Development of Membrane Models for Study of Drug Interactions

Master's Thesis

Master of Materials Science

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1. Introduction

Microbial contamination had been a leading threat to human health globally, affecting humans, animals and plants. Infectious diseases such as AIDS and Hepatitis B rooting from microbial pathogens are the leading cause of death worldwide. In 2010, approximately 48 million cases of pathogen-related diseases were reported in the United States alone. The spread of virus and bacteria through air, water and food had prompted researchers to find alternatives on minimizing and controlling the effect of microbial pathogens before it becomes a serious health hazard [1,2,3]. Development of new antibiotics, antiseptics and disinfectants are aimed at curbing bacterial population and preventing further proliferation.

Antibacterial agents are used as one of the measures in restricting the advancement of pathogenic bacteria. The mechanism of interaction between antimicrobial agents with the membrane of bacteria involves electrostatic attraction between positive charge moiety of the agent to the negatively charged acidic phospholipids comprising the bacterial membrane [4]. The antibacterial compound penetrates further into the membrane and disorganizes the structure of cell membrane which leads to the leakage of cellular content and subsequently the death of cell [5].

Langmuir technique proves to be one of the effective ways to measure the interaction between the antimicrobial agents with the cell membrane. The formation of ordered lipid monolayer by means of compression of lipid molecules mimic the bacterial cell membrane as a less complex model. The membrane affinity towards antibacterial agents are evaluated using penetration measurement in which the antibacterial agents introduced affects the change in surface pressure of the lipid monolayer. The degree of change of the surface pressure allows quantitative characterization of the molecular interaction and can be considered as a reasonable approach in estimating the efficacy of the antibacterial agents [6].

During my work, Langmuir technique was used to form ordered lipid monolayers with different lipid compositions and to investigate the membrane affinity of a series of selected agents as model drug compounds.

2. Literature review

2.1. Antibacterial agents

Antibacterial agents are a group of materials which destroys bacteria by killing or by interfering with its growth resulting in a decreased pathogenic effects towards the biological environment [7]. The most widely used membrane active agents are small molecules which dominates the market today especially in pharmaceutical industries. There are many advantages in using small molecule membrane active agents which includes relatively easy synthesis process, easily eliminated from the body and are easily processed in large scale. Due to the small size, they can easily pass through the cell membrane barriers [8].

The agents can be divided into two groups; bactericidal, which destroys bacteria, or bacteriostatic, which slows down the growth of bacteria [9]. Antibacterial agents can also be further classified into organic and inorganic agents. Extensive research had been done using organic antibacterial agents such as organic acids, lysozyme, bacteriocins and essential oils. It was found that the agents are highly influenced by the surrounding condition. Organic antibacterial agents are very sensitive towards high temperature and pressure, while in contrast, inorganic agents proven to be more stable under the particular condition. These agents, especially metal oxides, are more advantageous in terms of having strong antibacterial activity at lower concentrations and longer shelf-life [10].

Antibacterial agents are very crucial in combating infectious diseases. However, new health problems may emerge due to rise of resistant bacteria, where pathogen strains may transfer their antibiotic-resistant genes to other bacteria, creating multiple drug resistant bacteria [11]. One prominent disease related to transfer of antibiotic-resistant genes is Tuberculosis (TB). These bacteria are immune to antibiotics which makes it a challenge in the health industry to create a proper solution for the problem. The use of high-dose antibiotics was found to be unsuitable since it produced intolerable toxicity in the human body [9]. Thus, alternative methods were studied extensively to create a treatment towards the bacterial diseases.

2.2. Bacterial cell wall

In order to kill bacteria, the understanding of its properties and characteristics are crucial. Bacterial cell walls are divided into Gram-positive and Gram-negative bacteria depending on its structure, components and functions. The two cell walls differ in terms of the thickness in which Gram-positive bacteria species such as *Staphylococcus* and *Bacillus subtilis* have a thick, approximately 20-50nm peptidoglycan (PG) cell wall layer compared to Gram-negative cell wall which has a thinner layer as can be seen in Figure 1. Gram-negative bacteria such as *Escheria Coli* consist of an outer membrane which is resistant to hydrophobic compounds and detergents [9,12]. The outer membrane also has an additional coating of lipopolysaccharide (LPS) which covers 90% of the membrane and which is a polyanion [12].

(a)

(b)

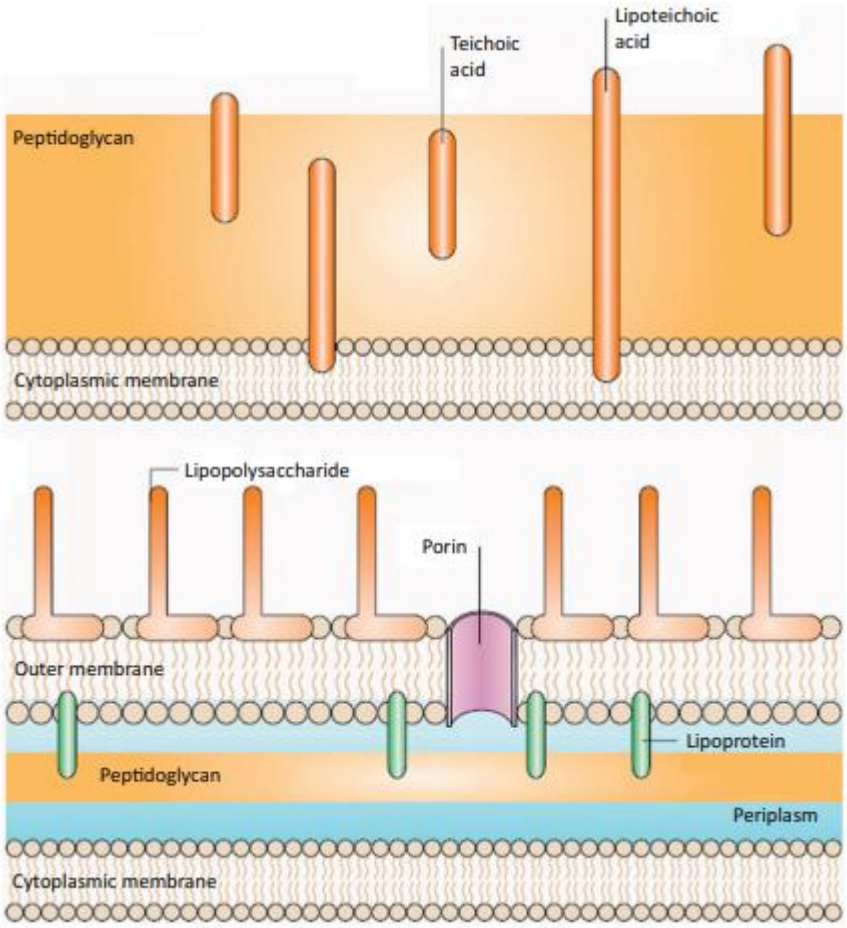


Figure 1: Bacteria cell membrane (a) Gram-positive cell wall (b) Gram-negative cell wall [9]

