cardiolipin content than the G-IM, at 8% per leaflet versus 2%. This would mean that the stabilising effects cardiolipin in the G+PM are stronger, reducing the degree of MG disruption and therefore requiring greater MG concentrations for loss of bilayer planarity to occur in a system.

PE and phosphatidylglycerol (PG) phospholipids have geometrical differences which may explain how disrupted the two systems are by MGs. In terms of lipid head groups, the G+PM model is dominated by phospholipids with PG head groups, while the G-IM is dominated by PE head group phospholipids. Compared to PE, PG headgroups are larger, with a cylindrical geometry, and are electronegative. In contrast, PE headgroups are zwitterionic and have a conical geometry, which promotes negative curvature. While the negative curvature of PE may counteract some MG disruption, PE head groups form tight packing, which creates a high lateral pressure environment. Therefore PE lipids are more significantly affected by insertion of MGs into a bilayer, as the presence of MGs in the bilayer disrupts hydrogen bonding between PE headgroups, causing increases in the lateral area per headgroup and therefore redistributing the pressure throughout the bilayer [287]. As PG headgroups are less tightly packed than PE headgroups, the addition of MGs did not cause as severe immediate destabilisation of the bilayer, therefore causing increased tolerance against MGs in the G+PM model.

The tails of lipids in these models also contribute to the susceptibility differences between models. Phospholipids found in the G+PM model were exclusively unsaturated, while 22 of the 100 phospholipids in each leaflet of the G-IM displayed unsaturation and a further 8 were unsaturated in two tails. Due to the unsaturation present in the G-IM, the packing of lipid tails in this bilayer model was less tight than the G+PM, and therefore the bilayer had higher base fluidity. Less rigid packing of lipids has been shown to allow greater transfer of stress across the bilayer, while hydrophobic cores with tighter packing have greater resistance to uniform deformation caused by headgroup lateral area expansion [288].

The data presented within this chapter suggests that POPC bilayers are more

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susceptible than G+PM to significant MG disruption, however this does not accurately reflect the true susceptibility of mammalian cell membranes. There are several key differences between bacterial and mammalian cells, other than their lipid compositions, which are not accounted for within these simulations that would explain this. Arguably the most important difference between bacteria and mammalian cells is the environment that they exist within, and the protection that provides. Planktonic (individual, free-floating) G+ bacteria have fewer barriers to prevent interaction of MGs with the plasma membrane than mammalian cells, despite G+ bacteria having a cell wall that acts as a diffusion barrier at the cell-surface interface that mammalian cells lack. The glycocalyx is an external layer outside of the bacterial cell envelope and the mammalian plasma membrane. Structurally, the glycocalyx differs significantly between bacteria and eukaryotes. In bacteria this is also referred to as the capsule or slime layer, and is specialised towards colonisation, facilitating surface adhesion and biofilm formation. In eukaryotes, the glycocalyx is a significantly more complex structure, forming an organised, dense mesh of glycoproteins and glycosaminoglycans which provides protection from shearing and facilitates immune signalling and mechano-transduction. The eukaryotic glycocalyx is typically around $0.5\ \mu\text{m}$ in comparison to the up to $10\ \mu\text{m}$ of the bacterial capsule [297,298]. Despite the mammalian glycocalyx having lower thickness on average than the bacterial glycocalyx, the mammalian glycocalyx has a higher density of negatively charged sugars, which creates greater steric repulsion of lipid nanoparticles [299]. This therefore could inhibit interactions between MGs and the mammalian plasma membrane. Additionally, mammalian cells organise into tissue layers which feature an extracellular matrix of collagens and fibronectin which slow amphiphile permeation, tight junctions between cells that prevent amphiphile diffusion through intercellular gaps, and interstitial fluid which dilutes molecule concentrations before they come into contact with the mammalian plasma membrane [300–302].

Another mechanism present in eukaryotic cells that bacterial cells lack is more robust and rapid cell membrane repair mechanisms which are activated in response to membrane damage. Damage to cell membrane integrity causes an influx of calcium ions from the external environment into the cell, which in turn triggers multiple

bilayer repair mechanisms. Ca^{2+} ions bind proteins on peripheral lysosomes present near the cell membrane, causing them to fuse with the cell membrane. This increases the surface area of the membrane, which can reduce the stresses placed on the membrane and releases acid sphingomyelinase enzymes which promote the formation of ceramide microdomains at the damaged area, promoting its later endocytosis, removing ruptures from the membrane. Ca^{2+} dependent phospholipid binding proteins annexins, and calpain proteases facilitate the controlled budding off of damaged sections of the membrane [303]. These mechanisms both repair physical damage caused by amphiphilic molecules in the membrane and revert membrane compositions, and therefore also its properties, towards its baseline characteristic prior to disruption. Repair of membrane damage in mammalian cells is a very fast process, occurring within a minute of the initial Ca^{2+} ion influx into the cell, with complete restoration of normal membrane tension and lipid compositions taking place over minutes [304].

No studies, experimental or computational, have been performed identifying that the above cell membrane repair mechanisms are utilised to repair damage caused by MGs, however non- Ca^{2+} -dependent membrane repair mechanisms have been observed in cells treated with saturated FAs. Repair mechanisms in response to FAs have not been reported to employ traditional mechanisms, as Ca^{2+} ions occur in response to structural disruption while FAs tend to primarily cause bilayer leakage. Instead, FA repair typically occurs through adapting to FA insertion by altering localised lipid ratios to restore bilayer homeostasis and is particularly driven by the endoplasmic reticulum, which can drive changes to phospholipid synthesis to replace damaged sections of the membrane [305,306]. MGs behave more detergent-like than FAs which could potentially cause enough direct damage to the bilayer to allow significant Ca^{2+} leakage through the bilayer. In comparison to mammalian membranes, bacteria lack many equivalent pathways utilised by mammalian cells, including all Ca^{2+} mediated repair mechanisms. Bacterial cells also lack an endoplasmic reticulum. Damage to the cell envelope of bacteria induces the upregulation of gene expression to maintain bilayer lipid compositions or upregulate genes to survive in a stationary phase [307,308]. Additionally, the electron transport chain energy production pathway is localised to the

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plasma membrane of G+ bacteria, so disruption of bilayer properties may destabilise energy production in a way that the bacterial cell cannot recover from where energy production in mammalian cells remains uninhibited due to being localised to the mitochondria. MGs are considered Generally Recognised as Safe, so MGs are generally considered to be less cytotoxic to mammalian cells than FAs [39].

It is important to note, however, that these protective advantages of MGs only occur when comparing mammalian cells to planktonic bacteria. Bacteria primarily exist in biofilms - stationary colonies of bacteria which produce a thick, difficult to permeate extracellular matrix - as this provides a significant fitness advantage for bacteria cells by allowing them to avoid environmental stress, avoid immune detection during infection, and allow metabolic conservativity between heterogenic species within the biofilm [309]. Biofilms are notoriously difficult to penetrate for many molecules that display antimicrobial potency towards planktonic bacteria, and it has been reported that to eradicate a biofilm colony concentrations magnitudes greater than the planktonic bactericidal concentration must be used [310]. Although planktonic bactericidal concentrations of detergents have been shown clinically to be tolerable by mammalian cells, concentrations high enough to destroy biofilms would likely fall outside of the therapeutic window for potential treatments and cause toxic side-effects on host cells [311]. Biofilm formation has been highly associated with bacterial infection; in particular chronic infections such as bronchitis, but also in some acute infections [312]. Although most detergents struggle with penetrating biofilms, C12-MG has been repeatedly reported in literature to both inhibit the formation of biofilms and either disrupt or eradicate established bacterial biofilms against *Staphylococcus epidermidis*, *S. aureus*, and *P. aeruginosa* [40,313,314]. All three of these species are highly clinically relevant, MDR pathogens which are well known for the colonisation of medical devices, and subsequently forming difficult to disrupt biofilms. Given this, it would seem likely that treatments using MGs would be primarily focused on clearing planktonic bacteria, with potential for combination treatments which would assist MGs in dispersing biofilms.

Despite these considerations which need to be made when interpreting the results

presented in this chapter, limitations of this series of simulations detailed in the section below, continuing to model more realistic bacterial lipid bilayers consistently allows for greater insight regarding how MGs may interact with cell membranes once they move past prior barriers in the bacterial cell envelope. As complexity within a model grows, it enables hypotheses generated from more simplified models to be reevaluated, and potentially provide stronger evidence of behaviours to complement experimental research. In the context of this thesis, the findings presented within this Chapter have further evidenced the hypotheses presented regarding factors associated with MG-mediated lipid bilayer disruption within the previous Chapter, and have enabled more robust investigation of mechanisms that may be used to relieve the stresses generated by MGs in lipid bilayers.

6.5.3 Limitations of Study

The work presented in this chapter is not without its limitations. To complete the investigations required for this chapter 20 systems needed to be simulated for a duration of 600 ns each, totalling 12 μ s of simulation time. This required a significant investment of both computational and real-world time to complete and therefore it was decided to forego replicates of each simulation due to the time limitations of this project. This was not ideal, as repeating simulations would have given significant insight into the likelihood of phenomena in these systems occurring. Similarly, the timescale of the simulations provided a maximum of 600 ns, however qualitative approaches would normally see simulations extended to a microsecond onwards. A 600 ns production run was deemed appropriate for sampling of events in this methodology in Chapter 5 as all bilayer systems, other than the 4 symmetrical ones in Chapter 5, within these two chapters are inherently in disequilibrium due to the asymmetric inclusion of MGs and therefore the early events in the trajectory where the bilayer attempts to create mechanical equilibrium were considered the most relevant to this study. Despite this, extending systems where budding occurred would have enabled an increased understanding of bud stability and likelihood of scission occurring, as scission was only observed in the POPC_200_GMCY and GNIM_200_GMCY systems, and in some

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systems disruption did not take place until 300 ns into the simulation. Ideally, smaller intervals in concentration would have also been simulated in order to gain greater insight into specific concentration thresholds for budding to occur, however this was likewise unfeasible to be conducted systematically within the context of this study.

Additionally, there is notable lack of data driven analysis performed on systems where budding occurs, which is a consequence of the limitations of tools used in this chapter for analysis. Some of the tools used for system analysis in this chapter rely on the planarity of bilayers in order to calculate system properties. Bilayer thickness is calculated by MEMBPLUGIN as the distance between phosphorus atoms in the upper leaflet and the lower leaflet. Budding caused a loss of planarity in bilayers which in turn confused measurements, as a region of very high thickness was present in the upper leaflet, and caused average thickness readings to be produced that did not fully reflect the thickness of the planar portion of the bilayer. Area per lipid values were calculated in two dimensions across the xy plane, so area could not correctly account for lipid placement in a bud on top of the bilayer. To calculate 1D partial density profile systems, trajectories needed to undergo post-processing to centre the bilayer centre of mass (COM) at $z=0$ to remove the natural drift of the bilayer within the water box. Bud formation caused the bilayer COM to shift in the calculation as the bud grew, which produced incorrect density profiles as the bilayer centre was not consistent between frames. Specialised Python packages for bilayer analysis such as FATS LIM and LiPyPhilic exist which may provide suitable tools for extreme bilayer morphologies, however these tools could not be explored in the time remaining for this project when these issues were discovered. These systems could be re-explored with these tools in any future work which follows on from this project to assess whether more detail on the buds could be extracted from them.

While phospholipid bilayer models for both major bacterial cell envelope architecture were used in this chapter, there are several aspects of the bacterial envelopes which are not accounted for within these simulations. No proteins were present in either bacterial membrane model. These were omitted due to size and complexity, and the understanding that MG mechanisms were overwhelmingly

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associated with cell membrane destabilisation leading to unnecessary increases in simulation time if included, however the presence of proteins in the bilayer would influence local properties and lipid partitioning of a living bacterial membrane. These lipid ratios also do not contain hopanoids, which are a bacterial equivalent to cholesterol found in eukaryotic cell membranes and is a regulator of membrane fluidity and stability. The absence of hopanoids may therefore alter the susceptibility of these bilayer models to MG in comparison to bacterial cell membranes. The cell wall of the G⁺ bacterial envelope was also not modelled despite its proximity to the cell membrane, which would likely influence the ease of MGs to escape the bilayer, and would prevent the simulation artefact of MGs crossing PBCs to interact with the inner leaflet of the bilayer. An additional important limitation of the bilayer models selected were the lack of curvature in the systems. Bacterial cell membranes are not planar and instead can take on several morphologies in nature, including spherical, cylindrical, and helical. It has been shown in biophysical studies that bilayer morphology can influence FA and MG susceptibility between planar and spherical models, so it is likely that the planar nature of the bilayer models used in this chapter do not necessarily capture the full intricacies of how MGs disrupt the structure in already curved regions of the bilayer.

More significantly, no simulations detailing the effects of MGs on the G⁻ outer membrane (G-OM) were presented within this chapter. The G-OM is an important barrier mechanism to MG interaction with the phospholipid bilayer which the molecules must pass through to cause the disruption observed in these systems, so simulating MG interactions with this bilayer would likely provide useful insight into why G⁻ bacterial species tend to be more resistant to MGs than G⁺ species. Simulations using the same lipid composition as the G-OM model detailed in Pogozheva *et al.* were attempted as part of this project, however these systems required a much larger system size to accommodate *E. coli* lipid A within the simulation box, which caused calculations for the trajectory to be significantly slowed. As this coincided with issues with equilibration of G-OM systems with pre-inserted MG, and issues with the tools used being unusable for the analysis of a bilayer consisting of a lipid A monolayer and

phospholipid monolayer, it was decided that these systems were to be abandoned for this chapter.

6.6 Conclusions

The results presented from this investigation into how MGs influence phospholipid bilayer destabilisation in models representative of two common phospholipid bilayer compositions provide further insight into the behaviours cell membranes undergo in response to high concentrations of MGs.

- The degree of MG destabilisation is influenced by the composition of the lipid bilayer they insert into. Cell membranes which display tighter packing of lipid tails and looser packing of head groups are less immediately affected by the disruptive effects of MGs. This provides additional evidence that the mechanical stress induced by MG insertion is a significant driving force in their ability to destabilise bilayers.
- Curvature induction in lipid bilayers appears to be a highly important mechanism to relief of asymmetric stresses from MG insertion. Bending in bilayers is known to reduce stress between leaflets, and the two extreme behaviours observed in simulations with high asymmetric MG concentrations - budding and mass flip-flop - were both linked to high curvature induction in the bilayer.

Chapter 7

Energy Barriers to Monoglyceride Movement Through Lipid Bilayers

7.1 Rationale

The previous two results chapters have highlighted interesting phenomena which occur in phospholipid bilayers upon exposure to MGs in high concentrations. Although the results discussed in these chapters provide novel evidence of experimental hypotheses regarding membrane disruptive mechanisms of MGs, some discussions of the results turned towards speculation, relying on assumptions regarding how MGs are likely to interact with different systems to explain the behaviours observed.

This chapter presents a study designed to utilise US as a means to better understand the thermodynamic energy barriers that influence MG interactions with lipid bilayer systems. Where possible, the energy barriers calculated are compared to the behaviours observed in the previous two chapters to better contextualise why these MG behaviours were observed, and produce a more complete picture from MD simulations of MG-mediated lipid bilayer disruption.

7.2 Chapter Aims

1. Determine a suitable protocol for the US of MGs and lipid bilayers.
2. Use US to calculate the energy barriers to the movement into, and through, different lipid bilayer models for various MGs.
3. Use calculated potential of mean force values to provide further context to the behaviour of MGs observed in previous results chapters.

7.3 Methodology

7.3.1 System Setup

Lipid Bilayers

POPC, G-IM, and G+PM lipid bilayers were used for the simulations performed in this chapter. For unloaded systems, the starting structures for each of these bilayers were taken from the final frame of the production run for control systems used in Chapter 5 and Chapter 6, to ensure that systems had already undergone sufficient equilibration. The parameters of these models have been validated prior to this chapter in Chapter 5 (POPC) and Chapter 6 (G+PM and G-IM). Two further US used systems with C8-MG and C18-MG already present inside the upper leaflet lipid bilayer respectively. Lipid bilayers for these systems were taken from the corresponding asymmetrical membrane system's production run. For each system, the membrane segment was saved as a pdb and psf file and moved to a separate directory for system creation.

Monoglycerides

C8-MG, C10-MG, C12-MG, and C18-MG were used for US simulations with the POPC system. C10-MG was used for US of the G-IM and G+PM. C8-MG and C18-MG were used in US of POPC bilayers already containing MG. MG structures were positioned in solution within the simulation box 45 Å above the membrane centre ($z=0$) and tails were aligned with the Z axis. For each system, a single MG molecule was present in

the system, which was pulled through the membrane for subsequent SMD simulations to produce the reference positions required for each US. Following the placement of the MG in the simulation box, the system was solvated with water and NaCl ions were added to the water box to neutralise the system charge where applicable and increase the concentration to 0.15 mol/L.

7.3.2 Steered Molecular Dynamics Simulation Setup

SMD simulations were performed using NAMD Version 3.0b. Dummy SMD atoms were attached to each atom of the MG molecule and were pulled at a constant velocity of 5×10^{-6} Å/fs in the -z direction. This velocity was selected after testing two additional velocities of 1×10^{-7} Å/fs and 2.5×10^{-6} Å/fs, discussed in Section 7.4.1 (Determining SMD Velocity). A harmonic constraint force of 1 kcal/mol/Å² was used for each SMD simulation. The pulling was performed for 25 ns using an NPT ensemble with a constant temperature of 298.15 K, controlled using a Langevin thermostat, and constant pressure of 1.01325 Bar, controlled using the Langevin Piston method. PBC and PME were employed. Prior to the pulling, a water minimisation with all atoms except those of the water molecules fixed was performed for 1 ns using an NPT ensemble with the same parameters as the pulling stage. A 1 fs timestep was used for both parts of the simulation.

7.3.3 Umbrella Sampling Setup

Generated SMD trajectories were used to obtain reference frames for each US. 36 windows were created for each US simulation spanning the Z axis range of 0 to 35 Å and were spaced at equal 1 Å intervals for all systems except POPC_GMS which ranged from 0 to 34 Å as this was the first US conducted before the methodology was refined. Figure 7.1 illustrates window the initial US setup and MG starting positions in the first and last windows. Each window was simulated independently for 50 ns with a 2 fs timestep except for POPC_GMS where each window was simulated for 40 ns. The total simulation time for each US was 1.8 μ s. Simulations were performed using an NPT ensemble with a constant pressure of 1.0135 bar, controlled by the langevin

piston method, and a constant temperature of 298.15 K. PME was employed with 1.0 grid spacing and a cut-off of 12.0 Å. The collective variable was defined as the Z-axis distance between the centre of mass (COM) of the SMD MG and the phosphorus atom of each phospholipid molecule in both leaflets of the lipid bilayer. A harmonic constant of $K = 0.025 \text{ kcal mol}^{-1}$ was used. PBC wrapping was not utilised in the simulation of US windows. US simulations in this chapter will be referred to as shown in Table 7.1.