The cell membrane is a complex system which differs from organism to organism, and it is separating the interior part of a cell from the outer. This biological membrane consist of proteins, carbohydrates, lipids and act as the main site for various events to occur including signaling, synthesis, adhesion and transport. The high complexity and continuous motion of the membrane had made it a challenge to study the components and the existing relationship between them. The key is to understand a membrane system described by the distinctiveness and complexity of each segment of the membrane, followed by the chemical composition, and lastly, the functional role of the segments. The composition of the membrane is in the focus of interest because it depends on the variability of the lipid head group and fatty acid chains [13]. On the other hand, cell wall is designed to provide strength while performing barrier activities such as controlling the permeability between the inner and outer part of the cell. The presence of antibacterial agents will disrupt the activities and function of the barrier and subsequently lead to the breakdown of the cell membrane [5].

2.3. Nanosize antibacterial agents

The development of nanotechnology has promoted nanosized material as the new antibacterial agents due to its enhanced properties compared to bulk-sized material. By limiting the size to nanometer range, the nanomaterial shows better mechanical strength, diffusivity, chemical reactivity and optical properties which is contributed by the large surface area to volume ratio. This had led to the discovery where the transport of antibiotics using nanosized carriers to be more effective in resisting infectious diseases including those antibiotic-resistant bacteria [9,10].

Antibacterial nanomaterials such as metal, metal oxide and organic nanomaterials possess distinct properties which contribute to the various mode of interactions with bacteria cell membrane. The toxicity of nanomaterials acts by these two approaches which transpire simultaneously in most cases; (1) interference in membrane potential and structural stability and (2) formation of free radical known as reactive oxidation species (ROS) upon interaction with nanoparticles [9,14]. Binding of nanomaterial on the bacterial cell membrane via electrostatic interaction disrupts the membrane potential which leads to depolarization and depletion of strength of the membrane. As a result, functions of the membrane such as transportation, respiration and

transduction of energy were impaired leading to cell lysis and death of cell. Burst of ROS caused by severe oxidative stress induces destruction of macromolecules comprising the cell which subsequently leads to peroxidation of lipid, change of proteins, enzyme hindrance and DNA impairment as shown in Figure 2 [14].

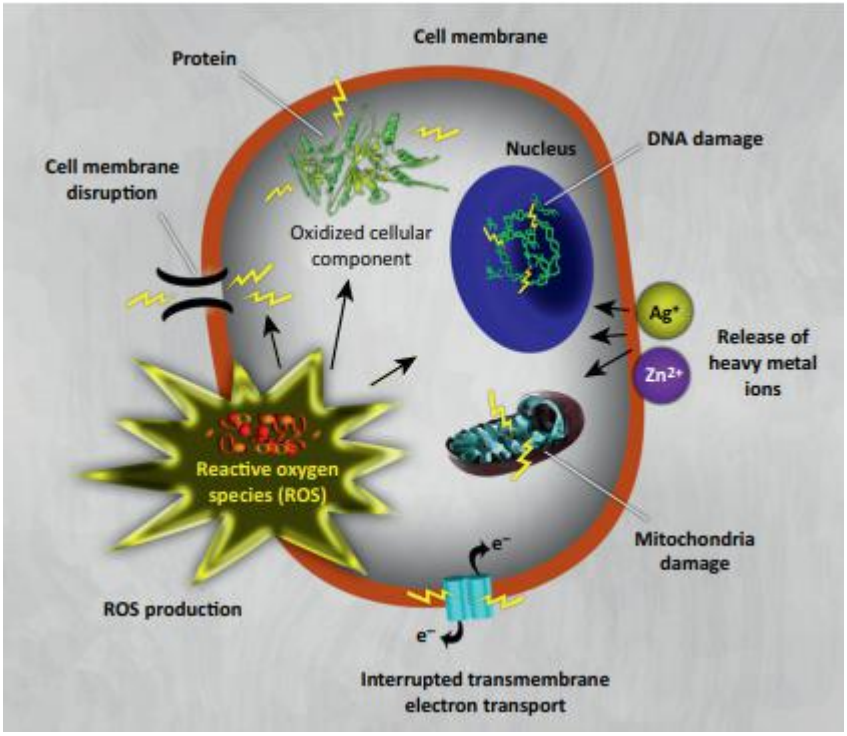


Figure 2: Nanoparticle toxicity mechanism against bacteria [9]

However, the mechanism of toxicity of nanoparticles is highly dependent on the variety of intrinsic properties, composition, surface modification and the type of bacteria. Some example of diversified mechanism against specific type of bacteria are presented in Table 1.

Bacteria	**Bacterial property**	**Nanoparticle composition**	**Mechanism of toxicity**
Gram-positive bacteria			
S.aureus	Biofilm formation, normal flora skin, production a matrix of exopolymeric substances	Ag-coated SPIONs (superparam-agnetic ironoxide)	Bacterial toxicity by penetration within the biofilm and increase of the bacterial toxicity in the presence of external magnetic field, ROS generation, electrostatic interaction and physical damage of bacteria.
		Ag-Au-coate-d SPIONs	

(Halophilic) *bacterium* sp. EMB4	Non-pathogen Gram-positive halophilic, has a thicker PG layer with higher percentage of neutral PG	ZnO	Electrostatic interaction, morphological changes in the presence of bulk and nano ZnO, increase in membrane permeability and ZnO accumulation in the cytoplasm
B.subtilis	Non-pathogen, protective endospore forming	Ag	Release of Ag^{+} and Cu^{2+}, electrostatic interaction, cell wall damage, rupture of the plasma membrane and disrupt biochemical process
		CuO	
Gram-negative bacteria			
P.aeruginosa	Normal flora of skin and intestine, biofilm formation	TiO_2	Photoactivation of TiO_2 promotes bactericidal effect Peroxidation of the polyunsaturated phospholipid of membrane, loss of respiratory activity
E.coli		NiO	Growth inhibition (in aqueous medium) Significant damaged cellular function, physical/mechanical stresses on cellular structure integrity (in aerosol exposure)
		Ag	Disturbed permeability, respiration and cell division Interacts with cell membrane and sulphur- and phosphorus-containing compounds
C.metallidurans CH34	Non-pathogen, Resistant in the presence of several forms of heavy metal	TiO_2	*C.metallidurans* CH34 is resistant to NPs. TiO_2 and Al_2O_3 can internalize in this bacterium but these NPs do not cause death
		Al_2O_3	

Table 1: Various nanomaterials and their toxicity against Gram-positive and Gram-negative bacteria [9]

Among the factors influencing the reaction of antibacterial nanoparticles against bacteria includes the growth rate of bacteria and the formation of biofilm. Bacteria growing at a faster rate are more responsive towards antibiotics and nanostructured particles compared to slow-growing bacteria with reasons concerning the specific type of bacterial strain. The formation of biofilm also leads to significant health problem such as bacterial infection. The adhesion of a certain type of complex microbes on a

surface leads to secretion of a matrix which envelops the pathogenic bacterial community and protects it against antibiotics and antibacterial agents [9].

2.4. Application of antibacterial agents

Antibacterial agents are also used widely in food industry particularly in food packaging and quality monitoring of food products [15]. Contamination on food products can easily occur during food processing and distribution which can compromise the physical, chemical and biological change of food. Proper packaging of food insulates the product from external contamination and prevents the food from deteriorate during processing and storage [16]. Active packaging (AP) emerge as an assist in preserving the quality while maintaining food safety. AP can be classified into two groups; i) immobilized AP, which serve its purpose without the need of movement and ii) active release packaging, which allows controlled movement of nonvolatile agents or a release of volatile component into the surrounding atmosphere of the food product. Responsive AP occurs by a trigger in the change of food product or packaging environment and causes the releasing of antimicrobial agent by several means as shown in Figure 3 [16].

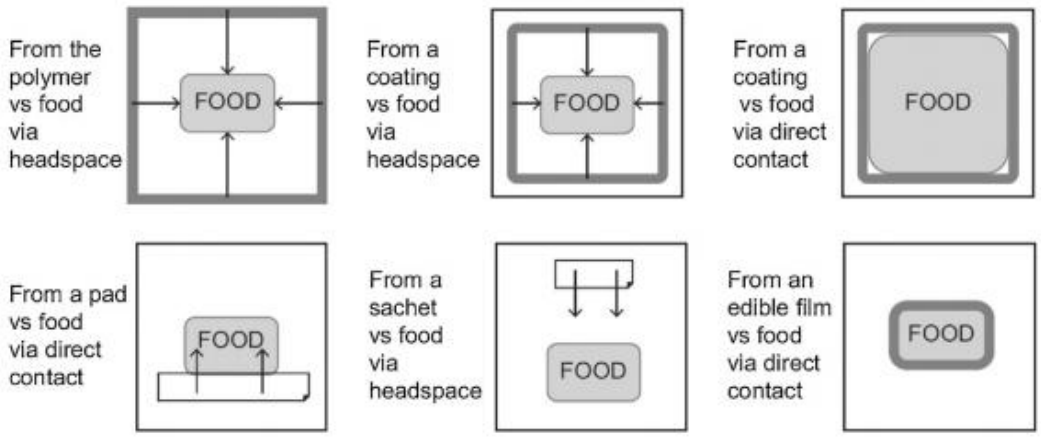


Figure 3: Antimicrobial releasing method in active packaging [16]

Nano-inorganic material had shown promising antibacterial properties. Metal oxides such as ZnO, MgO and CaO are marked as safe for human consumption and could benefit as it has antibacterial activities against certain pathogens**.** ZnO nanoparticles are reported to have higher antibacterial resistance towards

microorganisms compared to other metal oxide nanoparticles [10]. Zinc oxide is one of the main component widely used in food and drug industries. For example, ZnO quantum dots are used to reduce *Salmonella bacteria* in egg whites, application of ZnO powder in dermatological products such as creams and ointments as well as ZnO nanoparticle in drug carriers in which all of these applications are in reference to its antibacterial properties [15]. In food industries, ZnO is incorporated into food packaging, where coming into contact with the food, it will act on the food-borne microorganisms and slows their growth and thus extends the shelf life of the product itself [17].

Antimicrobial agents are also used directly in food to combat foodborne microbial pathogens. Among the Food and Drug Administration (FDA) approved antimicrobials there are various types of organic acids such as acetic, benzoic, sorbic, lactic and propionic acid, nitrites and nitrates, natural preservatives such as nisin and lysozyme, as well as paraben [18]. Some of the example of the use of antimicrobial compounds in food can be seen in Table 2.

Compound	Microbial target	Food Application
Acetic acid, acetates, diacetates	Yeasts, bacteria	Baked goods, condiments, dairy products, sauces
Lysozyme	*Clostridium botulinum,* other bacteria	Cheese, casings for frankfurters, cooked meat and poultry products
Parabens	Yeasts, molds	Beverages, baked goods, syrups, dry sausage
Sorbic acids, sorbates	Yeasts, molds and bacteria	Most foods, beverages, wine

Table 2: List of antimicrobials used in foods as approved by FDA [19]

The increase of awareness of public hygiene has also impacted the textile industry in terms of defeating harmful effect caused by microorganisms. Textiles are used very broadly in daily lives such as clothes, socks, towels, bed sheets and floor coverings are all very susceptible to contamination. In normal environment, textiles used will always be exposed to microorganisms and can cause undesirable effects such as unpleasant odor, staining and deterioration of fibers [20], however, the highest purity especially expected in hotels and hospitals due to the multiple usage of bed linens and

garments which increases the possibility of cross contamination and transmission of diseases between guests and patients [21].

Microorganisms generally accumulate and multiply on surfaces especially at suitable temperature and humidity, and in the presence of dust, sweat, oil and dead skin cells, thus it is very crucial to have textiles with modified surfaces to significantly reduce proliferation of microorganisms [20]. Application of antibacterial modification of surface is one of the used alternatives which utilizes the unique properties of metallic and inorganic nanomaterials into textiles with the aim to create an antibacterial surface which would prohibit the primary attachment of bacteria and subsequently hinder the formation of biofilm [22]. The surfaces can undergo chemical and physical treatments such as surface polymerization, functionalization and modification of surface structure. Surface polymerization is the modification of surface chemistry in order to adhere the antimicrobial agent onto a surface using techniques such as atom radical transfer or chemical bonding [22].

The production of textiles with electrical conductivity are emerging as it overcomes static charge accumulation on textile surfaces. Conjugated polymers or known as intrinsically conducting polymers (ICPs) has been of interest for its enhanced electrical conductivity, good environmental stability and easy to synthesis. ICPs such as polypyrrole (PPy) and polyaniline are used in coating of textiles and are used as static charge dissipation, electromagnetic interference shielding and even nowadays, applications such as military camouflage are adopted due to their ability to absorb fractions of microwave radiation [23]. The synthesis by oxidative polymerization of PPy introduces positive charges along its backbone chain which gives rise to its antibacterial properties and are further utilized as antibacterial agents in textiles [24].