Table 7.1: US system names, bilayer models, and MG species used to create US windows.

Name	Bilayer Model	MG Bilayer Content	Pulled MG
POPC_GMCY	POPC	None	C8-MG
POPC_GMC	POPC	None	C10-MG
POPC_GML	POPC	None	C12-MG
POPC_GMS	POPC	None	C18-MG
POPC_ASYM_GMCY	POPC	44 C8-MG Upper	C8-MG
POPC_ASYM_GMS	POPC	44 C18-MG Upper	C18-MG
GPPM_GMC	G+PM	None	C10-MG
GNIM_GMC	G-IM	None	C10-MG

7.3.4 Analysis

Free energy curves were calculated from US windows using the Weighted Histogram Analysis Method (WHAM) [229]. 300 bins, a convergence tolerance of 0.001, and temperature of 298.15 K were selected for histogram generation. For each free energy profile, ΔG_{exit} was defined as the free energy difference between the minimum PMF of the MG inside the bilayer and the rightmost peak around the +25 Å region of the curve, which represented the point of MG entry into the bilayer from solution, in kJ/mol (partition free energy). ΔG_{trans} was defined as the free energy difference between the minimum PMF of the MG inside the bilayer and the PMF of the membrane centre ($z=0$) in kJ/mol (translocation free energy). ΔG_{in} was defined as the free energy difference between $z=35$ and the rightmost peak in the PMF curve in kJ/mol (insertion free energy). The preferred position of the MG within the bilayer was determined as the minimum PMF of the MG inside the bilayer. All data was normalised such that $z=35$ was 0 kJ/mol to allow comparison between PMF curves. Estimated timescale for

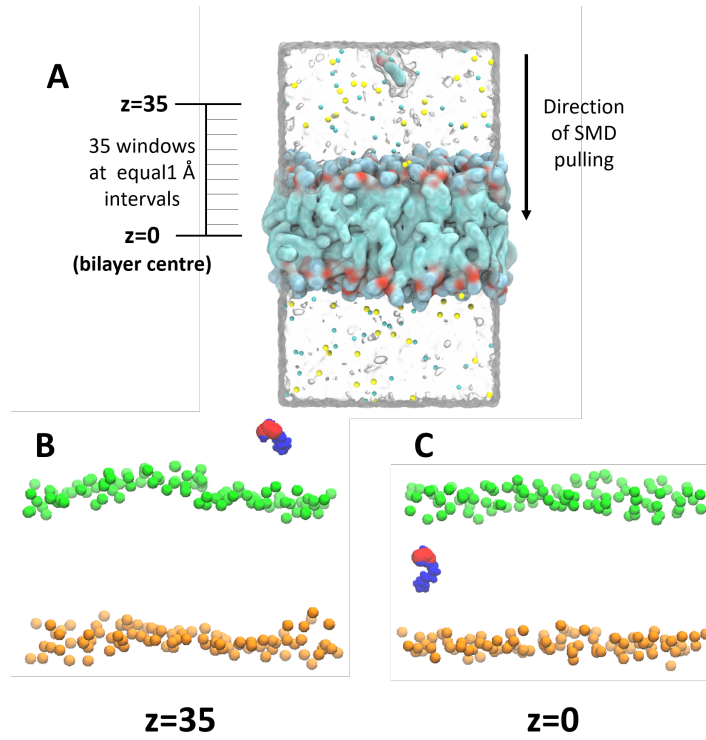


Figure 7.1: (A) Initial system setup for US. (B-C) Snapshots of starting C8-MG position at windows $z=35$ (B) and $z=0$ (C).

energy barriers being crossed was calculated as

$$\tau = \frac{1}{k} \quad (7.1)$$

where k is escape rate calculated using Kramer's Escape Rate

$$k = v e^{-\frac{\Delta G}{k_B T}} \quad (7.2)$$

where v represents escape attempt frequency (10^{11} s^{-1}) and $e^{-\frac{\Delta G}{k_B T}}$ represents the Boltzmann probability factor for a specified energy barrier ΔG .

7.4 Results

7.4.1 Determining SMD Velocity

Prior to the US, SMD simulations needed to be performed to provide the reference structure for each window. A constant K value of $1 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ was used, and three SMD velocities ($1 \times 10^{-5} \text{ \AA/fs}$, $2.5 \times 10^{-6} \text{ \AA/fs}$, and $5 \times 10^{-6} \text{ \AA/fs}$) were initially used during the SMD phase of the first two US (POPC_GMCY, and POPC_GMS) before a single velocity was decided upon to be used for all subsequent US. $1 \times 10^{-5} \text{ \AA/fs}$ was the first velocity tested. This velocity was deemed unsuitable after conducting a pulling of C18-MG. At this velocity, C18-MG did not have adequate time to properly orient itself with the membrane normal as the MG was translocated through the upper leaflet and lower leaflet (Figure 7.2 A). This made this velocity unsuitable for creating the starting structures for each US window, as incorrect alignment of the MG within the membrane would require additional equilibration time to fix the orientation.

This problem was not reflected in the lower two velocities, $5 \times 10^{-6} \text{ \AA/fs}$ and $2.5 \times 10^{-6} \text{ \AA/fs}$, as in both pulling molecules correctly oriented in the membrane (Figure 7.2 B). Although both provided a suitable reference point for US window generation, $2.5 \times 10^{-6} \text{ \AA/fs}$ requires a simulation a factor of two longer than $5 \times 10^{-6} \text{ \AA/fs}$ to cross the simulation box through the bilayer. Because of this, $5 \times 10^{-6} \text{ \AA/fs}$ was ultimately selected as the velocity to continue with for the remaining SMD simulations to improve computational efficiency, as differences between $5 \times 10^{-6} \text{ \AA/fs}$ and $2.5 \times 10^{-6} \text{ \AA/fs}$ for the purpose of providing a reference for US was negligible.

7.4.2 Umbrella Sampling Validation

It is important to validate each US before trusting the PMF values generated from the simulations. Validation can be done in two ways. These are convergence of PMF, and window overlap.

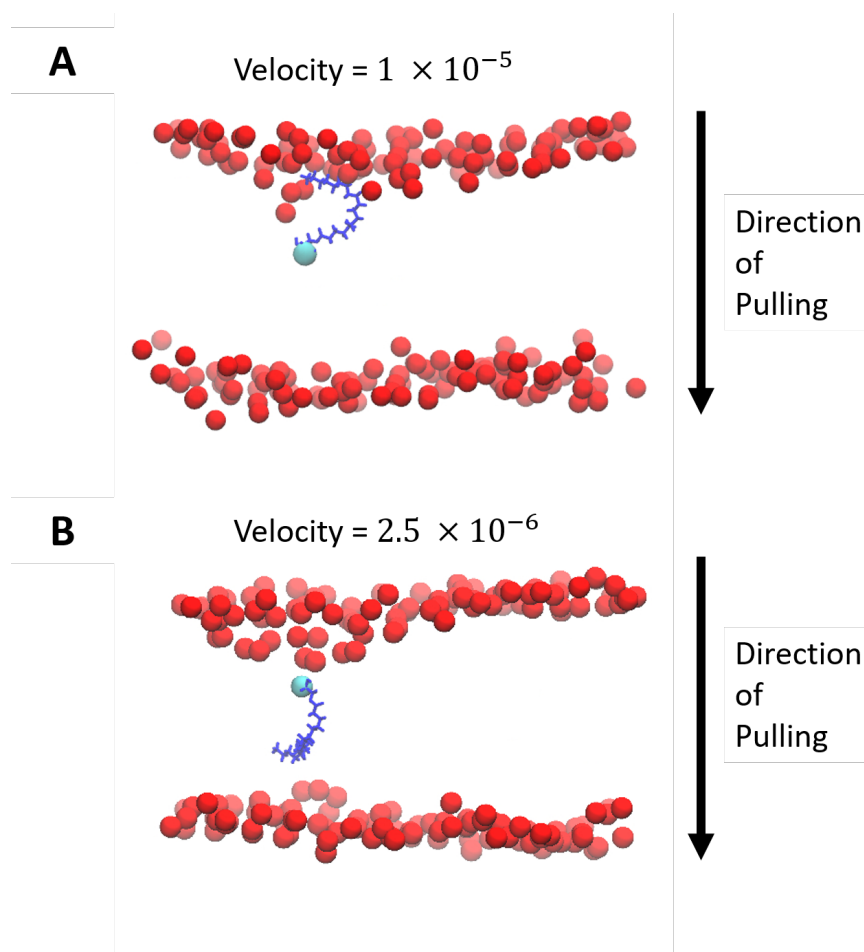


Figure 7.2: Alignment of CV-SMD pulled C18-MG with the bilayer normal at velocities 1×10^{-5} (A) and 2.5×10^{-6} (B). Red sphere represent the boundaries of the upper and lower leaflet. Dark blue region represents the C18-MG tail region. Light blue sphere represents the C18-MG headgroup. Both SMD pullings were performed in the -Z direction.

Umbrella Sampling Window Overlap

Window overlap within the histogram generated using WHAM is a measure of whether bias has been successfully removed to calculate an accurate PMF curve, by confirming all regions of the collective variable have received adequate sampling. Histograms produced by WHAM for each system are found in Figure 7.3.

Across all US, overlap between windows was very strong, indicating good sampling along the whole reaction coordinate. In addition, the umbrellas for each window displayed strong symmetry, which indicates that individual windows were also sampled

well. Strong sampling of the reaction coordinate prevents offsets in the continuous free energy profiles calculated by WHAM, as poorly sampled regions along the reaction coordinate requires WHAM to estimate PMF when removing bias, which can significantly offset calculated PMF values depending on the position of the poorly sampled region along the free energy curve. Count in these figures refers to the number of times in a window a specific position along the reaction coordinate was visited during the sampling, hence why the histogram for POPC_GMS (Figure 7.3 D), which was sampled for 40ns instead of the 50ns the windows of each other system was simulated for, displayed average count peaks around 20% lower than all other systems.

Chapter 7. Energy Barriers to Monoglyceride Movement Through Lipid Bilayers

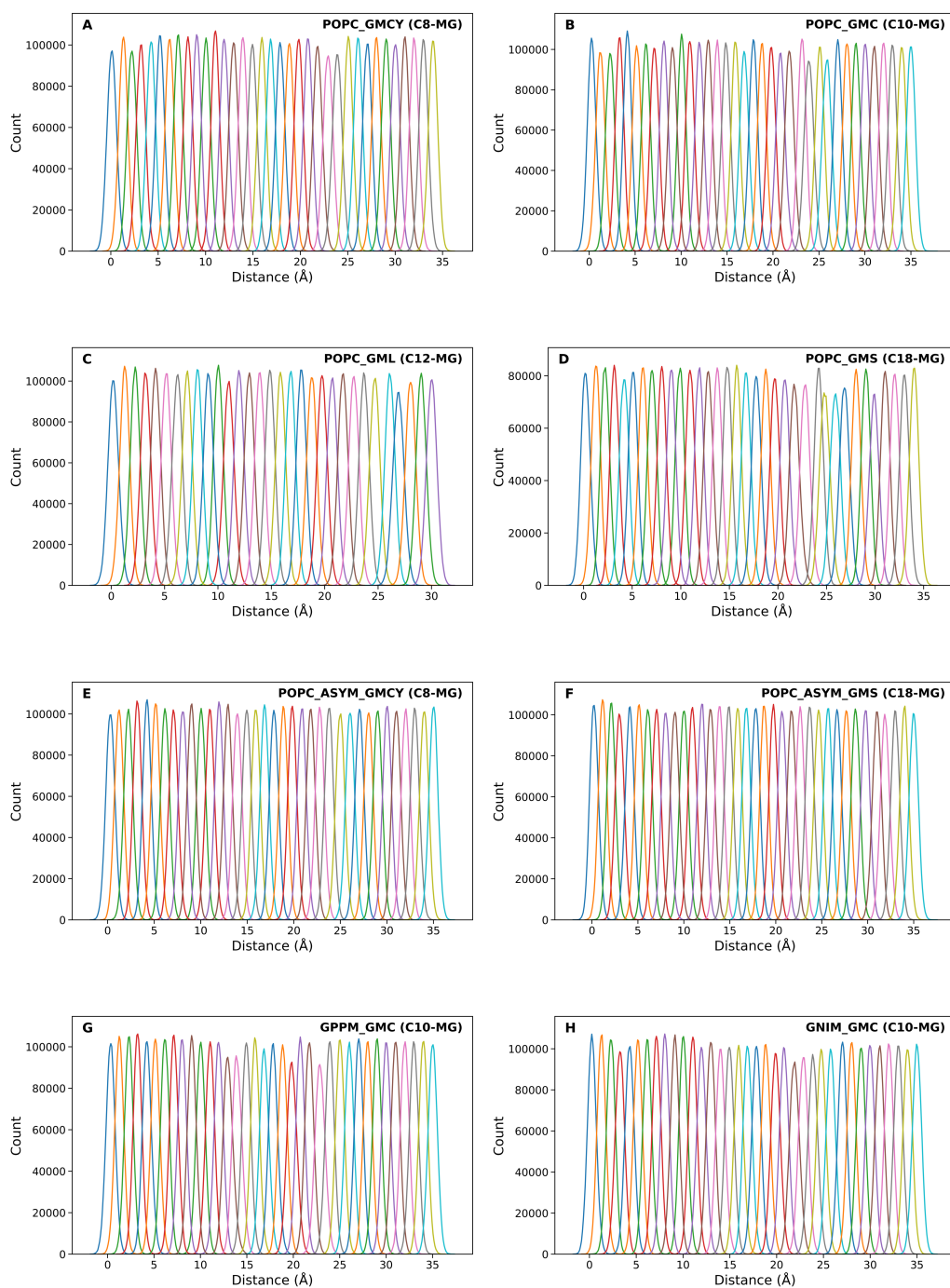


Figure 7.3: Histograms for US POPC_GMCY (A), POPC_GMC (B), POPC_GML (C), POPC_GMS (D), POPC_ASYM_GMCY (E), POPC_ASYM_GMS (F), GPPM_GMC (G), GNIM_GMC (H).

Umbrella Sampling PMF Convergence

PMF convergence was assessed by calculating the PMF curve across several blocks of time. PMF was calculated in several blocks for each system - the last 10/20/30/40ns of the system, and the full 50ns, except for system POPC_GMS, which was limited to 40 ns. Convergence curves are displayed in Figure 7.4. The extent of convergence varied significantly between systems. The curves for POPC_GML and POPC_ASYM_GMS displayed fairly strong overlap, with variance at the energy minimum being around 2.5 kJ/mol for POPC_GML and around 5 kJ/mol for POPC_ASYM_GMS. Other than the final 10 ns curve, POPC_ASYM_GMCY also displayed strong convergence with a variance of about 2 kJ/mol. Poor convergence in PMF curves is an indicator that more sampling is required, which would cast some doubt on the accuracy of the energy barriers calculated for a simulation. Each window was simulated for 50 ns (40 ns for POPC_GMS) as it was expected to be a sufficient simulation time to fully sample each region along the reaction coordinate, and it was not feasible within the confines of this study to uniformly extend these windows to the point where suitable convergence was observed. Therefore, results obtained in this chapter should be treated as indicative of potential energy barriers for MG movement through a specific bilayer model, rather than concrete evidence for exact energy barriers within the model bilayer systems. Ultimately, the PMF for the final 30 ns of each US was selected for analysis going forwards to enable consistency in comparisons between US. To determine the range of values the relevant free barriers along the PMF curves the minimum and maximum value between the different times for each system was recorded. Two of the curves were excluded as outliers for each system when doing so. The final 10 ns were not considered as this length of time was not long enough for the data to be considered viable. The curve sampling the full simulation time was also excluded to remove bias towards the initial starting structure in each window.

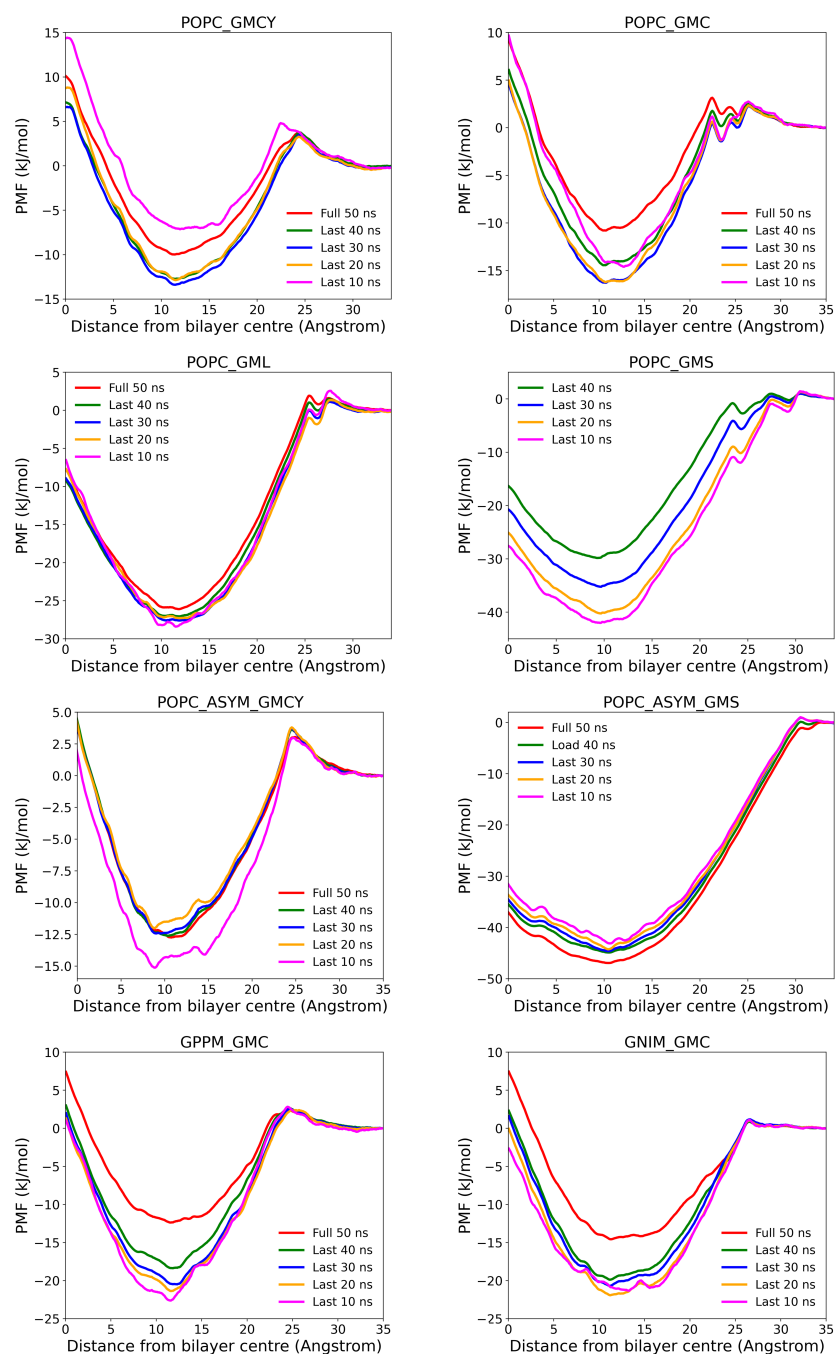


Figure 7.4: PMF convergence for US POPC_GMCY, POPC_GMC, POPC_GML, POPC_GMS, POPC_ASYM_GMCY, POPC_ASYM_GMS, GPPM_GMC, GNIM_GMC. Red represents 50 ns sampled, green represents the last 40 ns, blue represents the last 30 ns, yellow represents the last 20 ns, and pink represents the last 10 ns.