2.5. Antibacterial polymers

Polymeric antimicrobial agents are widely used as an alternative to conventional agents due to some additional advantages. Apart from having high molecular weight and low cost, polymeric antimicrobial agents are especially used for its chemical and environmental stability, the difficulty to diffuse through the skin, low toxicity, nonvolatile properties and desirable biocompatibility [24]. An antimicrobial polymer

is described as polymeric structure consisting of biocidal pendant group or biocidal repeating unit which is developed through polymerization of low molecular weight biocides. Among them, antimicrobial polymeric materials comprising of quaternary ammonium (QAS) and/or phosphonium salt (QPS) are the most widely investigated nowadays [24,25]. Polymeric QAS/QPS are more desirable compared to low-molecular weight QAS/QPS due to the higher positive charge density. Since most of the bacteria membranes are negatively charged, polymeric QAS/QPS promotes initial adsorption onto the membrane surface resulting in an enhanced antibacterial activity [1].

The antibacterial polymers with cationic groups are synthesized to mimic cationic antimicrobial peptides (AMPs) with enhanced feature. Synthetic polymers are easy to synthesized and possess good stability against enzymatic degradation as opposed to antimicrobial peptides [26]. The antimicrobial polymers were designed according to the chemical templates of natural antimicrobial peptides found in the immune system of the body (Figure 4) [27].

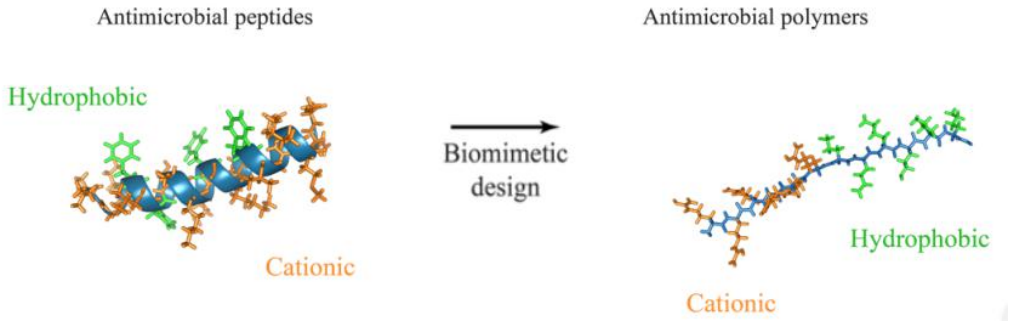


Figure 4: Biomimetic design of antimicrobial polymer according to the structure of natural peptide [27]

Antimicrobial cationic polymers generally contain two functional parts; cationic part and hydrophobic part in which the main positively charged component is the quaternary ammonium group yielding quaternary ammonium compound (QAC) [28]. QACs are used for various industrial purposes ranging from surfactants to antiseptics to anesthetics due to its desired physical properties in addition to its antibacterial characteristic. It is widely used in personal care industry because of its ability to adsorb onto organic surfaces and decrease the interfacial and surface tension, making it very desirable in hair care products and skin care. Formulations of lotions and hair conditioners mainly incorporate alkyl and polymeric QACs [29]. The destructive and

restriction effects of QACs are highly influenced by the environmental conditions where the probability to have significant interaction of QACs is reduced at high microbial density and also in the presence of organic material such as biofilm. The activity of QACs is also reduced in hard water, making high concentration of mineral salts to inactivate the QACs. However, the antibacterial activity is enhanced with increasing temperature and time of exposure [30].

QACs are classified as membrane active agents in which the interaction of the cationic agents with microorganisms results in the following events: (1) adsorption of agents onto bacterial cell surface (2) penetration into cell wall (3) binding and disorganization of cytoplasmic membrane (lipid or protein) (4) breaching of intracellular components (5) deterioration of proteins and nucleic acids (6) wall disintegration caused by autolytic enzymes. The loss of structural integrity and damaging effects towards the inner components causing the death of cell [3,31]. The destructive activity of QAC on virus is associated with the separation of virus from its envelop which causes the release of nucleic acid while exposure of non-encased virus leads to the formation of micelles, which are not destructive [30]. Some properties of QACs includes medium sized molecules weighing between 250 to 400g/mol, have high boiling points and low volatilization rate [29]. Common QACs include chlorine and bromine salts of cetrimonium (cetyl trimethyl ammonium) consisting of 16 alkyls chain length [4].

In general, QAC have the chemical structure $N^+R_1R_2R_3R_4X^-$ with positively charged nitrogen head bearing four bonds of R representing a hydrogen atom, a basic alkyl group or substituted alkyl group by other functional group such as methyl, benzyl or ester groups, while X represents an anion as shown in Figure 5 [4].

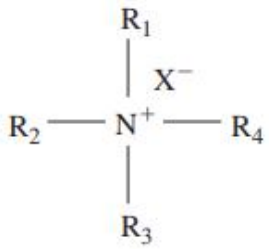
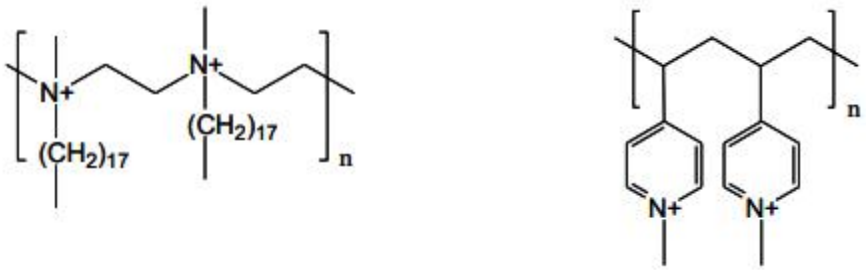


Figure 5: General structure of quaternary ammonium compound [29]

Quaternary ammonium groups are structured either as the main chain or as a side chain (Figure 6). Antimicrobial polymers with existing hydrophobic backbone does not require biocidal repeating units, on the contrary, polymer backbone with hydrophilic characteristic requires hydrophobic component accompanying the backbone, which is equipped by the hydrophobic side chain [32].



(a) (b)

Figure 6: Quaternary ammonium group (a) in the main chain (b) as a side chain [32]

Various cationic surfactants were investigated to reveal antibacterial mechanisms such as between monomeric amphiphile dodecyltrimethyl ammonium bromide (DTAB) and *Escherichia coli.* The effect of homologous cationic surfactant such as decyltrimethyl ammonium bromide (DeTAB), hexadecyltrimethyl ammonium bromide (HTAB), tetradecyltrimethyl ammonium bromide (TTAB), and octyltrimethyl ammonium bromide (OTAB) were also of interest in determining the influence of alkyl chain length towards the antibacterial properties [33].

Apart from polymers with cationic groups comprising the backbone, there are also polymers with biocide as the end group. Through polymerization, the macromolecule is terminated by a cationic surfactant making this technique advantageous in terms of controlling the groups existing at the two ends of the polymer. The non-active group distal to the antimicrobial end known as the satellite group as shown in Figure 7, is found to dominate the activity of the whole macromolecule [32]. Example was proven by previous studies on the antimicrobial activity of poly(alkyloxazoline) (POX) telechelic with quaternary ammonium group as one of the end group which exhibit about ten times lower activity against *S.aureus* cells as compared to low molecular weight compound dodecyl-trimethyl-ammonium chloride (DTAC) with the molecular weight of the polymer in the range 2000-12000 g/mol found to have no significant effect on the activity of the polymer [25].

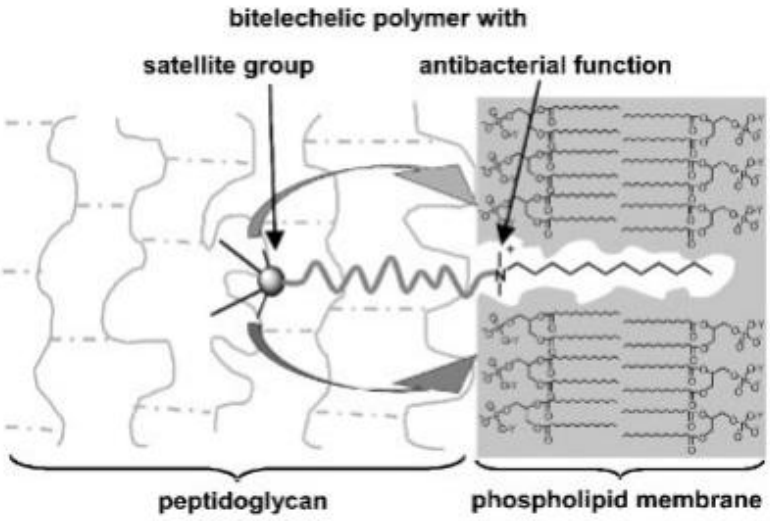


Figure 7: Telechelic polymer with satellite group [25]

The effect of various satellite group were investigated using POX with *N,N*-dimethyldodecylammonium (DDA) end group utilizing different satellite groups such as alkyl chain, DDA, aminohexyl, block of poly(2-phenyl-1,3-oxazoline and [tert-butoxycarbonyl)amino]hexyl with the hydrophobicity of the satellite end controlled by introduction of alkyl spacers of different lengths. The difference in membrane affinity with various satellite group dependence reveals the possible binding approach of the polymer onto the membrane. The first possibility is the penetration of both ends of the polymers at two distant points as shown in Figure 8(a). For example, the binding of POX-DDA derivatives at two sites should result in a higher affinity and demonstrate a much more active polymer, however, this mechanism is not relevant since it doesn't comply with the increase of polymer affinity with the addition of second DDA end group. The second possibility involves penetration at close proximity. This mechanism occur due to the formation of unimolecular micelles of polymers below the CMC value, in which the structure forms aggregates of hydrophobic chains enveloped by the hydrophilic polymer backbone. Sat-POX-DDA polymers consisting of hydrophobic satellite group will tend to form the micelle structure, and due to the aggregation of the end terminals, the penetration into bacterial cell membrane will occur on a common point as shown in Figure 8(b) [25].

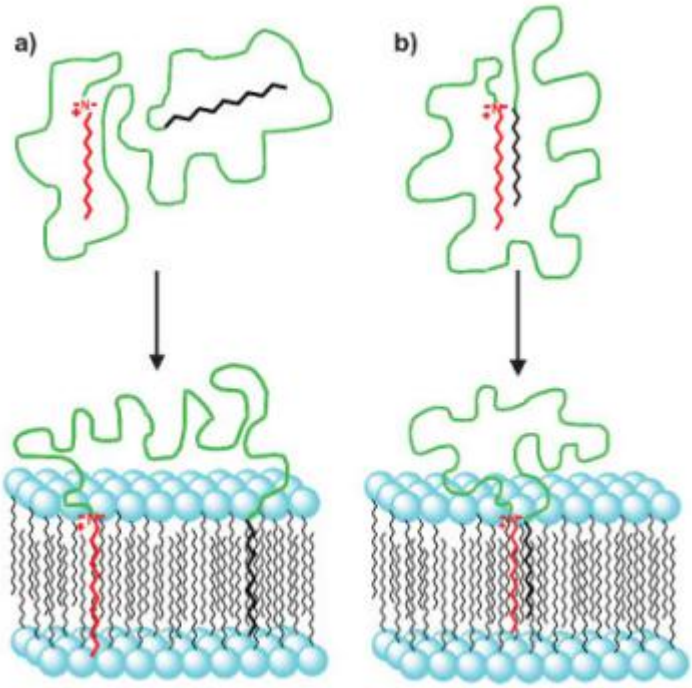
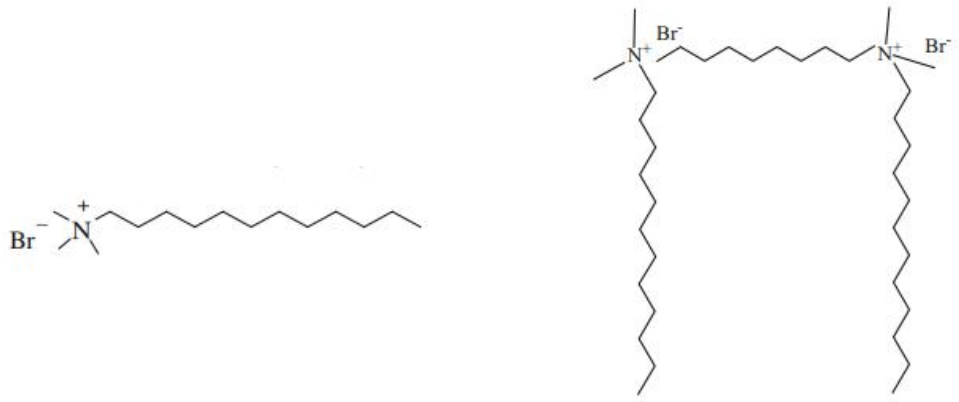


Figure 8: Penetration mechanism of alkyl-PMOX-DDA into a phospholipid membrane [25]

Cationic amphiphilic polymers were prepared by functionalizing branched poly(ethyleneimine) (PEI) using ethylene carbonates with cationic groups and different alkyl chain length [6]. Mono-quaternary ammonium amphiphiles containing one cationic head group and one alkyl chain are more popular in the commercial use comparing to dimeric amphiphiles regardless of its superior properties. Dimeric quaternary ammonium amphiphiles possessing two cationic head groups and two alkyl chains have lower critical micelle concentration and are more capable at reducing surface tension [34]. In a study, the effect of cationic dimeric amphiphile was determined by the antibacterial activity of octamethylene-1,8-bis(dodecyldimethylammoniumbromide) (12-8-12) against Gram-negative bacteria, *E.coli*, and compared with the activity of monomeric amphiphile dodecyltrimethylammonium bromide (DTAB). The difference in structures of both polymers are as shown in Figure 9.