7.4.3 Calculation of Free Energy Barriers Associated with Monoglyceride Insertion into Model Lipid Bilayers

7.4.4 POPC Systems

When interpreting the PMF curve of a leaflet, the position along the X-axis indicates the position of the MG within the leaflet, as is shown in Figure 7.5, The lowest point in the PMF curve represents the most energetically favourable position within the leaflet. As shown in this Figure the MG is very strongly aligned with the bilayer normal, which was expected as this is the most thermodynamically favourable conformation for the MG within the bilayer, and the region that MG tended to accumulate at, as shown in density profiles in previous chapters. The dotted horizontal line on Figure 7.6 represents $k_B T$, calculated as the product of the Boltzmann constant $k_B T$ (1.38×10^{-23} J/K) and temperature T at the temperature the US were conducted at (298.15K). $k_B T$ in this graph represents the thermal fluctuation of molecules at room temperature, and therefore the likelihood of a thermal barrier being spontaneously crossed. At this temperature, $k_B T$ is 2.479 kJ/mol, as marked on the Figure. There were four key free energy barriers calculated for understanding MG energetics, also marked on Figure 7.5. ΔG_{in} (green) was the initial free energy barrier required for a MG monomer to partition into a bilayer leaflet from the aqueous solution. ΔG_{par} (blue) was the partition free energy of the system, which represents the thermodynamic stability of the MG in its optimal position in the bilayer in relation to its stability in solution. For all curves presented PMF curves in this chapter the ΔG_{par} value was negative, which indicated that all MG molecules were more thermodynamically stable for MG monomers to exist within the bilayer than within solution. ΔG_{exit} (orange) was the free energy difference between the highest point the PMF curve and the lowest point of the PMF curve, which indicated the energy barrier required for a MG monomer to exit the bilayer. The final energy barrier, ΔG_{trans} (red) represented the energy barrier that needed to be crossed in order for the molecule to reach the centre of the bilayer, which would in theory be the most energetically unfavourable position within the hydrophobic core. In bilayers with complete symmetry between leaflets it is assumed that the free energy barriers are

identical between leaflets and therefore the free energy barriers calculated could also be mirrored to the lower leaflet, though as discussed later in this chapter in Section 7.5.1 subsection 2 (Potential Issues with Window Sampling) the free energy barriers calculated for ΔG_{trans} may have some inaccuracy.

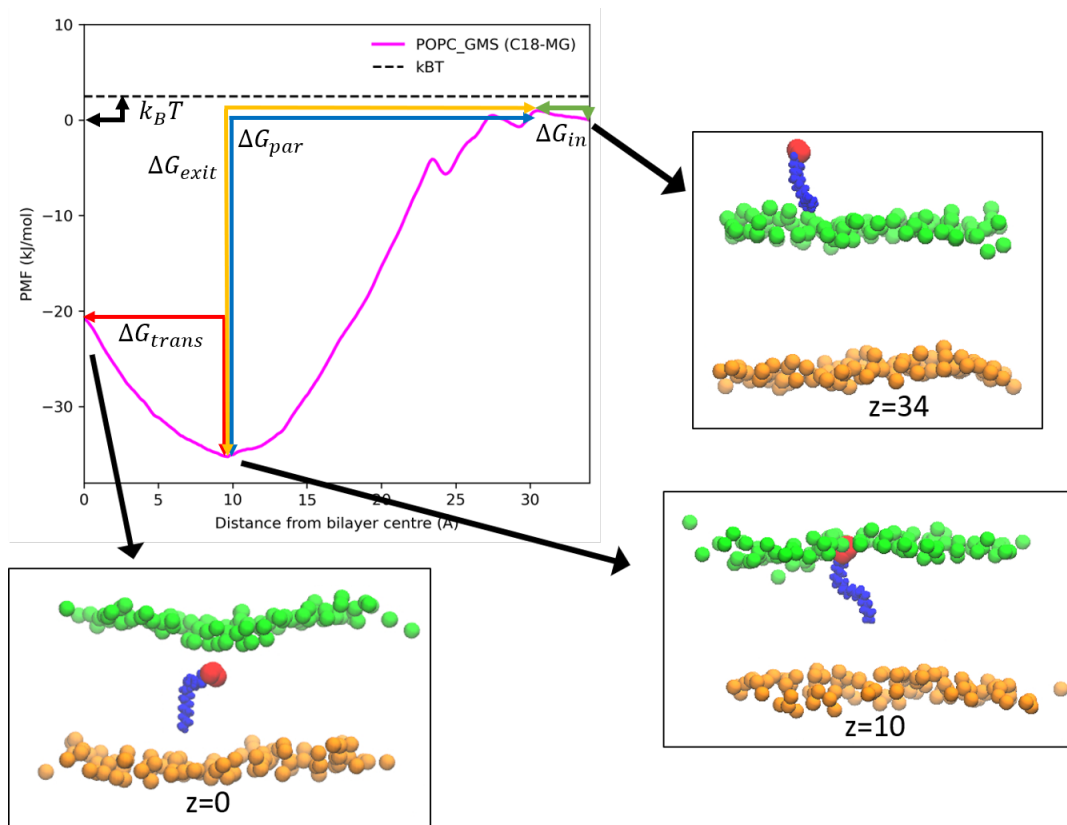


Figure 7.5: Positions of C18-MG at different positions along the reaction coordinate. $z=34$ shows the presence of the molecule outside the bilayer system. $z=10$ shows C18-MG at its most energetically favourable position within the bilayer. $z=0$ represents the position of C18-MG at the bilayer centre. Green spheres represent the headgroup region and outer boundary of the upper leaflet. Orange spheres represent the headgroup region and outer boundary of the upper leaflet. Red represents the headgroup of C18-MG. ΔG_{par} represents the point of MG entry into the bilayer from solution, in kJ/mol (partition free energy). ΔG_{trans} represents the free energy difference between the minimum PMF of the MG inside the bilayer and the PMF of the membrane centre ($z=0$) in kJ/mol (translocation free energy). ΔG_{exit} represents the free energy barrier required for the MG to exit the bilayer. ΔG_{in} represents the free energy difference between $z=35$ and the rightmost peak in the PMF curve in kJ/mol (insertion free energy).

The PMF curves for the 4 MG US using a pure POPC bilayer are displayed in

Figure 7.6, and reported free energy values in Table 7.2.

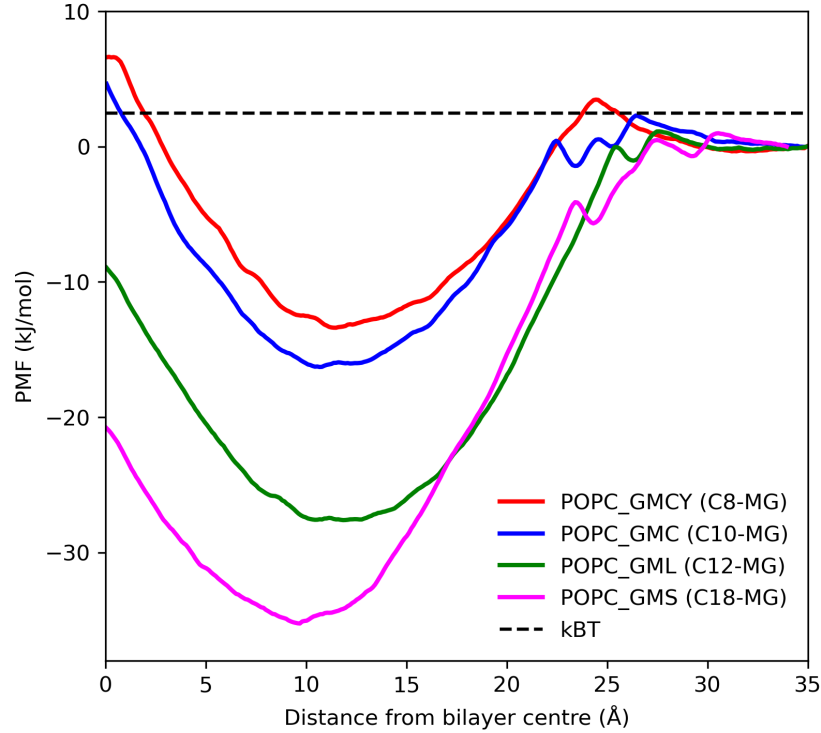


Figure 7.6: Normalised free energy curve for movement of C8-MG (red), C10-MG (blue), C12-MG (green), and C18-MG (pink) through the upper leaflet of POPC bilayer systems.

Table 7.2: Calculated energy barriers for MG entry into POPC bilayer leaflet (ΔG_{in}), MG exit from POPC bilayer leaflet (ΔG_{exit}), MG partitioning from solution into the bilayer (ΔG_{par}), and movement across the POPC bilayer leaflet (ΔG_{trans}). Brackets indicate the variation of ΔG energy barrier values obtained from the final 20 ns, 30 ns, and 40 ns of the trajectory (final 20 ns and 30 ns for POPC_GMS), with the lower and upper limits corresponding to the minimum and maximum calculated energy barriers respectively. All values are presented in kJ/mol.

System	ΔG_{in}	ΔG_{exit}	ΔG_{par}	ΔG_{trans}
POPC_GMCY	3.5 (3.3, 3.7)	16.6 (15.8, 16.6)	-13.1 (-13.1, -12.5)	19.8 (19.6, 21.3)
POPC_GMC	2.3 (2.3/2.6)	18.6 (17.0, 18.6)	-16.3 (-16.3, -14.4)	20.9 (20.5, 21.2)
POPC_GML	1.1 (1.1, 1.5)	28.7 (28.5, 28.7)	-27.6 (-27.6, -27.0)	18.6 (17.8, 19.5)
POPC_GMS	0.3 (0.3, 0.3)	35.5 (35.5, 40.5)	-45.2 (-45.2, -35.2)	14.4 (14.4, 15.1)

The collective variable region of 20-30 Å represented the simulation box region where the MG reached the lipid-water interface, and consequently any changes to free energy above the baseline 0 kJ/mol indicates potential energy barriers to MG entry into the

bilayer (ΔG_{in}). For C10-MG, C12-MG, and C18-MG, all peaks in this region occurred below $k_B T$ and C8-MG displayed an energy barrier of around 3.5 kJ/mol which is just $1.41 k_B T$. This indicates that energetically all four MGs displayed very low barriers to entry into POPC lipid bilayers, easily reachable through standard thermal fluctuation and movement into the membrane would be expected to occur spontaneously at a ps scale. This ease of MG insertion was observed in unbiased MD simulations presented in Chapters 5, where MG molecules that exited the bilayer were able to spontaneously re-insert into the a bilayer leaflet near immediately following contact with the structure.

An energy minimum was observed in each PMF curve in Figure 7.6, corresponding to the most energetically favourable position along collective variable (Z-axis distance between MG COM and phosphorus atom in both leaflets of the lipid bilayer) within the upper leaflet. These positions aligned with observed peaks for MG density in the partial mass density profiles presented in Chapter 5. This would explain why C8-MG, C10-MG and C12-MG tended to accumulate around the +10-11 Å position in the bilayer in simulations. The density profile for C18-MG displayed in Chapter 5 did not reflect the preferred position of +9 Å, though this may be due to the significant increase in leaflet order observed in the upper leaflet of POPC systems when high concentrations of C18-MG were present in the upper leaflet, causing greater alignment of C18-MG tails with the z axis and therefore more consistent positioning. Figure 7.5 provides an example of MG positions at various points in the PMF curve.

In reference to reported ΔG_{exit} and ΔG_{trans} values in Table 7.2, calculated energy barriers were several times greater than $k_B T$. Calculating estimated timescale required for energy barriers to be crossed using the escape rate from the Kramer’s Escape Rate formula, the energy barriers for MG expulsion from the membrane (ΔG_{exit}) indicated that in an unloaded POPC bilayer (without MG present) C8-MG and C10-MG would be expected to readily exit the bilayer at a nanosecond scale, with C8-MG having the lowest expected timescale of 80.9 ns. C10-MG was more than two times slower at crossing this energy barrier at an expected 181.4 ns, while C12-MG would take 10.6 μ s, and C18-MG 165.7 μ s, as calculated by $\tau = \frac{1}{k}$. These timescales reflect significant differences in the ease of MGs to leave a bilayer they insert into. Generally, this also

aligned with observed MG behaviour in POPC simulations relatively accurately, as throughout the 600 ns trajectories no expulsion of C12-MG and C18-MGs was observed, while C8-MG and C10-MG simulations displayed frequent expulsion of MGs from the upper leaflet.

For C12-MG and C18-MG, the energy barriers for flip-flop (ΔG_{trans}) were significantly lower than the energy barriers for exit, which would suggest that movement within the bilayer was much more energetically favourable than these molecules leaving the bilayer. This timescale would place the estimated time for movement across the centre of the membrane to be 181.4 ns for C12-MG and 33.3 ns for C18-MG, though this does not necessarily reflect behaviour observed in Chapter 5 systems, as only one instance of C12-MG movement was observed and no movement of C18-MG was observed in either the symmetrical or asymmetrical 100 MG loaded simulation for these MGs. As the leaflet composition of the POPC system was symmetrical, it would be expected that identical PMF curves would be produced through sampling the lower leaflet, so this may suggest that the presence of other MGs in a bilayer disrupts bilayer properties in a way that may alter energy barriers to movement within the bilayer, though this is discussed as potentially not being the case further in Section 7.1.5. The lower energy barrier for movement through the bilayer compared to exiting the bilayer for C12-MG may also explain why disruption of bilayer planarity across all systems tended to occur less with C12-MG than C8-MG or C10-MG, as exiting the bilayer would be much less energetically favourable.

The ΔG_{trans} barriers for C8-MG and C10-MG were greater than ΔG_{exit} by 3.2 kJ/mol for C8-MG and 2.3 kJ/mol for C10-MG, which aligns with the observation that these molecules exiting the bilayer was more preferred to movement towards the lower leaflet. This would also align with the hypothesised stress relief mechanisms for MG-induced bilayer disruption, as it would be more energetically favourable for these molecules to be expelled outwards, forming the bud structures observed on highly disrupted bilayers containing C8-MG and C10-MG, than relieve stress by mass movement of MGs to the lower leaflet, as was observed in some C12-MG containing systems. The readiness of C8-MG and C10-MG to exit the bilayer in comparison to

C12-MG may also explain some experimental differences between the molecules, in particular C10-MG and C12-MG. The expected time of $10.7 \mu\text{s}$ required for C12-MG to overcome the energy barrier to exit the membrane in comparison to 181.4 ns for C10-MG would likely result in high sustained concentrations of C12-MG within the bilayer, particularly in the upper leaflet, for longer periods of time than C10-MG, causing asymmetric stresses to accumulate in the leaflet for longer and impact bilayer function over a greater timescale, potentially causing greater disruption to essential bilayer processes despite the apparent higher counts of C12-MG molecules in a leaflet required to cause immediate degradation of the membrane structure.

7.4.5 MG-Loaded POPC Systems

To further explore whether the presence of MGs in a bilayer would affect energy barriers to MG movement through the POPC bilayer, two additional US were performed using lipid bilayers with 44 MG molecules pre-inserted into the upper leaflet for C8-MG and C18-MG (Figures 7.7, 7.8). These two MGs were selected as the two extremes of the molecules tested, and differed significantly in the extent that the presence of other MGs already within a bilayer affected their respective free energy curves.

For C8-MG (Figure 7.7, Table 7.3), the two curves are very similar in shape. ΔG_{in} values were near identical between the two US, with only a 0.3 kJ/mol difference. This indicated that the energy barrier C8-MG was required to overcome in order to enter into the bilayer was not significantly influenced by MG disruption of bilayer properties, as this difference was equivalent to a 53.2 ps increase in time expected for a C8-MG molecule to insert into a C8-MG loaded bilayer. In POPC_ASYM_GMCY, the most energetically favourable position along the reaction shifted approximately $2.5 \text{ \AA}$ towards the membrane centre. ΔG_{exit} was lower for POPC_ASYM_GMCY by 0.4 kJ/mol, which was a timescale of 68.9 ns in comparison to 80.9 ns for POPC_GMCY. This indicated that although the energy barrier to C8-MG entry was not affected by the existence of other MG molecules in the bilayer, disruption by C8-MG may cause individual C8-MG molecules to exit the bilayer slightly more readily, though based off expected minimum and maximum barriers calculated from convergence having very high overlap

this would likely not cause a significant difference between the two bilayer states. The increased rate of MG exit at higher concentrations was observed in Chapter 5 for C10-MG, so due to similarity in ΔG_{exit} values between the two MGs in undisrupted POPC bilayers in Figure 7.6 it could be assumed that the same would also be true for C8-MG. The difference in ΔG_{trans} free energy barriers between the two molecules was more significant, being 3.5 kJ/mol lower for C8-MG to move past the bilayer centre of the disrupted leaflet. This was 4.1 times quicker than in the undisrupted leaflet, which could explain why some movement of MG to the lower leaflet occurred simultaneously with bud initiation in systems very severely disrupted by C8-MG. ΔG_{trans} being slightly lower than ΔG_{exit} in POPC_ASYM_GMCY when the opposite was true for POPC_GMCY further evidences that disruption caused by the presence of MG in a bilayer may affect the behaviour exhibited by MG molecules inside lipid bilayers.