(a) (b)

Figure 9: Chemical structure of monomeric amphiphile DTAB (a) and 12-8-12 dimeric amphiphile (b) [34,35]

The activity of both amphiphiles are measured by means of minimum inhibitory concentration (MIC), which is the smallest concentration needed of antibacterial agent in impeding the growth of bacteria. The antibacterial activity of 12-8-12 is significantly larger by showing 1/20 of the MIC reading comparing to DTAB and it was concluded that this effect was due to the difference in their chemical structures. The antibacterial activity of the QAC strongly relies on the charge strength of the head groups as well as the hydrophobicity of the alkyl chains, thus, 12-8-12 will exhibit better antibacterial activity contributed by the two attached quaternary ammonium cations with two alkyl chains compared to monomeric DTAB [34].

One of the major concerns of antibacterial agents is the selectivity of the agents towards mammalian and bacterial cell membrane. The driving force of the membrane-disruption properties of antibacterial polymers relies strongly on its hydrophobicity, although the same factor could effect in the lysis of human red blood cell [36]. Agents with good antimicrobial efficacy and low cytotoxicity towards the host cell is desired. Polymer-induced lysis of red blood cell (RBCs) or hemolysis has been widely used in the measure of polymer toxicity towards the membrane [37]. The hydrophobicity of polymer were interrelated with the hemolytic activity based on the optimization of the cationic-hydrophobic ratio of the antibacterial polymer. Cationic polymer with absence of hydrophobic side chains are inclined to bind with anionic bacterial membrane over zwitterionic human membrane cells, however, with the incapability to rupture the membrane. If the polymer is too hydrophobic, non-selective binding will occur towards all cell membranes including the host cells.

Thus, tailoring the amphiphilic balance could result in a polymer with selective electrostatic binding to bacteria and membrane-rupture ability through hydrophobic interactions [36]. Net cationic charge of antibacterial polymer had displayed selective binding towards bacterial cells compared to the host cells due to the lack of anionic lipids on RBCs membrane surface [37].

2.6. Antibacterial activity/efficacy

The antibacterial efficiency of water-soluble QAC polymers are determined by using two main methods: (1) minimum inhibitory concentration (MIC), the minimum antibacterial concentration to restrain microbial growth and (2) minimum bactericidal concentration (MBC), the least concentration needed to kill microorganisms [1,38]. The MIC method involves the cultivation of selected microorganisms of Gram-positive and/or Gram-negative bacteria in a sterilized nutrient medium. Selected antibacterial polymer were transferred into the agar or broth medium and incubated for a duration of time at a specified temperature. The extent of inhibition could be determined by measuring the optical density (OD) of the medium. The MIC value corresponds to the lowest concentration with no significant change in turbidity compared to the control sample [6]. The primary advantage of adopting MIC method is that it is relatively easy to perform, therefore many species or QACs could be analyzed simultaneously. However, the method is only applicable for QACs with low susceptibility due to the precipitation of QACs in the nutrient medium at high concentration [30].

Biocidal tests are similar to MIC tests in terms of exposure to antibacterial polymer, however, after a period of time, the medium is neutralized and the number of available microbes are determined. The MBC value is defined as the lowest concentration which produced no colonies after a time, usually longer than in MIC. This method is advantageous in testing all types of disinfectants with the effects of temperature and is easy to conduct, however, this method requires a longer time and more difficult to reproduce than the MIC tests [30,39].

Hemolytic activity were also conducted in most antibacterial activity measurement to determine the selectivity of antibacterial agents towards bacterial and human cell membrane. This measurement involves the introduction of antimicrobial polymer in a

buffer solution into human red blood cell solution at body temperature for a duration of time. The supernatant collected from the solution is measured for the release of hemoglobin by absorbance and compared with blank and positive control. The hemolytic activity corresponds to the polymer concentration which causes 50% hemolysis in the RBC compared to the positive control [6]. The selectivity of antibacterial agents are usually expressed by the HC_{50}/MIC value [28].

2.7. Membrane modelling

Biological membranes consist of lipid bilayers comprising of numerous amphiphilic lipid molecules as fundamental unit. The bilayers which act with fluid movement are adhered together by hydrophobic interactions of the acyl chains [40]. The study of natural cell membrane is often time-consuming and not cost-effective as the complexity of the membrane often causes systematic investigations to be very difficult. Nowadays, biomimetic membrane models with different complexities are used to generate the basic ideas to help answering questions regarding the membrane system (Figure 10) [41].

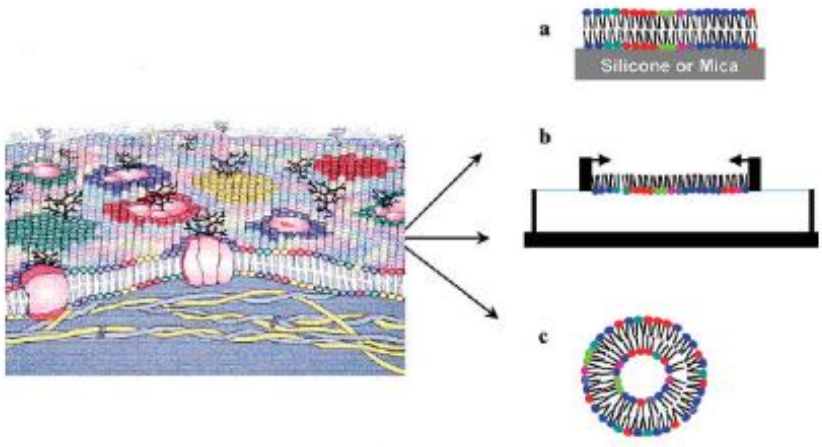


Figure 10: Illustration of cell membrane (left) and membrane model (right). Lipid arrangement in a cell membrane supported lipid bilayer (a), lipid monolayer (b) and liposome (c) [40]

Membrane models including liposomes or vesicles, Langmuir monolayer and supported lipid bilayers each has its own advantages and disadvantages. None of the models will be as accurate as in the real membrane, however, under specifically

controlled environment, simulated membrane can mimic the characteristics of a natural membrane in a very accurate manner.

Langmuir technique is one of the widely used method in the investigation of cell membrane due to its simplicity and it is able to investigate complex biological processes. In this technique, a monomolecular layer is produced by spreading the lipid solution on the top of the surface of the subphase in a Teflon trough. Due to the amphiphilic character of the lipid, it will act as a surfactant which will be adsorbed at polar-apolar interfaces to form a single monolayer and reduce the surface tension with their hydrophobic tails oriented to the air while the heads are submerged in the water as shown in Figure 11 [42]. This system added advantage in terms of homogeneity, stability, defined geometry of the lipid packing and controllable lateral packing density of the monolayer [13].

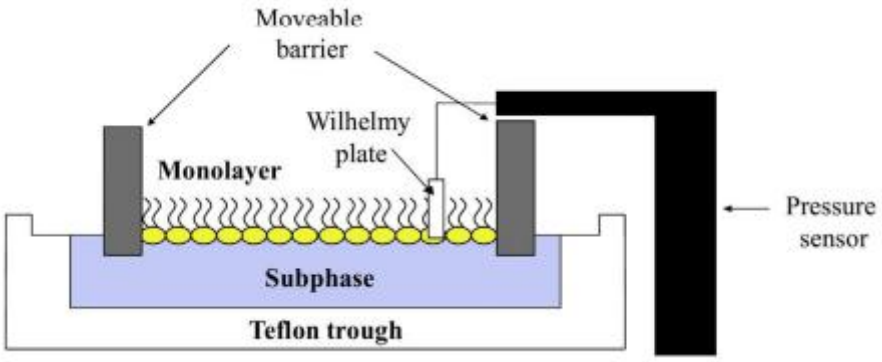


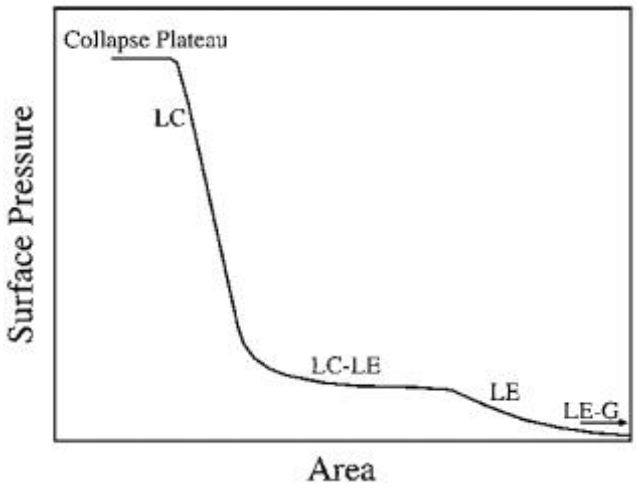
Figure 11: Schematic design of Langmuir through with lipid monolayer forming on air/liquid interface [12]

The correlation between the surface pressure and the surface area of the lipid assembly provides information on the thermodynamic properties on the phase behaviour of the monolayer [43]. By moving the barriers, the monolayer can be compressed and expanded. The result of the compression is the so called surface pressure-mean molecular area isotherm (π-A). During the compression, the area per lipid molecule decreases and the monolayer can exhibit the following phase transitions: liquid-expanded and gaseous phase (LE-G), liquid-expanded (LE), liquid condensed-liquid expanded phase (LC-LE) and liquid-condensed phase (LC). The surface pressure (π) is calculated using

$$\pi = \gamma_o - \gamma$$

where γ_o is the surface tension of pure water and γ is the surface tension of monolayer at air/water interface [44].

(a)



(b)

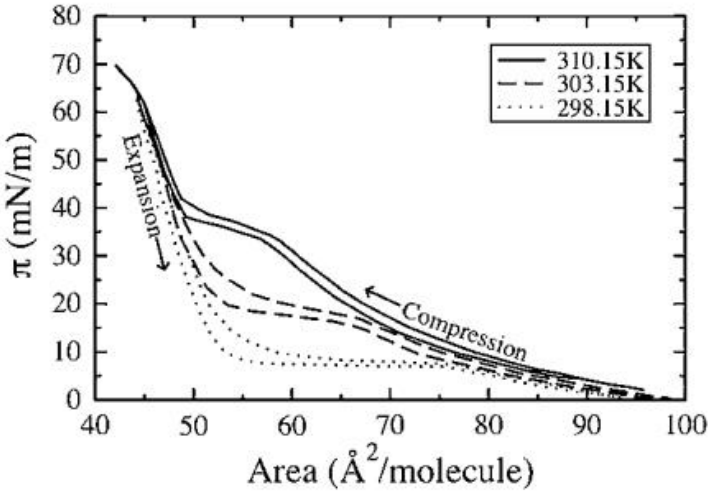


Figure 12: Phase behaviour in surface pressure-area isotherm of DPPC (a) and the effect of temperature on shape of compression and expansion of isotherms (b) [44]

The monolayer becomes relatively incompressible as it is compressed into the condensed phase. During this stage, the surface pressure is high and slight change in area creates a very steep slope in the isotherm as shown in Figure 12(a). The isotherm also responds to changes in the temperature in which a growth leads to an increase in thermal motion of the chains and as a result, the isotherms are shifted to higher surface areas as shown in Figure 12(b) [44]. In practice, the isotherms may vary considerably according to the apparatus used, rate of compression, type of solvent,

ionic strength, pH and experimental effects such as leakage and impurities. However, varying the dynamic compression rate and presence of small concentration of ions does not produce significant effects on the isotherm of zwitterionic monolayers [44].

Lipid monolayers are often used to imitate bacterial membranes regardless of some difference in Gram-positive and Gram-negative bacteria cell structure. The composition plays an important role in determining the extent of interaction with antibacterial agents. Gram-negative bacteria are generally comprised of zwitterionic lipid, phosphatidylethanolamine (PE) and some anionic group [12]. Monolayer formation can be used to study various things such as the specifity of a peptide in a mix of phospholipid or even in a specific phospholipid itself. The characterization of headgroup of a phospholipid is an important aspect as it is the first thing an antibacterial agent will encounter as it reaches the membrane. Thus, the charge, size and polarity of the headgroup will significantly affect the selectivity of the antibacterial agents [12].