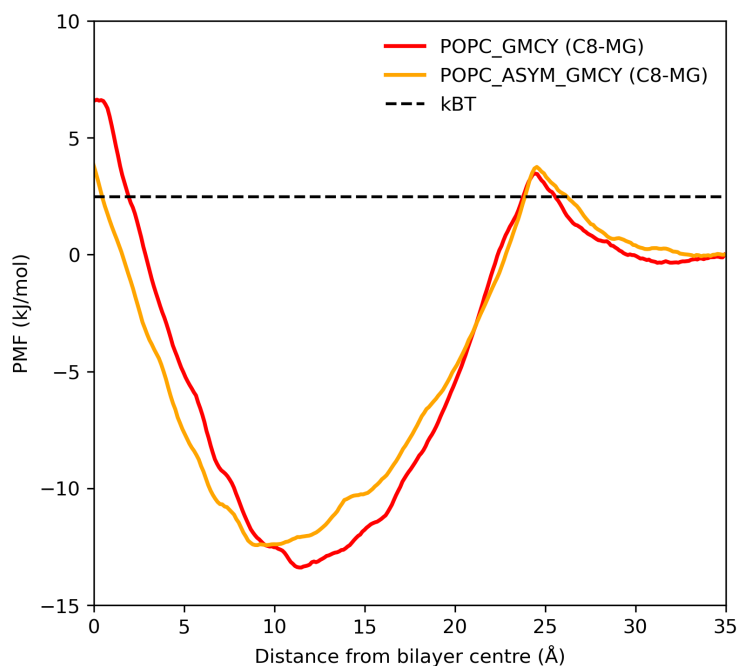


Figure 7.7: Normalised free energy curve for movement of C8-MG through the upper leaflet of a pure POPC bilayer system (red) and loaded POPC bilayer (orange).

C18-MG displayed significant differences between the pure POPC and MG loaded bilayers inside the bilayer, however the ΔG_{in} barrier to C18-MG entry into the bilayer was entirely unaffected by the prior changes to leaflet properties by C18-MG (Figure

Table 7.3: Calculated energy barriers for MG entry into POPC bilayer leaflet loaded by MG (ΔG_{in}), MG exit from POPC bilayer leaflet (ΔG_{exit}), MG partitioning from solution into the bilayer (ΔG_{par}), and movement across the POPC bilayer leaflet (ΔG_{trans}). Brackets indicate the variation of ΔG energy barrier values obtained from the final 20 ns, 30 ns, and 40 ns of the trajectory, with the lower and upper limits corresponding to the minimum and maximum calculated energy barriers respectively. All values are presented in kJ/mol.

System	ΔG_{in}	ΔG_{exit}	ΔG_{par}	ΔG_{trans}
POPC_GMCY	3.5 (3.3, 3.7)	16.6 (15.8, 16.6)	-13.1 (-13.1, -12.5)	19.8 (19.6, 21.3)
POPC_ASYM_GMCY	3.8 (3.6, 3.8)	16.2 (15.8, 16.3)	-12.4 (-12.6, -11.9)	16.3 (16.3, 17.0)

7.8, Table 7.4). The PMF curve for POPC_ASYM_GMS was much shallower than POPC_GMS, displaying a fairly linear decline in free energy from around 30 Å to 15 Å where the curve in POPC_GMS had a fairly shallow and uneven free energy curve from 30 Å to 23 Å before becoming more linear. This may have been caused in part by the poor sampling of the POPC_GMS system, however. The energy minimum for C18-MG within the leaflet moved 1 Å further from the bilayer centre, being located at +11 Å in POPC_ASYM_GMS and 10 Å in POPC_GMS. This may be due to the expansion in leaflet P-P thickness consistently observed in systems containing C18-MG. The ΔG_{exit} energy barrier for both systems were significantly higher than their respective ΔG_{trans} values, however the difference between ΔG_{exit} and ΔG_{trans} was not proportional between the two curves. In POPC_GMS there was a difference of 21.1 kJ/mol between the two, meaning a 2.5 times greater ΔG_{exit} barrier while POPC_ASYM_GMS had a 35.3 kJ/mol and 4.5 times difference between the two barriers. The ΔG_{exit} barrier estimated crossing timescale was extremely high relative to those estimated for MCMGs for both systems, with an expected time of 165.7 μ s for POPC_GMS and 9.7 ms for POPC_ASYM_GMS. This significantly slower crossing time aligns with our knowledge of C18-MG as a stabiliser molecule that increases order in bilayers and therefore increases the energy barriers required for molecules to cross the bilayer and aligns with the lack of C18-MG movement observed in Chapter 5 simulations [123]. The calculated ΔG_{trans} values appear less accurate, however, as no movement was observed across leaflets in any simulations despite the calculated energy barrier suggesting this to occur at a ns timescale.

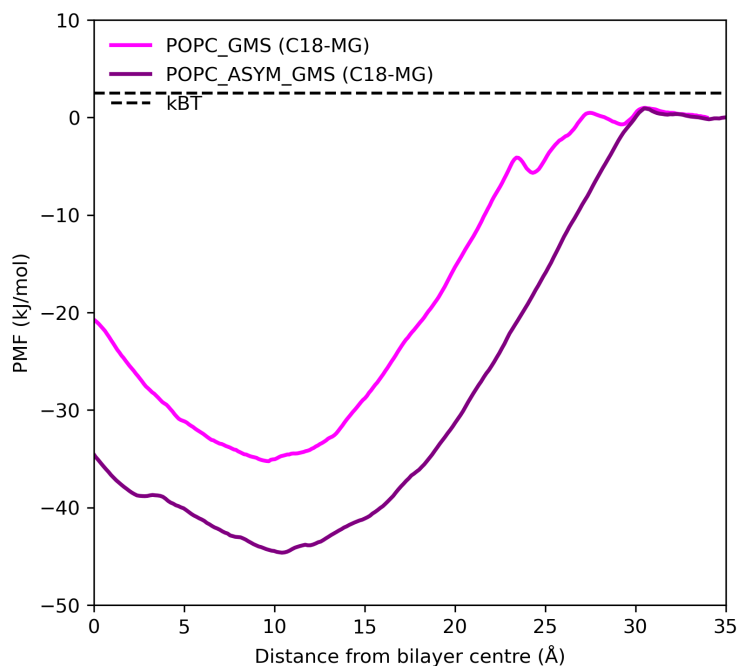


Figure 7.8: Normalised free energy curve for movement of C18-MG through the upper leaflet of a pure POPC bilayer system (pink) and C18-MG loaded POPC bilayer (purple).

Table 7.4: Calculated energy barriers for MG entry into POPC bilayer leaflet loaded with C18-MG (ΔG_{in}), MG exit from POPC bilayer leaflet (ΔG_{exit}), MG partitioning from solution into the bilayer (ΔG_{par}), and movement across the POPC bilayer leaflet (ΔG_{trans}). Brackets indicate the variation of ΔG energy barrier values obtained from the final 20 ns and 30 ns of the trajectory, with the lower and upper limits corresponding to the minimum and maximum calculated energy barriers respectively. All values are presented in kJ/mol.

Name	ΔG_{in}	ΔG_{exit}	ΔG_{par}	ΔG_{trans}
POPC_GMS	0.3 (0.3, 0.3)	35.5 (35.5, 40.5)	-45.2 (-45.2, -35.2)	14.4 (14.4, 15.1)
POPC_ASYM_GMS	0.9 (0.3, 1.0)	45.6 (45.2, 45.6)	-44.6 (-44.9, -44.2)	10.2 (9.2, 10.4)

7.4.6 Bacterial Membrane Model Systems

Two final US were performed to identify potential energy barrier differences for MG movement between the unloaded POPC bilayer and unloaded bacterial membrane models (Figure 7.9). C10-MG was utilised in these studies due to it sitting between the two other MCMGs in terms of disruption observed in Chapter 5 and Chapter 6, and had experimental backing in terms of antimicrobial activity against bacterial species

which is not consistently reported for C8-MG [164]. For both bacterial membrane models, ΔG_{in} values indicated that these models also had negligible energy barriers to MG entry into the bilayer, as they are all below $1K_B T$. GNIM_GMC displayed the lowest energy barrier, while GPPM_GMC displayed a similar barrier to POPC_GMC. Peaks differed in location between the three systems. The initial free energy peak for GPPM_GMC occurred at +24.7 Å while the other two sat at +26.5 Å. The free energy minima of C10-MG appeared to sit at +10 Å for POPC, +11 Å for G-IM, and +12 Å for G+IM, though poor sampling in the free energy curve for the bacterial membrane US windows makes this difficult to confirm.

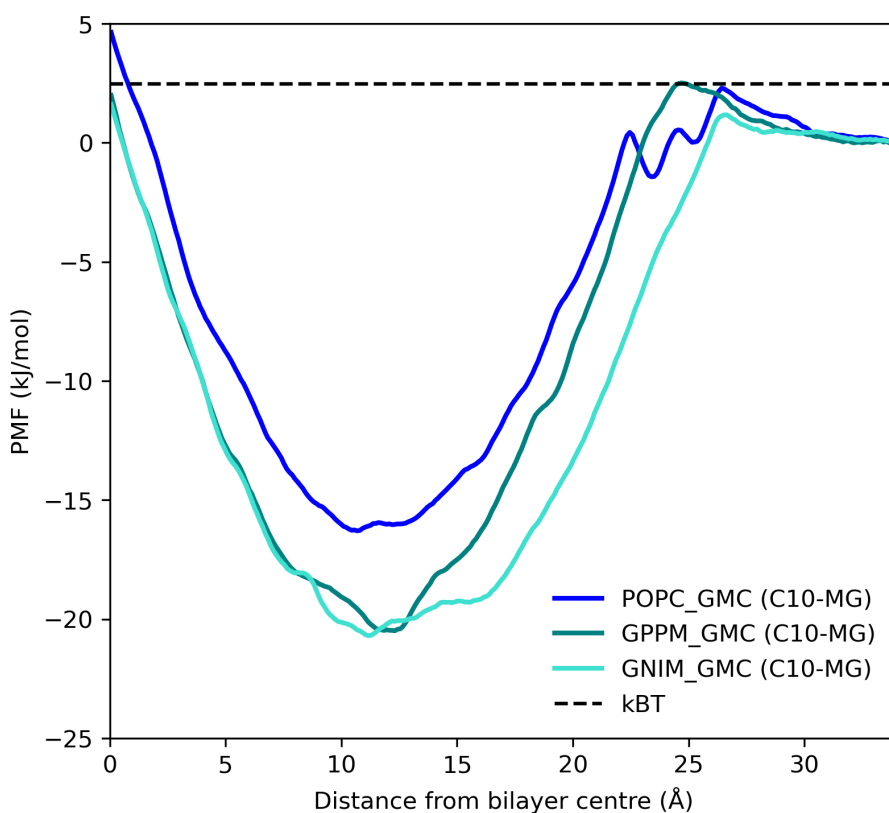


Figure 7.9: Normalised free energy curve for movement of C10-MG through the upper leaflet of a pure POPC bilayer system (blue), pure G+PM bilayer model (turquoise) and pure G-IM bilayer model (teal).

Free energy barriers for the above three US are displayed in Table 7.5. As described above, ΔG_{in} barriers were negligible, all implying timescales of tens of ps scale,

estimated at 252.9 ps, 233.3 ps, and 162.3 ps for POPC_GMC, GPPM_GMC, and GNIM_GMC respectively. In terms of movement inside the bilayer, POPC_GMC had the lowest barriers for both exit from the leaflet and movement past the hydrophobic core, with an expected timescale of 181.4 ns and 458.7 ns for ΔG_{exit} and ΔG_{trans} respectively. ΔG_{exit} and ΔG_{trans} values for GNIM_GMC and GPPM_POPC were similar, displaying identical ΔG_{exit} barriers and GNIM_GMC displaying a ΔG_{trans} barrier 0.5 kJ/mol greater than the GPPM_GMC system.

Table 7.5: Calculated energy barriers for C10-MG entry into lipid bilayer leaflet (ΔG_{in}), MG exit from lipid bilayer leaflet (ΔG_{exit}), MG partitioning from solution into the bilayer (ΔG_{par}), and movement across a lipid bilayer leaflet (ΔG_{trans}) for POPC, G+PM, and G-IM bilayer models. Brackets indicate the variation of ΔG energy barrier values obtained from the final 20 ns, 30 ns, and 40 ns of the trajectory, with the lower and upper limits corresponding to the minimum and maximum calculated energy barriers respectively. All values are presented in kJ/mol.

System	ΔG_{in}	ΔG_{exit}	ΔG_{par}	ΔG_{trans}
POPC_GMC	2.3 (2.3/2.6)	18.6 (17.0, 18.6)	-16.3 (-16.3, -14.4)	20.9 (20.5, 21.2)
GPPM_GMC	2.1 (2.1, 2.3)	21.9 (19.9, 22.9)	-19.8 (-20.7, -17.8)	21.8 (20.8, 22.0)
GNIM_GMC	1.2 (0.9, 1.2)	21.9 (20.7, 22.9)	-20.6 (-21.7, -19.7)	22.3 (21.8, 22.3)

The difference in ΔG_{trans} barriers between bacterial models corresponded to a 20% slower estimated energy barrier crossing speed, and timescale difference of around 147 ns (807 ns for GNIM_POPC in comparison to 660 ns for GPPM_POPC). This was 3-3.8 times longer than the time required to exit the bilayer in the POPC system. The shared ΔG_{exit} energy barrier was equivalent to 687 ns. This could in part explain why the G-IM was generally more susceptible than POPC to MGs in terms of causing severe disruption, as C10-MG would be more likely to remain contained to a single leaflet for longer, therefore causing continuous asymmetric stress in the bilayer, however this is unlikely to be a significant contributor to the mechanism as nanosecond scale differences between molecules are likely negligible at the minute to hour timescales that MG-mediated bilayer disruption is observed at experimentally. This logic would also not follow with the G+PM simulations in the previous chapter showing lower comparative susceptibility to C10-MG than the POPC bilayer model, as reported energy barriers were slightly higher for both ΔG_{exit} and ΔG_{trans} . It should be noted that all three of these bilayer models are undisturbed by MG, and Section 7.4.5

identified that energy barriers for C8-MG and C18-MG differed to varying degrees in leaflets where the respective MG was already present in the upper leaflet. Therefore, it may be that more significant differences in energy barriers may emerge between the bacterial bilayer models when other MG molecules are present in the leaflet.

7.5 Discussion

7.5.1 Summary of Findings

US is a highly valuable enhanced sampling technique for MD simulations that provides direct calculation of relevant energy barriers for systems. There has been a distinct lack of US performed using MGs published in literature to provide direct comparison points for the PMF curves. The only published US of a MG was performed in Gahan *et al.* (2021) which calculated the PMF of C12-MG as a function of distance along a 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) lipid bilayer [191]. Comparisons between this US with POPC_GML (Table 7.6) indicated a consistent trend that C12-MG has higher free energy barriers required for the molecule to exit the bilayer than to cross into the lower leaflet of the bilayer.

Table 7.6: Comparison of calculated free energy barriers for C12-MG exit from energy minimum within leaflet and movement across the bilayer centre ($z=0$) for a POPC bilayer (presented in this chapter in Figure 7.6) and a DOPC bilayer presented in Gahan *et al.* (2021). Brackets for POPC_GML represent maximum/minimum ΔG values across the last 20/30/40 ns. Brackets for Gahan *et al.* represent standard error.

Source	ΔG_{par} (kJ/mol)	ΔG_{trans} (kJ/mol)
POPC_GML	-27.6 (-27.6, -27.0)	18.6 (+14.4, 15.1)
Gahan <i>et al.</i>	-34.34 (± 0.96)	22.7 (± 0.96)

The ΔG_{exit} and ΔG_{trans} free energy values calculated by Gahan *et al.* were both greater than those calculated for the POPC_GML US, even considering potential error, with a 19.65% increase for ΔG_{exit} and 22.04% increase for ΔG_{trans} in comparison. This is counter-intuitive to what would be expected in differences between these two bilayer models, as DOPC bilayer are generally more fluid than POPC bilayer due to containing two cis-monounsaturated tails in comparison to POPC's one saturated tail and one cis-monounsaturated tail, which causes greater splaying of lipid tails thereby reducing

packing [315]. Additionally, The DOPC US was conducted at a constant temperature of 310.15 K in comparison to the 298.15 K of POPC_GML, which would further increase the relative fluidity of the DOPC bilayer. Given this, it would be expected that the DOPC US would produce lower ΔG values, which did not happen. Given apparent issues with convergence between windows, this may explain this discrepancy. Despite the differences in calculated values, the higher energy barrier to expulsion than flip-flop for C12-MG remains consistent between the two US.

Though no other US simulations have been performed using MGs, US using other molecules that share key structural traits with MGs, namely the amphiphilic single tailed structure, have been performed, so some comparisons can be made to these molecules. Table 7.7 displays calculated energy barriers for a variety of molecules published in literature.

Table 7.7: Published ΔG_{par} and ΔG_{trans} free energy barriers for single tailed amphiphiles 3-oxo-C12-AHL, lauric acid, myristic acid, palmitic acid, steric acid, and miltefosine published in literature. Brackets represent standard deviation.

Molecule	Formula	Model	ΔG_{par} (kJ/mol)	ΔG_{trans} (kJ/mol)	Ref
3-oxo-C12-AHL	$C_{16}H_{27}HO_4$	DOPC	-26.29 (0.32)	35.57 (0.45)	[191]
C12-FA (anionic)	$C_{12}H_{23}O_2^-$	DOPC	-21.71 (3.61)	43.97 (4.77)	[191]
C14-FA (neutral)	$C_{14}H_{28}O_2$	DOPC	-43.51 (2.09)	12.56 (4.61)	[316]
C14-FA (anionic)	$C_{14}H_{27}O_2^-$	DOPC	-30.12 (2.5)	68.66 (11.30)	[316]
C16-FA (neutral)	$C_{16}H_{32}O_2$	DOPC	-47.28 (0.42)	12.56 (0.42)	[316]
C16-FA (anionic)	$C_{16}H_{31}O_2^-$	DOPC	-33.05 (1.67)	65.31 (10.47)	[316]
C18-FA (neutral)	$C_{18}H_{36}O_2$	DOPC	-55.65 (2.93)	13.82 (3.35)	[316]
C18-FA (anionic)	$C_{18}H_{35}O_2^-$	DOPC	-46.44 (2.09)	69.08 (7.12)	[316]
Miltefosine	$C_{21}H_{46}NO_4P$	POPC	-43.9	15.2	[317]
Miltefosine	$C_{21}H_{46}NO_4P$	DOPC	-43.4	28.2	[317]

The 12 carbon 3-oxo-C12-AHL C12-MG displayed a lower ΔG_{trans} energy barrier and a more significant energy barrier for flip-flop, while the inverse was reported both within this chapter and Gahan *et. al* for C12-MG. The energy barrier for flip-flop was greater than either reported value, while the partition energy barrier was lower than both. These energy barrier differences likely correspond to structural differences between the molecules, despite equal tail lengths. The headgroup of 3-oxo-C12-AHL is homoserine lactone ring, which is bulkier than the glycerol headgroup of C12-MG and lacks hydrogen-bond donor groups, containing only hydrogen bond acceptors. These

differences in headgroup structure therefore alter the capability for these molecules to translocate across the hydrophobic core of the bilayer, with a bulky headgroup that cannot form intramolecular hydrogen bonding hindering its translocation across the hydrophobic core. Among the FA listed in Table 7.7 it was consistent that deprotonated FAs also displayed greater ΔG_{trans} energy barriers than ΔG_{par} , which is likely due to the anionic headgroup of the molecules. Charged headgroups present a significant barrier to crossing the hydrophobic core, which is dense with non-polar hydrophobic tails which polar molecules cannot stabilise within.

In contrast, neutral FAs at the same tail length as the anionic FAs more readily translocate across the bilayer than exit the bilayer, which is due to the lack of electrostatic interactions in neutral headgroups, which prevents hydration by water molecules. Similar interactions are responsible for the greater energy barrier for exiting the bilayer than flip-flop in MGs. The energy barrier to flip-flop reported for neutral C18-FA was similar to the calculated flip-flop energy barrier for C18-MG. As both molecules being similarly sized and non-polar they would likely have similar thermodynamic profiles within the hydrophobic core, which aligns with the reported ΔG_{trans} values. C18-FA displayed a greater energy barrier for exiting the bilayer than C18-MG which is likely attributed to differences in headgroup structure. Despite both being neutrally charged, the headgroup of C18-MG is bulkier and therefore causes greater packing disruption when inserted into the bilayer than C18-FA. Consequently, greater mechanical stress is placed on the bilayer by C18-MG in comparison to C18-FA due to its greater geometric mismatch with the bilayer, which would reduce the thermodynamic barrier to exiting the bilayer.

The trend within both neutral and anionic FAs, where both ΔG_{trans} and ΔG_{par} energy barriers increased as tail length increased was also present for the MG sampled within this chapter. Due to the high structural similarity of FAs with MGs this suggests that likely C14-MG and C16-MG would also follow pattern, bridging the gap in this chapter caused by sampling only C8-MG, C10-MG, C12-MG, and C18-MG. With a 16 carbon tail, miltefosine displayed a similar energy barrier to insertion across both POPC and DOPC bilayer models, however flip-flop energy barriers differed significantly. This

is likely because DOPC is composed of two unsaturated oleoyl tails and a PC headgroup, while POPC is composed of a saturated palmitoyl and unsaturated oleoyl tail, and PC headgroup. Consequently, the lipid-water interface is identical between these models, but the structure of the hydrophobic core differs as the DOPC core displays looser packing. This is counter-intuitive to the greater energy barrier for crossing the core of the DOPC bilayer calculated in Maltades *et al.* (2015) and therefore may suggest that increased fluidity in the bilayer core does not necessarily correlated to a lower energy barrier for molecules to translocate across it. This emphasised that differences in bilayer composition should be considered when comparing calculated energy barriers between different models.

When comparing MG free energy barriers in the POPC US, calculated energy barriers may have provided additional justification for MG behaviours observed in previous chapters. As discussed above, the ΔG_{exit} energy barrier for C12-MG was over 10.1 kJ/mol higher than ΔG_{trans} . This aligns with the behaviour of C12-MG observed in previous MD simulations, where only flip-flop of C12-MG was observed, which suggested that the preference of C12-MG to remain in the bilayer and translocate to the lower leaflet was indeed more energetically favourable. C8-MG and C10-MG had fairly similar energy barriers to each other to move from their preferred position in the upper leaflet, though C10-MG had consistently slightly higher barriers by around for both ΔG_{exit} and ΔG_{trans} . Both C10-MG and C8-MG also displayed lower barriers for exiting the bilayer, which would explain why expulsion was the more common of the two stress relief processes observed in POPC bilayer simulations. All three of these MGs had near negligible energy barriers for individual molecule entry into POPC bilayers from water, though the peaks indicate that C8-MG has the greatest difficulty inserting into the bilayer. Bilayer simulations containing C8-MG highlighted that C8-MG molecules readily re-insert into all simulated bilayer models from the water box if a molecule comes in contact with the bilayer from the water box which, along with the expected picosecond timescale of this energy barrier, indicated that that this is a negligible energy barrier for all MGs to overcome.

The low energy barriers for C8-MG to overcome for exiting the bilayer in comparison

to C12-MG could be explained by the geometry of the MGs. As discussed in previous Chapters, MGs have conical geometry due to their single tail and comparatively large headgroup. This geometry becomes more extreme when the distance between the headgroup and end of the MG acyl tail is lesser, so C8-MG was considered the most conical of the MGs investigated in this thesis. A highly conical structure is less compatible with the more cylindrical phospholipid molecules that compose the bilayer, and therefore MGs induce curvature, causing mechanical stress in the structure. This mismatch also likely causes the MG to be less stable in the leaflet structure, and therefore C8-MG would have a lower energy barrier to leaving the bilayer than longer MGs such as C12-MG, and especially C18-MG, which can better tolerate the bilayer structure. To an extent, this may explain why C12-MG displays more consistent destabilisation of lipid bilayers *in vitro* and in living organisms than C8-MG, as individual molecules of C12 are expected to leave the outer leaflet of the bilayer at a timescale of approximately $1.06 \mu\text{s}$ once inserted - 132 times slower than the 8.1 ns time calculated for C8-MG to move from its preferred position in the leaflet to outside the bilayer. In theory, this could mean that even if extremely high MG concentrations of C8-MG could cause severe stress on the bilayer structure, C12-MG would retain more stable high concentrations of MG at the same position within the bilayer for a longer period of time, which may allow for more sustained alterations to bilayer properties over longer periods of time. This could therefore increase the time required for asymmetric stress caused by MGs to be relieved and potentially inhibit membrane processes for long enough to induce cell death.

The US of C8-MG through either pure or loaded POPC bilayers displayed virtually no difference between ΔG_{in} energy barriers. This was also true for the C12-MG US system. This is a strong indication that despite the evidence that MGs disrupt bilayer properties in POPC bilayers this does not increase the ease of further MG molecules in permeating the membrane. Given that energy barriers for individual MG insertion were negligible even in undisrupted bilayers, this is not impactful to the ease of MG partitioning into bilayers, and the insertion of molecules not increasing the insertion of further molecules has also been reported in MD simulations of FAs and MGs with

a ceramide bilayer [169]. Though ΔG_{in} barriers were not affected, energy barriers for C8-MG movement across the leaflet and into the water box were reduced in the loaded bilayer. This potentially indicates that disruption of bilayer properties does lower the energy barriers required for MCMGs to leave the bilayer after insertion. This may be due to the overall increase in bilayer fluidity, though Chapters 5 and 6 indicate that even when bilayer fluidity is reduced by MCMGs the order within the loaded leaflet does increase if the MG distribution is asymmetric. Unlike in POPC_ASYM_GMCY, there were significant change to energy barriers in the POPC_ASYM_GMS free energy curve in comparison to the pure POPC bilayer. This may be because C18-MG is a well known stabiliser molecule, which was reported in Chapter 5 to significantly reduce upper leaflet fluidity. This decrease in fluidity would in turn make movement through the leaflet more difficult, explaining the much higher energy ΔG_{exit} value. POPC_ASYM_GMCY had a 2.5 kJ/mol decrease in the energy barrier required to move past $z=0$ on the PMF curve, which made ΔG_{exit} and ΔG_{trans} roughly equal for this curve. Due to this, it would be expected that C8-MG flip-flop and expulsion could be observed at roughly the same rate, though the simulation the bilayer structure was taken from had a preference for C8-MG exiting the bilayer. Likewise, the expected timescale for C18-MG to move past the bilayer centre in the disrupted POPC bilayer also appeared significantly off compared to the rate observed in bilayers highly disrupted by C18-MG which suggests that the ΔG_{trans} energy barrier of this curve may be inaccurate.

The differences in ΔG_{exit} and ΔG_{trans} observed between MGs may play a role in influencing the behaviour exhibited in systems with very high MG concentrations. The two major asymmetric stress relief mechanisms utilised to reduce the stress caused by extreme MG concentrations in a single leaflet that were identified within systems discussed in this thesis were outer leaflet budding and mass movement of MGs to the lower leaflet over the timespan of a few 10s of ns. Systems containing C12-MG were consistently the most resistant to budding occurring than systems containing C10-MG and C8-MG, which would align with the significantly higher energy barriers required for C12-MG to exit the bilayer in comparison to C10-MG and C8-MG. Additionally, the lower energy barrier to movement across the membrane centre for C12-MG indicates

that movement of MGs across leaflets is more energetically favourable for this molecule. This may explain why budding was less likely to be observed in C12-MG systems, as movement of MG to the lower leaflet may be a lower energy solution to relieve the leaflet curvature induced by C12-MG.

Evidence was also presented that the observed differences in MG susceptibility between bilayer models in Chapter 6 may be associated with free energy barrier differences. It was discovered that ΔG_{exit} and ΔG_{trans} energy barriers for both bacterial membrane models were higher than in the POPC leaflet. When considering the compositions of these specific bilayer models, this does make sense for undisrupted bilayers, as the PE and PG headgroups that dominate bacterial bilayers have tighter headgroup packing than POPC, and a higher degree of tail saturation in these models also causes tighter packing in the hydrophobic core. This could suggest that MG energy barriers for the bacterial bilayer models may be more significantly impacted by MG disruption due to greater packing disruption caused by MGs in these system. Though no highly disrupted bacterial membrane models were employed in US within this chapter to evaluate this, this does align with our understanding of the role hydrophobic mismatch and geometric stress likely play in driving lipid bilayer disruption. The lack of disrupted bacterial models in this Chapter was because the GPPM_GMC and GNIM_GMC US were the final simulations conducted for this project, and therefore further investigation of this could not be performed with the time remaining in this project. Energy barriers for MG insertion into the bilayer remained negligible between the three systems, which indicates that differences in MG susceptibility between mammalian cells and different bacterial species is unlikely to stem from issues with MG entry into the cell membrane directly, and therefore is likely associated with either the membrane lipid composition, or other barriers MGs are likely to come into contact with prior to membrane interaction. For G- bacterial cells this is particularly in reference to the outer membrane which has been known to influence the antimicrobial activity of MGs between G+ and G- cells [35].

Consistently, energy barriers were slightly lower or of negligible difference for C10-MG exiting the bilayer than for the molecule moving to the lower leaflet, which was

also observed in the POPC systems. This may explain why there appeared to be a preference for budding as a stress relief mechanism in leaflets heavily disrupted by C10-MG in Chapter 6 rather than mass flip-flop, though energy barrier differences were not so significantly different that movement of some molecules to the lower leaflet was not observed. As no other MGs were tested, it is unknown whether the same would be true of the energy barriers for the other MGs relevant to the work conducted throughout this thesis. Although it could be assumed that it would be based on the data presented in POPC US, proving so with further US with the more realistic bacterial models would significantly strengthen this argument and would serve as a direct route for the continuation of the presented research aims in the future. Ultimately, the comparisons between lipid bilayer models provide novel insight into the potential influence of MG movement within a bilayer in causing disruption both within a leaflet and across the bilayer structure. Iterating upon the methodology used to produce these preliminary findings would likely provide greater clarity on the usefulness of this data given some issues with sampling detailed below.

7.5.2 Potential Issues with Window Sampling

It is important to discuss potential issues with sampling within the presented free energy curves in order to determine the appropriate level of confidence in the presented results. Seemingly minor differences in sampling within windows can cause unanticipated consequences in the produced free energy curves, and therefore offset calculated ΔG_{in} , ΔG_{exit} and ΔG_{trans} values. The SMD simulations used as the reference for each window were pulled from the water box through the lipid bilayer with SMD dummy atoms aligned in a +z direction. Because of this, windows through to $z=0$ all start with the MG oriented towards the upper leaflet. This means less sampling was required to explore the energy landscape at the headgroup region of the leaflet, where this alignment was preferred, than at the bilayer centre where different positioning of the MG would be expected. Across the 50 ns simulation time of the $z=0$ window in all US, the MG remained aligned with the upper leaflet, which suggests the 50 ns simulations time of this window was likely not long enough to explore the entire

energy landscape at this position. This has likely impacted the PMF of this window and therefore ΔG_{trans} values are likely under-estimated in this work. Running the $z=0$ window for much longer to see whether orientation changed over a longer period of time may have confirmed whether this was the case, though due to this requiring an indeterminate amount of computational time to achieve it was not performed in this study.

To assess whether the poor sampling of $z=0$ was likely to affect predicted ΔG_{trans} , a small range of $-z$ windows (-1 to -4) were created and sampled for 50 ns in the POPC_ASYM_GMCY system, and from these windows it appears that the PMF curve continues to increase past $z=0$, potentially reaching a peak at -4 Å, though it is unclear whether this is the case due to no continued sampling past this point (Figure 7.10). The increase of this energy barrier, rising from 16.3 kJ/mol to 24.8 kJ/mol, significantly impacted the expected timescale required for C8-MG to overcome ΔG_{trans} , increasing from an estimated timescale of around 71.7 ns to around 2.2 μ s, which is around 30.7 times slower. It is unclear why this potential peak occurs in the negative range collective variable range rather than $z=0$, however based on its existence, it would appear wise to sample some of the lower leaflet when performing any MG US to determine whether this is consistent in other systems. Combining this with the potential issues with window sampling highlighted already within in this chapter, it should be considered that reported ΔG_{exit} values are more reliable than ΔG_{trans} values.

7.5.3 Limitations of Study

US is a time intensive sampling process, and therefore this technique could not be utilised to its fullest extent within the time limitations of this project in order to fully understand energy barriers associated with MG translocation through bilayers. The average time for the production run of each window was 60 ns/day, which therefore required 20 GPU hours per window, or 720 GPU hours per US. For the 8 US conducted for in this chapter, this was equivalent to 8 months of computational time required to complete this series of simulations, which was a significant time investment, even considering the ability to simulate 3-4 windows simultaneously on ARCHIE-WeSt in

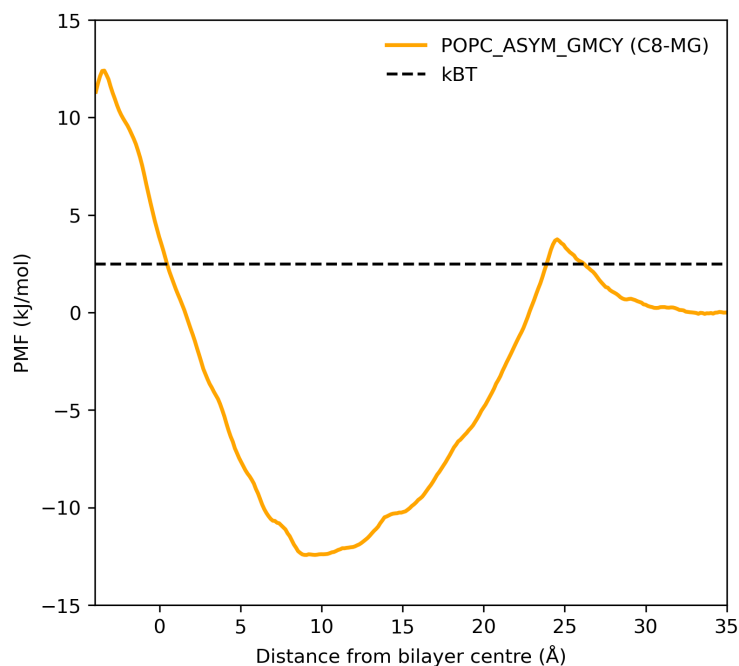


Figure 7.10: Normalised free energy curve for movement of C8-MG through the upper leaflet of a POPC bilayer system loaded with 44 molecules of C8-MG in the upper leaflet sampled from windows $z=35$ to $z=-4$.

optimal conditions. As US was conducted as the final phase of this project, there was not enough time to fully investigate all desired systems. This has meant that there is a lack of evidence to back up the likelihood of some behaviours discussed in this chapter, particularly investigating the movement of MGs through the bacterial models. Ideally US would have been conducted for all MCMGs in loaded bilayers and both bacterial membrane systems, rather than only C8-MG and C10-MG respectively, to better understand potential differences in energy barriers between molecules within these systems. MG-loaded bacterial systems would have also been sampled to confirm whether the observations in energy barrier changes found in the POPC systems were also reflected in more realistic bacterial models.

Another aspect of the methodology which had to be foregone due to time constraints was only sampling the $+z$ range of the collective variable. The lack of negative collective variable sampling was justified within the methodology as, due to $z=0$ being the centre of the hydrophobic core of the bilayer, this should be the least energetically

favourable position and therefore the point in the PMF curve where the energy barrier to translocation to the lower leaflet would be at its highest. However, as discussed in the section above, inconsistencies in what energy barriers indicate in comparison to behaviour observed in other simulations may suggest that the PMF curve may continue to climb past 0, and ΔG_{trans} barriers for some of these US may be higher than reported, potentially due to poor sampling near the $z=0$ region. Sampling the negative range of the reaction coordinate would alleviate concerns regarding this.

As already discussed in this chapter, sampling of some windows in this Chapter may have been inadequate, leading to inaccuracies in free energy curves. This problem was especially associated with the $z=0$ window of each US, as no reorientation of MG headgroups or tails with the lower leaflet occurred in these windows, though in several free energy curves the convergence of windows may be insufficient. Extended sampling of these windows would allow better sampling of phase-space in these region and therefore produce more accurate ΔG_{trans} values, though it is unclear how much additional time this window would need to be extended by to enable this. This could be combined with negative reaction coordinate sampling to improve reliability of energy barrier calculation for MGs between leaflets. Extension of all windows could also be performed to ensure full convergence of free energy curves and ensure calculated energy barriers could be analysed with greater confidence, though this would be far more computationally intensive.

A final limitation of this study was focusing on individual MG molecules for the sampling. Most relevant industrial and medical applications of MGs utilise LNP formulations rather than free MGs [283]. As these structures are much larger than individual MGs, it is fairly likely that energy barriers for MG entry into the bilayer would be influenced by the differences in initial interaction between molecules and the bilayer between these two forms. In particular any potential transfer of MG monomers from the micelle into the bilayer were not modelled, though the use of a single MG molecule could be considered representative of monomer partitioning from free MG from aqueous solution, which occurs as MG monomers are exchanged between aqueous solution and the micelle aggregate. At the all-atom scale that these US

were conducted, this could not be accounted for as a lack of prior MG nanoparticle simulations in previous chapters would cause difficulty in determining the correct behaviour of the nanoparticle while interacting with the bilayer. Additionally, the G- outer membrane model was initially planned to be included in this series of US. Ultimately this was excluded, as the G- outer membrane was not successfully simulated in the previous chapter, and the system size required to accommodate the G-OM model would significantly slow window simulation speeds to the point where it was not feasible to perform in addition to the other US featured in this chapter. Similarly, the G+ cell membrane is directly anchored to the G+ cell wall, which would likely have significant impacts on energy barriers required for MGs to insert into the bilayer. This was also not feasible to factor into the all-atom simulations in this thesis, as it would require the z-axis length of the system to be extended by a further 200-800 Å, which would significantly extend simulation times for each window, much as the lipopolysaccharide in the G-OM would.

7.6 Conclusions

An US series of 8 systems where C8-MG, C10-MG, C12-MG, or C18-MG were pulled through POPC, G+PM, or G-IM lipid bilayer models was performed successfully in this chapter. The calculation of PMF from these systems provided novel insight into how MG behaviour displayed in previous chapters may be influenced by energy barriers associated with MG movement once inserted into a bilayer.

- Calculated energy barriers to movement from the upper leaflet to lower leaflet, and exiting from the bilayer for C8-MG, C10-MG, C12-MG, and C18-MG align with movement behaviour of MGs observed in previous chapters, though calculated timescales for MGs to cross lipid bilayers did not always correspond accurately. This may be due to some unanticipated issues with sampling within windows.
- Energy barriers to MG entry into bilayers from solution are negligible, and prior insertion of MG into a bilayer structure does not appear to reduce the energy barriers required for entry of further MGs.

- Differences in energy barriers for exiting the bilayer and moving between leaflets may explain differences in stability and preferences between budding and mass flip-flop for bilayers experiencing disruption by different MCMGs. Differences in expected timescale to exit the bilayer may also influence the duration of bilayer property destabilisation at timescales that extended past those that have been simulated in this thesis.
- Trends in C10-MG free energy barriers appear consistent between POPC and bacterial model bilayers, though the lack of US of bacterial models using C8-MG and C10-MG, and poor sampling of regions of the G+PM reaction coordinate preclude strong conclusions from being drawn regarding this.

Chapter 8

Discussion and Conclusions

8.1 Summary of Results

Within this work, several experimental findings and hypotheses regarding factors related to MG antimicrobial efficacy were investigated using MD simulations at an atomistic level of detail in an attempt to gain novel insight into them. The first of these hypotheses was that MG disruption requires a high localised concentration of MG within the bilayer, as disruption is only observed above CMC experimentally. Utilising different MG concentrations across Chapters 5 and 6 identified that changes to bilayer properties were not linear with increasing MG concentration, and disproportionate disruption occurred at very high MG concentrations.

How MG structure influences bilayer destabilisation was computationally assessed for the first time by comparing the MCMGs C8-MG to C12-MG. Despite experimental observations that C10-MG and C12-MG were the most disruptive experimentally, at equal concentrations within the bilayer C8-MG consistently displayed more severe disruption of lipid bilayers across three different lipid bilayer compositions. This may suggest that the increased antimicrobial potency observed experimentally in C10-MG and C12-MG relative to C8-MG may be due to factors prior to MG insertion, for example lower C8-MG nanoparticle stability would cause lower concentrations to reach the bilayer, thereby influencing antimicrobial efficacy, though such factors were not studied within the context of this thesis.

Furthermore, the results obtained from research conducted in this thesis also shed light on the importance of MG placement in the structure. Asymmetry in MG concentration between leaflets consistently produced more system-wide destabilisation of bilayer properties in comparison to equal MG counts placed evenly across leaflets. This strongly supports the hypothesised role of asymmetric stresses accumulating in one leaflet of the bilayer due to MG insertion driving remodelling of the bilayer. This is further exemplified by MG behaviour within the bilayer at high concentrations, where severe curvature occurred, and in some cases budding of the upper leaflet formed. Additionally, a potential stress relief mechanism for MG-induced disruption was observed in some systems, where mass-movement of MG from the upper leaflet to the lower over a timescale of nanoseconds caused reductions in the extent of bilayer disruption for the remainder of the simulation time. This gives atomistic level insight into how MGs behave within highly disrupted bilayers and the ways that bilayers respond to stress induced by their presence.

To confirm whether the trends in MG disruption observed above were consistent in more complex models, and to better link observations to antimicrobial activity in MGs, further phospholipid bilayer models closer representing phospholipid bilayers found in G+ and G- bacteria were simulated with various MCMGs in Chapter 6. General trends in MG disruption and differences in the extent of disruption between MCMGs were observed which were similar to those of the POPC bilayer in Chapter 5, though there were differences in susceptibility between the three bilayer models. The G+ model was less susceptible than the POPC bilayer to significant MG disruption, which suggests that cell conditions outside the bilayer, such as external barriers and cell membrane repair mechanisms which could not be accounted for in simulations, likely have an important role in determining antimicrobial susceptibility for MGs. The G- bacterial membrane model was more susceptible than the Gram-positive bilayer despite G- bacteria being less susceptible to MGs. This likely indicates that the G- outer membrane and other parts of the G- cell envelope likely is highly important to MG resistance in G- bacteria, which aligns with experimental reports that damage to G- bacteria by MGs manifests through damage to the bacterial cell wall [35].

Finally, energy barriers were calculated for a variety of systems using umbrella sampling, which were the first time that these combinations of MGs and bilayer systems had been performed. Energy barriers indicated differences in expected timescales for MGs to remain in bilayers, which may affect the timescale that MG-induced disruption affects cell membranes and which asymmetric stress relief mechanisms are favoured in bilayers disrupted by different MG species. Differences in energy barriers between different MGs were reflected across all models, and the existence of other MCMGs within a bilayer may reduce energy barriers required for molecules to exit the bilayer or flip to the opposite leaflet, but did not reduce the energy barrier required for MGs to insert into a leaflet of a cell membrane, though there was a lack of evidence to fully back up this hypothesis.

8.2 Conclusions and Future Work

The overall aims of this thesis were:

- To provide a review of our current understanding of antimicrobial activity in monoglycerides, and provide the historical context which has led to increased interest in monoglycerides as a potential method of overcoming antibiotic resistance.
- To study how monoglyceride insertion into phospholipid bilayers influence bilayer properties and may lead to bilayer destabilisation using molecular dynamics simulations.
- To understand how differences in monoglyceride structure and bilayer lipid composition may influence the degree of destabilisation that lipid bilayers undergo using molecular dynamics simulations.

Chapters 1 and 2 successfully achieved the first of these aims by providing a comprehensive discussion regarding the development of antimicrobial resistance in bacteria and its impacts on global health. Further, Chapter 2 also discussed the potential of MGs as novel antimicrobial molecules, outlining existing understanding of

Chapter 8. Discussion and Conclusions

their mechanisms of action, the degree of bacterial susceptibility to monoglycerides, and current gaps in experimental knowledge that could be investigated using the computational methodologies employed in this thesis.

The effects of monoglyceride structure and bilayer composition were investigated in Chapters 5 and 6. Chapter 5 investigated three important experimental hypotheses regarding the monoglyceride antimicrobial mechanism of action using simplified model systems in order to generate novel evidence for or against these hypotheses by characterising changes to bilayer structure and key properties to determine the extent of monoglyceride-induced disruption. The novel insight into bilayer destabilisation by antimicrobial monoglycerides at an atomistic level therefore successfully met the second aim of this thesis.

Chapter 6 modelled MG-induced destabilisation of two additional bilayer models to the one investigated in Chapter 5, confirming that differences in lipid bilayer composition does influence susceptibility to monoglycerides, providing further evidence of important aspects of the antimicrobial mechanisms of MGs. Throughout both Chapter 5 and 6, three different medium chain monoglycerides were used in simulations, identifying trends between monoglyceride length and the degree of disruption.

The research presented in this thesis was fundamentally designed to provide novel insight into how MGs drive the destabilisation of biological membranes at an atomistic level of scale. The methodology employed for this objective allowed the creation of model bilayer systems to evaluate experimental hypotheses, providing strong evidence that MG disruption is associated with high asymmetric concentrations of MCMGs. These trends can be seen within both simplistic lipid bilayer systems, and in systems which more closely represent bacterial cell membranes. This alignment of computational findings with experimental hypotheses allow the merging of knowledge from these two research fields, creating an overall stronger understanding of what is needed for MGs to inhibit biological membrane properties. By producing a more accurate picture of the antimicrobial mechanisms of MGs, this in turn can be utilised in research and industry for the more intelligent design of novel MG-based therapeutics and disinfectants, improving efficacy.

This project is not without its limitations, as is true for any body of research. A primary limitation of the work carried out is the small scale of MD systems requiring many of the complexities of biological membranes - in particular local clustering of lipid domains, membrane-bound proteins, and membrane curvature - to be foregone in order for simulations at this scale to be run. As bilayer curvature has been shown to influence bilayer disruption in mixed MG/LA solutions the lack of these details are likely to influence some aspects of disruption mechanisms, even if fundamental understandings such as the influence of asymmetric stress and packing disruption through hydrophobic mismatch are likely to remain consistent across all bilayer morphologies. Additionally, some of the simulations performed in this thesis contain unrealistic MG concentrations within the modelled region of the bilayer compared to what would likely be plausible biologically. Within the context of this thesis, extremely high MG counts such as 150 and 200 MG within the system were performed to test the bilayer under extreme induced stress and gain greater insight into how the bilayer accommodates it, however as micelles tend to be composed of around 50-100 molecules these concentrations are unlikely to ever be reached in a region of a membrane in biological contexts as significant bilayer disruption would likely be reached prior to these count thresholds. As such, some of the more extreme deformations observed across this thesis grant interesting insight into processes that lipid bilayers may undergo when faced with high levels of disruption by MGs, but are not directly compared to real-world studies. Despite this, these simulations provide strong evidence of trends related to MG count within a bilayer, and benefit the core arguments of this thesis significantly.

Biomolecular simulations are an increasingly prominent research field which offers great potential for investigating aspects of experimental hypotheses that are difficult to examine with current experimental technique, as demonstrated in this thesis. An obvious pathway to further explore how MGs impact bilayer properties is to expand the system scale of the simulations from all-atom to coarse grained molecular dynamics in order to investigate MG entry in the form of micelles, or other stable nanoparticle formulations, which could not be performed at the all-atom scale used in this thesis. As this thesis is focused on the impacts of MGs that are already in a lipid bilayer,

understanding how LNPs containing MGs partition into membrane systems, and how this affects the distribution of MG molecules within a leaflet would allow interesting comparisons to how bilayer properties are affected in these systems in comparison to the more uniform distribution of MGs in simulations performed in this thesis. Coarse grained simulations would enable not only larger system sizes to be simulated in order to accommodate for the size of LNPs, but also allow for longer timescales to be simulated, which would allow better determination of whether the bilayer property destabilisation caused by MGs is retained at a scale of microseconds and beyond. Expanding the xy dimensions of lipid bilayers through coarse graining would also allow the observed budding behaviour within this thesis to be better characterised, by determining whether this phenomenon would be localised to specific regions of the membrane in systems as at an all-atom scale observed curvature crossed PBCs. To ensure coarse grained simulations are accurate, validation against similar all-atom models would be performed, which the MG parameters and atomistic MD systems created for this thesis would aid in.

Some aspects of this project could also be directly continued to further explore the findings displayed in this thesis. As discussed previously, the effects of MGs on bacterial cell membrane models could not be fully explored due to issues with simulating the Gram-negative outer bacterial membrane. This leaves a significant gap in the understanding of MG destabilisation of G- bacteria as the G-OM appears to act as a significant barrier to the antimicrobial activity of MG, based on the observed high susceptibility of the G-IM presented in Chapter 5. Successful simulation of the G-OM would provide a much clearer picture of how G- bacteria may resist the effects of MGs. Similarly, the cell wall anchored to the G+PM would likely impact its susceptibility to MGs, so simulations utilising this component of the cell membrane could give further insight into how bilayers respond to MG disruption and give a more accurate picture of the energy barriers a MG would have to cross to insert into the lipid membrane. All systems investigated also used only one type of MG in the systems, therefore studies on mixed MG compositions with a similar methodology to the one used in this thesis could be employed to investigate any potential synergy between

different lipid species, including between FAs and MGs, for which some synergy has been reported in literature, and synergy between MG species [185,186]. Potentially MGs could also be simulated in combination with antibiotic drugs known to have synergy with MGs to better understand whether such reported synergies are associated with MG-mediated bilayer destabilisation [152,187]. The atomistic molecular dynamics presented in Chapters 5 and 6 could also be extended to at least a microsecond, or be repeated to assess how consistently the behaviours observed within these systems occurred. Finally, umbrella sampling windows could be extended further to improve convergence and therefore calculate more accurate free energy barriers, particularly for MG flip-flop between leaflets.

In conclusion, the work presented in this thesis successfully met the aims it has set out to achieve, and presented novel insight into the antimicrobial mechanisms of monoglycerides, which fills existing gaps in understanding within this research area. The key findings of this thesis can therefore be utilised to inform the design of future computational studies investigating the antimicrobial capabilities of monoglycerides, or validate existing experimental hypotheses within the field.

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Appendix A

Additional Background Information

A.1 Development of Novel Traditional Antibiotics

The discovery of new traditional antibiotic drugs is a key component to combating the increasing prevalence of antimicrobial resistance. Many antimicrobial compounds originate from bacterial species, in particular *Streptomyces spp.* Many modern-day antibiotics used in medicine have roots in *Streptomyces* natural product isolates, such as streptomycin, the first member of the aminoglycoside family of antibiotics. [318,319] Traditionally, antibiotics were discovered through bacterial susceptibility testing using isolated compounds from bacteria. While this method has been successful in discovering novel antibiotics in the past, its usefulness has diminished over time as the quantity of undiscovered natural products from culturable bacteria diminishes. Since the 1940s-60s, which was considered the golden age of antibiotic discovery, there has been a significant reduction in the quantity of new antimicrobial compounds discovered - no new classes of antibiotics have been discovered using traditional susceptibility testing methods from bacterial products since the 1980s. [320] This is further amplified by the increasingly limited mechanisms of action to kill a microorganism already resistant to many existing mechanisms of antibiotic activity without producing toxicity towards the infected host's cells if used as a treatment. Additionally, the inability to test

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for bioactivity in viable-but-not-culturable (VBNC) bacterial-derived compounds using traditional methods impedes the ability to assess potential molecules. This is especially relevant to bacteria from marine environments, which research is turning towards for new sources of antibiotic compounds, as few have been successfully cultivated. [321]

Modern methods of antibiotic discovery increasingly utilise computational methods to analyse potential candidate drugs. A major method of utilising computational biology for this function is through genomic analysis. Genomic analysis is a method of discovering antibiotics. The sequenced genome of an organism is searched for indicators of genes which code for the production of molecules. Specific features in candidate antimicrobial compounds have been associated with antimicrobial activity, which can make screening for relevant compounds faster, such as functional groups with nucleophilic reactivity - for example phenol groups - and structural features which enable target binding. [322] Confirmation of antimicrobial activity from these genes can then be assessed by stimulating *in vitro* gene expression of the relevant pathway to overexpress the molecule and collect it in a high enough concentration to facilitate antimicrobial susceptibility testing. [323] Unlike traditional detection methods, genome mining enables assessment of antimicrobial activity in VBNC bacteria. [324]

Semi-synthetic derivatives of naturally occurring antibiotics can also be designed to overcome antibiotic resistance mechanisms. Semi-synthetic antibiotics are chemically modified structures of naturally-derived antibiotics which enhance existing characteristics to improve efficacy, reduce host toxicity, improve drug binding to the target, or alter the structure to overcome a specific resistance mechanism. Semi-synthetic derivatives of vancomycin, for example telavancin, has been found to show antibiotic activity in some vancomycin-resistant strains. [325]

A small selection of antimicrobial drug classes are entirely derived synthetically. All members of the sulphonamide and quinolone classes of drugs are entirely synthetic. [326] The most recent of these antibiotic classes, the tethered macrocyclic peptides, which were discovered as recently as 2024 and inhibit LPS-transport machinery LptBFGC, allowing targeting of antibiotic resistance *A. baumannii*. [327] While synthetic antibiotics prove useful in developing novel methods of targeting

infection, the research process for novel antibiotics is long and costly, and often development leads to a net negative profit to the developer, as novel antibiotics are generally kept as last-resort antibiotics, leading to reduced incentives for researchers and investors to develop novel compounds. [328]

A.2 Evolution and Transfer of Antibiotic Resistance Genes

Usually antibiotic resistant bacteria resist some, but not all, antibiotic drugs. Some species have natural resistance to an antibiotic due to their cellular architecture or the production of bacterial products. This is known as their intrinsic resistance. [3] The OM of G- bacteria creates an additional permeability barrier which inhibits antibiotic diffusion across the cell envelope into the cellular interior. The IM and OM have different permeabilities which can prevent antibiotics which penetrate the outer membrane from entering the cytosol and instead become sequestered in the periplasmic space. [79] This is particularly detrimental to the efficacy of tetracyclines and aminoglycosides which act intracellularly. [93, 94] Natural products produced by bacteria can inactivate antibiotics, for example the enzyme β -lactamases produced by *Staphylococcus aureus*, which inactivate β -lactam class antibiotics. [329] Antibiotic resistance mechanisms carried by bacteria are not always active, with some genetic pathways associated with antibiotic resistance induced by the exposure of a population to a drug. Efflux pump systems carried by bacteria can be overexpressed following antibiotic exposure, facilitating more effective transport of antibiotic drugs out of the cell to prevent toxic effects occurring. [330]

Antibiotic resistance is not always intrinsic and can also be acquired through various methods. Accumulation of single-nucleotide polymorphisms that naturally occur during, for example, DNA replication during cell division, can cause mutations in targets of antibiotics, reducing their efficacy. These resistance genes are often transmitted between generations by vertical transmission, as binary fission is an asexual process. [331]

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Resistance genes can alternatively be transferred from one cell to another within a generation through horizontal gene transfer. Bacteriophage viruses mediate the transfer of genetic information from one bacterium to another by the process of transfection. Bacteriophages adhere to bacteria and incorporate their genome into the bacterial genome using lysins to enable replication of the viral genome using the bacterial cellular machinery. [332] This process can lead to the formation of defective bacteriophage virions which contain bacterial DNA instead of viral DNA, which in turn can infect other bacteria and inject DNA from the original bacterium, potentially incorporating resistance mechanisms into the receiver cell genome. [333] Bacteria can also transfer genetic information directly between themselves through conjugation. Conjugation enables the movement of mobile genetic elements within the accessory genome, where resistance genes are typically found in bacteria, from one bacterium to another through the formation of a sex pilus on a donor bacterium, which then conjugates with a recipient bacterium. [334] Horizontal gene transfer of resistance genes is a process that provides a fitness advantage in response to an antibiotic molecule in the environment to a bacterial population by facilitating the spread of a resistance gene throughout a population. [335]

Antibiotic drugs will also select for antibiotic-resistant bacterial phenotypes within a population. Upon exposure of an antibiotic to the population, all bacteria which are susceptible to the drug will be killed or inhibited, while those which can resist the antibiotic can survive and outcompete susceptible bacteria. This therefore causes a rapid increase in population of daughter cells originating from the surviving bacteria, which possess the relevant resistance mechanism (Figure A.1). This can also be seen in sub-therapeutic antibiotic concentrations, as resistant bacteria have less inhibited growth and therefore can outcompete susceptible bacteria. [336] Other bacteria, referred to as "persistent bacteria", are susceptible to the antibiotic but have slight metabolic differences, which lead to their growth being inhibited rather than being outright killed. These cells resume growth after the antibiotic treatment course has ended, which may cause re-emergence of the infection, though the extent of their contribution towards this is debated. [337, 338]

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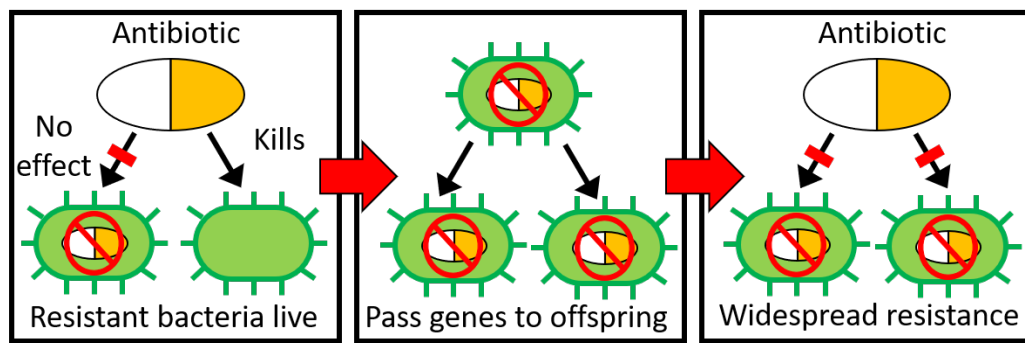


Figure A.1: Spread of antibiotic resistance genes within a bacterial population in response to the selection pressure of an antibiotic drug.

If multiple different antibiotic drugs are used in the treatment of an infection, a multidrug-resistant (MDR) phenotype can emerge in a bacterial strain. [339] While many microorganisms display levels of resistance to antimicrobial compounds, a small group of clinically significant pathogenic bacteria have been of particular concern due to this characteristic. The ESKAPE pathogens: *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.* are a group of bacteria of interest due to displaying MDR phenotypes (Table A.1). [340]

Table A.1: Summary of the characteristics and antibiotic resistances of the mentioned bacteria.

Species	Gram Stain	Morphology	Resistances	Ref
<i>Enterococcus faecium</i>	Positive	Sphere	Vancomycin, quinolones, cephalosporins, β -lactams.	[341–343]
<i>Staphylococcus aureus</i>	Positive	Rod	Methicillin, vancomycin, β -lactams.	[344–346]
<i>Klebsiella pneumoniae</i>	Negative	Rod	Carbapenems, colistin, β -lactams.	[347–350]
<i>Acinetobacter baumannii</i>	Negative	Rod	β -lactams, quinolones, oxacillin, aminoglycosides.	[351,352]
<i>Pseudomonas aeruginosa</i>	Negative	Rod	β -lactams, aminoglycosides, quinolones, cephalosporins, carbapenem.	[353–362]
<i>Enterobacter spp.</i>	Negative	Rod	Cephalosporins, carbapenems, colistin.	[363–367]

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The ESKAPE pathogens are of clinical significance as they have severely limited treatment options due to containing multiple resistance mechanisms and are common causes of nosocomial infection. [368] The emergence of ESKAPE pathogens has consequently altered the medical world significantly as it is becoming more common to need to use last resort antibiotics to treat MDR infections, risking development of antibiotic resistance against those drugs too. [369] Due to the declining numbers of antibiotics suitable to treat these infections, there is a significant focus in research towards the development of new antibiotics to keep up with the antibiotic resistance arms race against these pathogens.

A.3 Alternative Antimicrobial Compounds

While novel antibiotics are still being produced, with a potential new class of antibiotics being discovered as recently as 2024, there are still concerns that continuing to develop new antibiotic drugs will not be enough to combat the rise of antimicrobial resistance. Because of this, an offshoot of research into antimicrobial compounds has emerged, which instead attempts to overcome antibiotic resistance using molecules that are not considered traditional antibiotics but still have the ability to either enhance the antimicrobial efficacy of antibiotics or act as antimicrobial compounds themselves.

A.3.1 Antibiotic Enhancers

Antibiotic enhancer molecules typically aim to improve the efficacy of antibiotic drugs already in use. This can be achieved through inhibiting bacterial mechanisms that prevent the mechanisms of action of antibiotics or improving the targeting or accumulation of antibiotic at the target bacterium.

β -lactamase inhibitors are drugs that are typically administered alongside β -lactam class drugs in the treatment of infections caused by bacteria which produce β -lactamase enzymes due to the prevalence of β -lactam resistance in bacteria. [370]. β -lactamase inhibitors function by inhibiting one of the two mechanisms of action of the enzymes; catalysing the β -lactam ring of the antibiotic using a serine residue in the active site

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(serine β -lactamase) or using zinc ions (metallo β -lactamase), however, the majority of β -lactamase inhibitors only function against serine β -lactamase enzymes. [371]. This method of increasing antibiotic susceptibility of targets has limitations however, as resistance mechanisms that cause structural changes in antibiotic targets cannot be deactivated as readily by a drug administered alongside an antibiotic.

An alternative method of overcoming antibiotic resistance is the use of nanotechnology for the delivery of antibiotic molecules. For successful antibiotic treatment of an infection, the most basic requirement is for the antibiotic to reach the target in sufficient quantities to cause a bactericidal effect. A potential method of increasing drug accumulation at the target is through the use of a drug delivery system designed to target the bacterium the drug is meant to kill or deliver the drug at a high concentration. There are two major categories of drug delivery systems used for overcoming antimicrobial resistance: conjugated drug delivery systems and loaded drug delivery systems.

Loaded systems deliver antibiotic contained within a carrier, often in the form of a NP (Figure A.2). The behaviour of loaded drug delivery systems depends on NP type and formulation. Metal nanoparticles can be positively charged, so are attracted to, and able to diffuse through the negatively charged bacterial cell envelope to facilitate direct drug delivery into the cell, which increases the efficacy of neutrally charged antibiotics that struggle to permeate the bilayer. [372] Antimicrobial-loaded nanoparticles can also target a specific organism or host tissue using targeting mechanisms such as antibody conjugates, which are specific to a target antigen at the site. [373,374] reducing the toxicity of the antibiotic and extending the half-life of the drug. Vancomycin-loaded NPs have been shown to be effective at targeting *Staphylococcus aureus*. [375] Mesoporous silica NPs (MSNPs) are silicon-based nanoparticles which have a porous structure, enabling loading of compounds into them. MSNPs have a modifiable pore size and surface area to tailor its efficacy for a specific compound, and lack toxicity at low concentrations, making them excellent candidates for low-dosage drug formulations. [376,377] Like metal nanoparticles, MSNPs can be tailored towards a specific target and can also be effective against biofilms, which are notoriously hard for antibiotics to

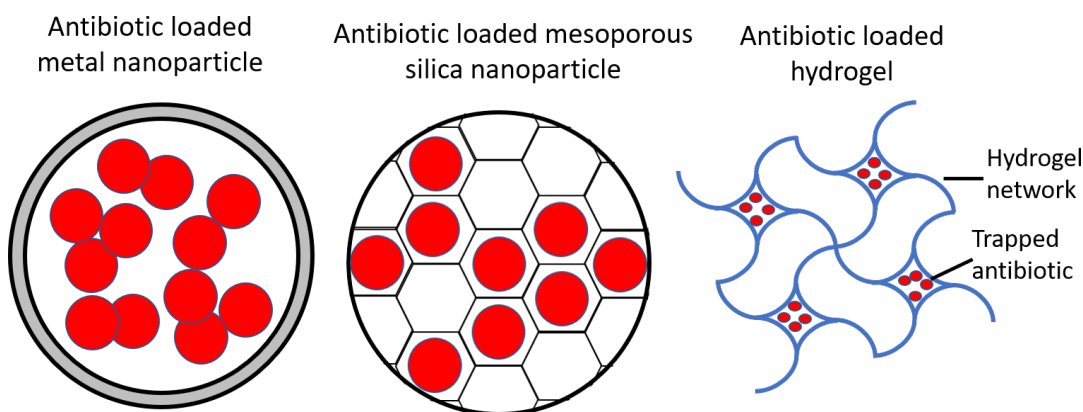


Figure A.2: Common loaded antibiotic drug delivery systems.

penetrate. [378]

Hydrogels, though some may display innate antimicrobial resistance, typically display antimicrobial activity through antimicrobial loading. [379] Although these approaches can aid in overcoming some methods of partial antibiotic resistance, they are still inherently limited by the spread of resistance mechanisms, particularly those that convey full tolerance, among bacterial populations. Hydrogels can function as either conjugated or loaded drug delivery systems. Hydrogels are hydrophilic, cross-linked polymers which are able absorb large quantities of water while maintaining their molecular structure. Naturally occurring hydrogels include fibrin and collagen. [380] Hydrogels are structurally similar to tissue and therefore can act as a scaffold during wound healing as their hydrophilic structure promotes immunological activity at the site of administration. [381] Hydrogels can be synthesised alongside antibiotic drug to capture the antibiotic within the structure, creating a loaded system. Alternatively, molecules can be covalently bonded to the hydrogel structure, creating a conjugated system where the antibiotic is released by the degradation of the hydrogel structure. [382]

Conjugated systems deliver antimicrobials by creating conjugates between an antibiotic and a carrier, for example, siderophore-antibiotic conjugates (Fig A.3). Siderophores are high-affinity iron-binding molecules secreted by bacteria to harvest iron, which are then internalised upon recognition by a bacterial receptor. [383] This

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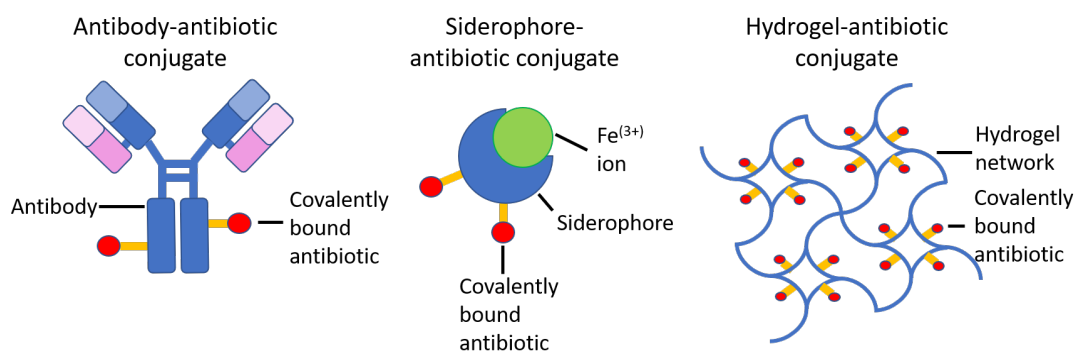


Figure A.3: Common conjugate antibiotic drug delivery systems.

can be exploited for internalisation of antibiotic-bound siderophores, such as the catechol and cephalosporin conjugate cefiderocol, which is used for treatment of MDR urinary tract infections by the NHS. [384]

Antibody-antimicrobial conjugates are an antibody and antibiotic joined together using a linker. Upon administration of the conjugate, the antibody portion of the conjugate will bind to the antigen complementary to its epitope, allowing delivery of the conjugated antimicrobial compound to a specific desired target site. Through the utilisation of monoclonal antibodies in these conjugates, the treatment is able to be tailored specifically to a single pathogen, increasing drug efficacy by reducing its spectrum of potential targets significantly to reduce exposure of an antibiotic to non-target microorganisms, slowing their rate of resistance evolution against it. [385] Antibody-antibiotic conjugates are limited by the need for a target antigen to be expressed for binding to occur, so effectiveness as a drug delivery system may be limited against bacteria which display antigenic variation, though antibodies which target static antigens on such bacteria, such as *Neisseria gonorrhoeae*, have been proven to be successful in treatments. [386]

A.3.2 Alternative Antimicrobial Molecules

Alternative antimicrobial molecules are molecules which display antimicrobial potential through mechanisms which are not shared with traditional antibiotics. Often these mechanisms differ significantly to the typical pathways utilised by these traditional

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antibiotics and therefore little resistance has emerged against them in bacterial populations, making them promising routes for the development of novel bactericidal drugs. There are many molecules which have been researched for this application.

Chitosan is a derivative of chitin, found in both animal and insect exoskeletons and fungal cell walls. [387] Chitosan-based hydrogels utilising chitosan dextran have been identified as producing antibacterial activity towards several clinically relevant bacteria, including *Pseudomonas aeruginosa*, *E. coli*, and *S. aureus*, manifesting through cell wall disruption. [388] Hydrogels can be synthesised from chitosan polymers, creating a hydrogel structure which displays inherent antimicrobial activity. Polycarbonate and polyethylene glycol have also been found to inhibit bacterial growth when used in hydrogel networks. [389]

Metal NPs typically range from 10-150 nm which, as described above, can be engineered to easily diffuse through biological membranes and also have some known antimicrobial potential. [390, 391] Oxidative stress is a major mechanism of toxicity caused by metal nanoparticles. Through this method of action, reactive oxygen species are generated by the release of metal ions from the nanoparticles, which disrupt bacterial processes or interact with oxygen in the bacterium, leading to the accumulation of reactive oxygen species. [392] This in turn leads to the initiation of the oxidative stress response, which, at high levels, can cause damage to bacterial DNA and activate cellular apoptosis. [393] Metal NPs can also denature ribosomes through binding to the metal ions and interrupt DNA replication. [394] Silver nanoparticles have been used in dentistry to prevent plaque biofilm development on dentures and gold nanoparticles have been found to be effective against enteric bacteria. [395, 396] Metal NPs display greater efficacy against G- bacteria than G+ as the negatively charged LPS attracts the positively charged metal nanoparticles and have a thinner cell wall to pass through. [397]

Another potential antimicrobial alternative to existing agents is dendrimers. Dendrimers are synthetic polymers that were first discovered in 1978. Dendrimers are large branched macromolecules which lead out from a ‘core’ molecule within the structure. [398] While dendrimers are often applied as a delivery system, some

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dendrimer structures have been reported to display inherent antimicrobial activity. Polyamidoamine dendrimers can function as both a drug delivery system and an antimicrobial. G5-PAMAM has been noted to display toxicity towards *Pseudomonas aeruginosa* and *Staphylococcus aureus*. [399]

Antimicrobial peptides (AMPs) are a significant field in the research of alternative antimicrobial compounds. There are several types of AMP, that can be categorised by source, activity, characteristics, and amino acid species. AMPs are usually cationic and have between 10 and 60 amino acid residues. [400] Mechanisms of AMPs can be divided into membrane targeting and non-membrane targeting mechanisms. Membrane disruption may occur through the carpet model, which sees the accumulation of AMPs on the surface of a membrane, resulting in the lysis of the bilayer structure. The Barrel Stave model sees AMP aggregates insert into the bilayer, forming a channel through the membrane. Other AMPs can affect different bacterial mechanisms, such as inhibiting protein synthesis by targeting ribosomes, inhibiting bacterial metabolism by inhibiting proteases, and preventing cell division by inhibiting DNA replication. [401]

A.4 Synthesis of Fatty Acids and Monoglycerides

Animal FAs are obtained either through synthetic pathways carried out by the animal or obtained through diet. *De novo* synthesis of FAs occurs in the cytosol of animal cells, primarily in adipose tissue or the liver. [402] *De novo* FA synthesis is mediated by the enzymes acetyl-CoA carboxylase and fatty acid synthase, and synthesis is a multistep process (Figure A.4).

FA synthesis requires acetyl-CoA and NADPH as precursors to the process. Acetyl-CoA is primarily obtained from decarboxylation of pyruvate within the mitochondria and transported into the cytoplasm, while NADPH is obtained from the pentose phosphate pathway. [403] FA synthesis begins with two molecules of acetyl-CoA. Acetyl-CoA carboxylase first catalyses the carboxylation of its biotin cofactor via the biotin carboxylase subunit, which is then transferred to one molecule of acetyl-CoA through the carboxyltransferase enzyme subunit, forming malonyl-CoA. [404] FA synthesis is rate limited by this step. [405]

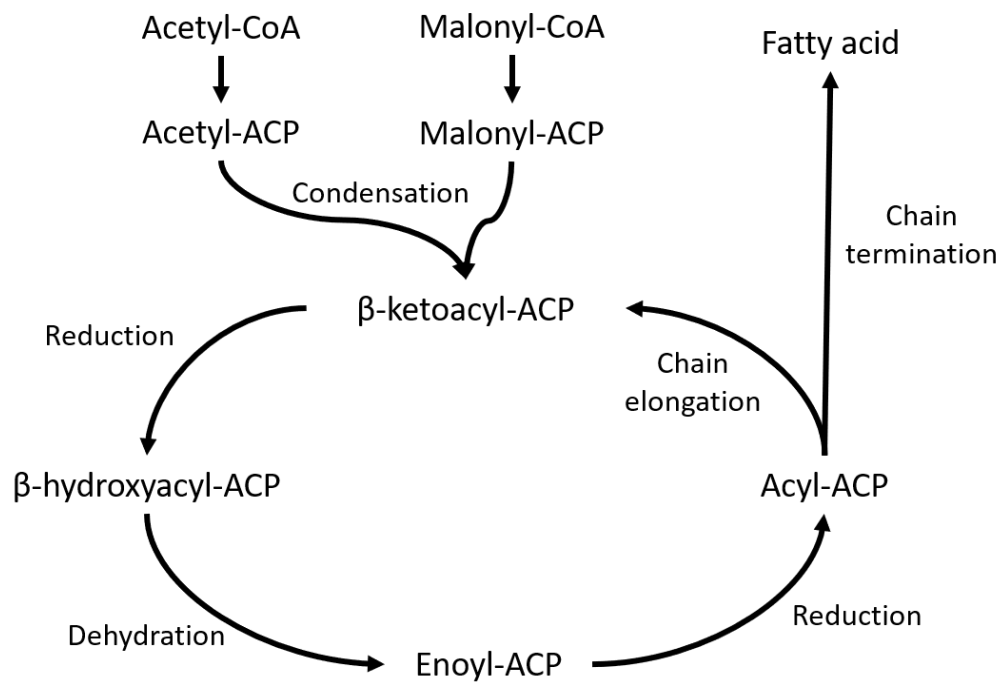


Figure A.4: Simplified stages of fatty acid synthesis.

Both the remaining molecule of acetyl-CoA and the synthesised malonyl-CoA are transferred to the fatty acid synthase complex to continue the process by an acyl carrier protein (ACP), which replaces each molecule's CoA group. Both these molecules are combined in a condensation step catalysed by β -ketoacyl-ACP synthase to produce β -ketobutryl-ACP. [406] This structure is then reduced to β -hydroxybutryl-ACP by β -ketoacyl-ACP reductase and then dehydrated crotonyl-ACP and finally reduced by enoyl-ACP reductase to produce the saturated butyryl-ACP. This four step cycle represents the elongation of the chain by one acetyl-ACP molecule. At the end of each cycle, the process is either restarted to continue growing the FA chain length, or the molecule is released from the cycle. Thioesterase facilitates the release of the FA chain from the complex by hydrolysing the thioester bond between the FA and ACP. [403] FAs in animals are primarily released from the FA synthase complex once they reach the length of palmitic acid (C16:0-FA). As FAs are extended by two carbons at a time, fatty acids produced by this process predominantly have an even acyl chain length.

Once released from the complex, FAs may undergo further modification to their

Appendix A. Additional Background Information

structure. Further elongation of the FA structure is mediated by enzymes in the endoplasmic reticulum in a multistep process similar to the cycle described above. [407] FA desaturases introduce double bonds into the saturated fatty acid structure to create monounsaturated or polyunsaturated fatty acids. [408] These bonds are more commonly cis-double bonds than trans. FA desaturases can only act on specific positions, and not all necessary desaturases are produced by all animals. Therefore, some vital FAs for animals must be obtained through diet, for example omega-3 fatty acid for humans. [409] FAs may also be incorporated into other molecules from this point, for example with proteins to create lipoproteins. [410]

The processing of fatty acids into TGs occurs in the endoplasmic reticulum to create a long term energy storage molecule. TGs are primarily synthesised from fatty acids in the liver, small intestine, and adipose tissue. The major method of triglyceride production in animals is through the glycerol-3-phosphate pathway. In hepatocytes this begins with the phosphorylation of the *sn-3* carbon by glycerol kinase. In adipocytes, as these cells do not express glycerol kinase, glycerol-3-phosphate is instead produced from dihydroxyacetone phosphate from the glycolysis pathway, which is reduced to glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase. [411] Glycerol-3-phosphate is then acylated at the *sn-1* position to form lysophosphatidate, then a second acyl group added to the *sn-2* carbon to form phosphatidate. Finally, the phosphate group is cleaved by a phosphatase to form diacylglycerol and a *sn-3* acyl chain is added to form a triacylglycerol. In the intestines, dietary fats are broken down via hydrolysis, which can also be utilised for triglyceride synthesis. MGs from this process can be acylated by monoacylglycerol O-acyltransferase into diacylglycerol, and then into triglycerides by diacylglycerol O-acyltransferase into diacylglycerol in enterocytes. [412]

Plants and microorganisms also have the ability to synthesise FAs and glycerides. Although the steps of *de novo* FA synthesis are generally shared with the animal synthesis pathways, the structure of fatty acid synthase differs from animal cells. Where animal FA synthase is a protein complex, referred to as Fatty Acid Synthase Type I, fatty acid synthase type II (FASII) is instead a series of discrete enzymes. FASII has the ability to terminate fatty acid synthesis early, which allows a greater range of FA

Appendix A. Additional Background Information

chain lengths to be produced. [413] FASII is found in plants, bacteria, some fungi, and mitochondria, where it is located in the plastids of plants, and the cytoplasm of plants and fungi. [414]

MGs are typically formed only as an intermediate product of triglyceride hydrolysis. This is because triglycerides are more efficient energy storage molecules, and MGs need to be broken down further into fatty acids to be converted into acetyl-CoA for use in the citric acid cycle. Fats ingested by diet are typically in the form of triglycerides, which are broken down within the small intestines into molecules with greater solubility. In the small intestine, bile salts facilitate fat digestion by trapping fats and causing micellisation of fats into small micelles. [415] Pancreatic lipase then catalyses the hydrolysis of triglycerides within these micelles, breaking them down into fatty acid and monoglyceride subunits which are then taken-up by enterocytes to be synthesised into triglycerides. Triglyceride hydrolysis also occurs in adipose tissue through a series of three lipase-mediated hydrolysis reactions. [416] Adipose triglyceride lipase catalyses the hydrolysis of triglycerides to diglycerides, followed by hydrolysis into monoglycerides by hormone sensitive lipase, and finally fatty acids by monoglyceride lipase. [417]

Industrially, monoglycerides are usually synthesised through glycerolysis. This reaction is typically mediated by an alkali catalyst at a temperature of 200-260 °C, and sees the transesterification of triglycerides with excess glycerol to produce mono- and diglycerides. [418] This process is performed usually using vegetable oils as the source of triglycerides. [418–420]

Appendix B

Monoglyceride Parameters

B.1 C8-MG.rtf

```
1      read rtf card append
2      * Topology for glycerol monocaprylate
3      *
4      36 1
5
6      AUTOGENERATE ANGLE DIHE
7
8      RESI GMC1          0.00 ! Glycerol monocaprylate (1-isomer)
9      !
10     GROUP
11     ATOM C1    CTL2    0.05 !
12     ATOM HA    HAL2    0.09 !          HA
13     ATOM HB    HAL2    0.09 !          |
14     ATOM O11   OHL     -0.65 !          |
15     ATOM HO1   HOL     0.42 !          HB---C1---O11--HO1
16     GROUP          !          |
17     ATOM C2    CTL1    0.14 !          HS---C2-----
18     ATOM HS    HAL1    0.09 !          |          |
19     ATOM O21   OHL     -0.65 !          HO2--O21          theta3
```

Appendix B. Monoglyceride Parameters

20	ATOM H02	HOL	0.42	!			
21	GROUP			!			
22	ATOM C3	CTL2	0.08	!			
23	ATOM HX	HAL2	0.09	!		HX---C3---HY	
24	ATOM HY	HAL2	0.09	!			gamma1
25	ATOM O31	OSL	-0.49	!		O32 O31	
26	ATOM C31	CL	0.90	!		\\ /	gamma2
27	ATOM O32	OBL	-0.63	!		C31	
28	ATOM C32	CTL2	-0.22	!			gamma3
29	ATOM H2X	HAL2	0.09	!		H2X---C32---H2Y	
30	ATOM H2Y	HAL2	0.09	!			
31	GROUP			!			gamma4
32	ATOM C33	CTL2	-0.18	!			
33	ATOM H3X	HAL2	0.09	!		H3X ---C33---H3Y	
34	ATOM H3Y	HAL2	0.09	!			
35	GROUP			!			
36	ATOM C34	CTL2	-0.18	!			
37	ATOM H4X	HAL2	0.09	!		H4X ---C34---H4Y	
38	ATOM H4Y	HAL2	0.09	!			
39	GROUP			!			
40	ATOM C35	CTL2	-0.18	!			
41	ATOM H5X	HAL2	0.09	!		H5X ---C35---H5Y	
42	ATOM H5Y	HAL2	0.09	!			
43	GROUP			!			
44	ATOM C36	CTL2	-0.18	!			
45	ATOM H6X	HAL2	0.09	!		H6X ---C36---H6Y	
46	ATOM H6Y	HAL2	0.09	!			
47	GROUP			!			
48	ATOM C37	CTL2	-0.18	!			
49	ATOM H7X	HAL2	0.09	!		H7X ---C37---H7Y	

Appendix B. Monoglyceride Parameters

```

50      ATOM H7Y  HAL2   0.09  !
51      GROUP
52      ATOM C38  CTL3  -0.27  !
53      ATOM H8X  HAL3   0.09  !      H8X---C38--H8Y
54      ATOM H8Y  HAL3   0.09  !
55      ATOM H8Z  HAL3   0.09  !      H8Z
56
57      ! Glycerol Backbone
58      BOND  C1   O11      O11  H01
59      BOND  C1   HA       C1   HB      C1   C2
60      BOND  C2   O21      O21  H02
61      BOND  C2   HS       C2   C3
62      BOND  C3   HX       C3   HY      C3   O31
63      ! Chain #3
64      BOND  O31  C31
65      BOND  C31  C32
66      DOUBLE C31  O32
67      BOND  C32  H2X      C32  H2Y      C32  C33
68      BOND  C33  H3X      C33  H3Y      C33  C34
69      BOND  C34  H4X      C34  H4Y      C34  C35
70      BOND  C35  H5X      C35  H5Y      C35  C36
71      BOND  C36  H6X      C36  H6Y      C36  C37
72      BOND  C37  H7X      C37  H7Y      C37  C38
73      BOND  C38  H8X      C38  H8Y      C38  H8Z
74
75      IMPR C31 O31 C32 O32
76
77      END

```


B.2 C10-MG.rtf

```

1      read rtf card append
2      * Topology for glycerol monocaprte
3      *
4      36 1
5
6      AUTOGENERATE ANGLE DIHE
7
8      RESI GMC1          0.00 ! Glycerol monocaprte (1-isomer)
9      !
10     GROUP
11     ATOM C1    CTL2    0.05 !
12     ATOM HA    HAL2    0.09 !          HA
13     ATOM HB    HAL2    0.09 !          |
14     ATOM O11   OHL     -0.65 !          |
15     ATOM HO1   HOL     0.42 !          HB---C1---O11--HO1
16     GROUP          !          |
17     ATOM C2    CTL1    0.14 !          HS---C2-----
18     ATOM HS    HAL1    0.09 !          |          |
19     ATOM O21   OHL     -0.65 !          H02--O21          theta3
20     ATOM HO2   HOL     0.42 !          |
21     GROUP          !          |
22     ATOM C3    CTL2    0.08 !          |
23     ATOM HX    HAL2    0.09 !          HX---C3---HY
24     ATOM HY    HAL2    0.09 !          |          gamma1
25     ATOM O31   OSL     -0.49 !          032  031
26     ATOM C31   CL      0.90 !          \\ /          gamma2
27     ATOM O32   OBL     -0.63 !          C31
28     ATOM C32   CTL2    -0.22 !          |          gamma3
29     ATOM H2X   HAL2    0.09 !          H2X---C32---H2Y

```

Appendix B. Monoglyceride Parameters

30	ATOM H2Y	HAL2	0.09	!		
31	GROUP			!		gamma4
32	ATOM C33	CTL2	-0.18	!		
33	ATOM H3X	HAL2	0.09	!		H3X ---C33---H3Y
34	ATOM H3Y	HAL2	0.09	!		
35	GROUP			!		
36	ATOM C34	CTL2	-0.18	!		
37	ATOM H4X	HAL2	0.09	!		H4X ---C34---H4Y
38	ATOM H4Y	HAL2	0.09	!		
39	GROUP			!		
40	ATOM C35	CTL2	-0.18	!		
41	ATOM H5X	HAL2	0.09	!		H5X ---C35---H5Y
42	ATOM H5Y	HAL2	0.09	!		
43	GROUP			!		
44	ATOM C36	CTL2	-0.18	!		
45	ATOM H6X	HAL2	0.09	!		H6X ---C36---H6Y
46	ATOM H6Y	HAL2	0.09	!		
47	GROUP			!		
48	ATOM C37	CTL2	-0.18	!		
49	ATOM H7X	HAL2	0.09	!		H7X ---C37---H7Y
50	ATOM H7Y	HAL2	0.09	!		
51	GROUP			!		
52	ATOM C38	CTL2	-0.18	!		
53	ATOM H8X	HAL2	0.09	!		H8X ---C38---H8Y
54	ATOM H8Y	HAL2	0.09	!		
55	GROUP			!		
56	ATOM C39	CTL2	-0.18	!		
57	ATOM H9X	HAL2	0.09	!		H9X ---C39---H9Y
58	ATOM H9Y	HAL2	0.09	!		
59	GROUP			!		

Appendix B. Monoglyceride Parameters

```

60      ATOM C310 CTL3  -0.27 !
61      ATOM H10X HAL3   0.09 !
62      ATOM H10Y HAL3   0.09 !
63      ATOM H10Z HAL3   0.09 !
64
65      ! Glycerol Backbone
66      BOND C1 O11 O11 H01
67      BOND C1 HA C1 HB C1 C2
68      BOND C2 O21 O21 H02
69      BOND C2 HS C2 C3
70      BOND C3 HX C3 HY C3 O31
71      ! Chain #3
72      BOND O31 C31
73      BOND C31 C32
74      DOUBLE C31 O32
75      BOND C32 H2X C32 H2Y C32 C33
76      BOND C33 H3X C33 H3Y C33 C34
77      BOND C34 H4X C34 H4Y C34 C35
78      BOND C35 H5X C35 H5Y C35 C36
79      BOND C36 H6X C36 H6Y C36 C37
80      BOND C37 H7X C37 H7Y C37 C38
81      BOND C38 H8X C38 H8Y C38 C39
82      BOND C39 H9X C39 H9Y C39 C310
83      BOND C310 H10X C310 H10Y C310 H10Z
84
85      IMPR C31 O31 C32 O32
86
87      END

```

B.3 C12-MG.rtf

```

1      read rtf card append
2      * Topology for glycerol monolaurate
3      *
4      36 1
5
6      AUTOGENERATE ANGLE DIHE
7
8      RESI GML1          0.00 ! Glycerol monolaurate (1-isomer)
9      !
10     GROUP
11     ATOM C1   CTL2   0.05 !
12     ATOM HA   HAL2   0.09 !           HA
13     ATOM HB   HAL2   0.09 !           |
14     ATOM O11  OHL   -0.65 !           |
15     ATOM HO1  HOL    0.42 !           HB---C1---O11--HO1
16     GROUP                !           |
17     ATOM C2   CTL1   0.14 !           HS---C2-----
18     ATOM HS   HAL1   0.09 !           |           |
19     ATOM O21  OHL   -0.65 !           H02--O21           theta3
20     ATOM HO2  HOL    0.42 !           |
21     GROUP                !           |
22     ATOM C3   CTL2   0.08 !           |
23     ATOM HX   HAL2   0.09 !           HX---C3---HY
24     ATOM HY   HAL2   0.09 !           |   gamma1
25     ATOM O31  OSL   -0.49 !           032  031
26     ATOM C31  CL    0.90 !           \\ /   gamma2
27     ATOM O32  OBL   -0.63 !           C31
28     ATOM C32  CTL2  -0.22 !           |   gamma3
29     ATOM H2X  HAL2   0.09 !           H2X---C32---H2Y

```

Appendix B. Monoglyceride Parameters

30	ATOM H2Y	HAL2	0.09	!		
31	GROUP			!		gamma4
32	ATOM C33	CTL2	-0.18	!		
33	ATOM H3X	HAL2	0.09	!		H3X ---C33---H3Y
34	ATOM H3Y	HAL2	0.09	!		
35	GROUP			!		
36	ATOM C34	CTL2	-0.18	!		
37	ATOM H4X	HAL2	0.09	!		H4X ---C34---H4Y
38	ATOM H4Y	HAL2	0.09	!		
39	GROUP			!		
40	ATOM C35	CTL2	-0.18	!		
41	ATOM H5X	HAL2	0.09	!		H5X ---C35---H5Y
42	ATOM H5Y	HAL2	0.09	!		
43	GROUP			!		
44	ATOM C36	CTL2	-0.18	!		
45	ATOM H6X	HAL2	0.09	!		H6X ---C36---H6Y
46	ATOM H6Y	HAL2	0.09	!		
47	GROUP			!		
48	ATOM C37	CTL2	-0.18	!		
49	ATOM H7X	HAL2	0.09	!		H7X ---C37---H7Y
50	ATOM H7Y	HAL2	0.09	!		
51	GROUP			!		
52	ATOM C38	CTL2	-0.18	!		
53	ATOM H8X	HAL2	0.09	!		H8X ---C38---H8Y
54	ATOM H8Y	HAL2	0.09	!		
55	GROUP			!		
56	ATOM C39	CTL2	-0.18	!		
57	ATOM H9X	HAL2	0.09	!		H9X ---C39---H9Y
58	ATOM H9Y	HAL2	0.09	!		
59	GROUP			!		

Appendix B. Monoglyceride Parameters

60	ATOM C310 CTL2	-0.18 !					
61	ATOM H10X HAL2	0.09 !				H10X---	C310--H10Y
62	ATOM H10Y HAL2	0.09 !					
63	GROUP	!					
64	ATOM C311 CTL2	-0.18 !					
65	ATOM H11X HAL2	0.09 !				H11X---	C311--H11Y
66	ATOM H11Y HAL2	0.09 !					
67	GROUP	!					
68	ATOM C312 CTL3	-0.27 !					
69	ATOM H12X HAL3	0.09 !				H12X---	C312--H12Y
70	ATOM H12Y HAL3	0.09 !					
71	ATOM H12Z HAL3	0.09 !					H12Z
72							
73	! Glycerol Backbone						
74	BOND C1 O11	O11 H01					
75	BOND C1 HA	C1 HB	C1 C2				
76	BOND C2 O21	O21 H02					
77	BOND C2 HS	C2 C3					
78	BOND C3 HX	C3 HY	C3 O31				
79	! Chain #3						
80	BOND O31 C31						
81	BOND C31 C32						
82	DOUBLE C31 O32						
83	BOND C32 H2X	C32 H2Y	C32 C33				
84	BOND C33 H3X	C33 H3Y	C33 C34				
85	BOND C34 H4X	C34 H4Y	C34 C35				
86	BOND C35 H5X	C35 H5Y	C35 C36				
87	BOND C36 H6X	C36 H6Y	C36 C37				
88	BOND C37 H7X	C37 H7Y	C37 C38				
89	BOND C38 H8X	C38 H8Y	C38 C39				

Appendix B. Monoglyceride Parameters

90	BOND	C39	H9X	C39	H9Y	C39	C310
91	BOND	C310	H10X	C310	H10Y	C310	C311
92	BOND	C311	H11X	C311	H11Y	C311	C312
93	BOND	C312	H12X	C312	H12Y	C312	H12Z
94							
95	IMPR	C31	O31	C32	O32		
96							
97	END						

Appendix C

Input Files and Scripts

C.1 Input File Repository

Relevant files are available at

<https://doi.org/10.15129/087cdb53-5dd9-4307-8cfe-9e4aec935ec3>