Plasma membrane generally consists of heterogeneous mixture of lipid molecules with different lipid ratios representing the membrane of various species such as mammalian or bacterial cell membrane. A closely related study of the lipid organization involves the understanding on pulmonary surfactant. Pulmonary surfactant is a mixture of lipids and proteins found on the air/water interface in mammalian lungs which acts in decreasing the surface tension during the breathing process [45]. An effective lung surfactant must be able to exhibit rapid adsorption, capable to be compressed to near-zero surface tension upon exhaling followed by active re-spreading upon lung expansion [46]. The primary component of the lung surfactant is phospholipids, of which ~80% are phosphatidylcholine (PC), and from that, it consists primarily of dipalmitoylphosphatidylcholine (DPPC). The formation of pure DPPC with reduced surface tension facilitates respiration and is considered one of the most crucial component in lung surfactant [45]. The other major phospholipid component is the dipalmitoylphosphatidylglycerol (DPPG) with anionic head group [47].

DPPC with the structure shown in Figure 13, has been used as model for bio-membrane due to its stability, elasticity and permeability properties which mimics the functions of natural membrane. Therefore, it is very suitable in investigating adsorption and penetration of agents. The DPPC possesses a transition temperature of 41°C with zwitterionic head group [48].

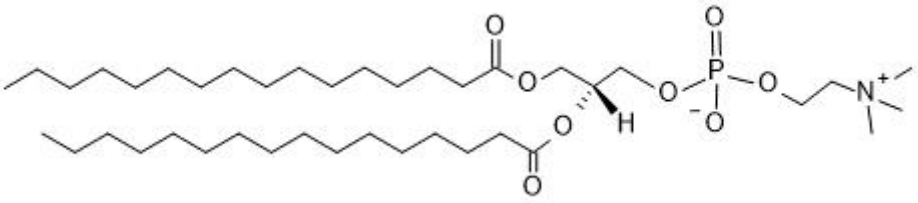


Figure 13: Chemical structure of DPPC

Lipid mixture has been extensively studied in order to mimic the lipids in natural lung surfactant. For example, in DPPC/DPPG lipid mixture, 80:20 ratio was used as the ratio was most commonly found in mammalian lung surfactant, while others applied 70:30 ratio to examine the effect of surfactant proteins such as SP-A, SP-B, SP-C and SP-D [47]. DPPC and DPPG were also adopted in the investigation of molecular interaction of bacterial lipid with model lipid layer. DPPC were constructed to mimic a model lipid layer whilst the insertion of DPPG with anionic charge mimics a bacterial layer allows the evaluation on the disorganization effect on the membrane as it is tested with antimicrobial polymer [6]. Other variety of lipid mixture were also analyzed such as DPPC with sphingomyelin and cholesterol since they were the dominating components of the inner and outer leaflets of a membrane [49]. Thus, the mixture of DPPC with either one of the component could reveal the specific interaction towards the particular component.

Sphingomyelin (SM) belongs to the sphingolipid groups and and makes up about 85% of the sphingolipids in humans [50]. It is zwitterionic in charge and are composed of long-chain bases, commonly sphingosine or 1,3-dihydroxy-2-amino-4-octadecene, and fatty acids (Figure 14). The long saturated hydrocarbon chains in SM tend to adopt solid-like phase in membrane organization. Sphingomyelin usually accumulate at the outer leaflet of the cell membrane, thus, the high density of SM will interact with sterols and create a rigid barrier from the outer cellular environment [51,52,53].

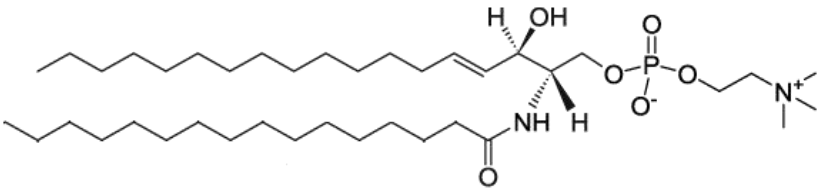


Figure 14: Chemical structure of SM

Cholesterol is a non-polar lipid which plays a major role as regulators in lipid organization. Sterol group also affects the fluidity and membrane permeability which is a significant contribution to the overall function of the cell membrane [54]. The presence of cholesterol induces phase separation and form liquid-ordered domains called rafts within the membrane layer. This raft generally contain a higher concentration of cholesterol and sphingomyelin compared to the remainder parts of the membrane. Studies on the rafts adopted equimolar mixture of the two components with phosphatidylcholine to allow formation of phase separation [55]. The effect of dual or ternary mixtures of these lipids provide valuable information on the organization of lipids, the ability to form a close-packed lipid structure, phase separation and formation of ordered phase. By comparing a single with mixed-component monolayer, information regarding the miscibility and component interaction could also be assessed [55,56].

The study of infectious disease such as Tuberculosis (TB), one of the most fatal disease in the world, could also be explored further using Langmuir monolayer method. In reducing the harmful effects caused by the *Mycobacterium tuberculosis* (*Mtb*) pathogen in TB, sufficient knowledge on the cell envelop is required in inhibiting the progress of the bacteria. Mycolic acid is the major component comprising the outer layer of *Mtb* cell envelop with almost 40% composition. Mixed monolayer of DPPC and mycolic acid allows the characterization of *Mtb* cell membrane and subsequent treatment with drugs in order to inhibit its growth [57].

There are two main alternatives in characterizing the activity between the model membrane and antibacterial agents; (1) holding the surface area of the monolayer constant while observing changes in surface pressure for a duration of time (2) maintaining constant surface pressure while observing an increase in the surface area as a result of penetration of antibacterial agent into the monolayer [12]. The injection of antibacterial agent using the first alternative results in an increase of surface pressure ($\Delta\pi$) which indicates the activity between the two components as shown in Figure 15.

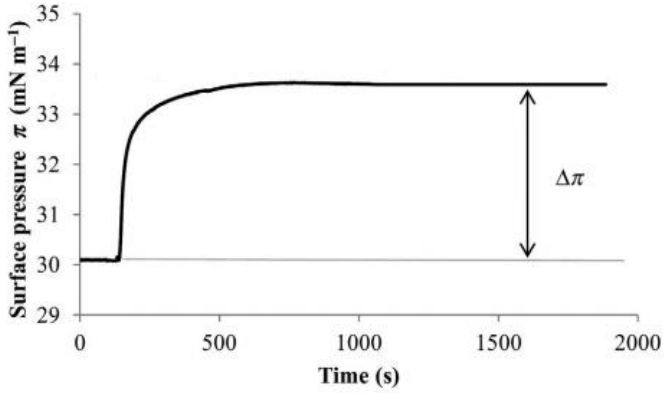


Figure 15: Change in surface pressure of lipid monolayer after injection of antibacterial agent [12]

3. Objectives of the thesis

The main aim of this study is to get an overview of antibacterial agents as well as its applications in different industries such as food and textiles. Various types of antibacterial agents were used in industries nowadays with different chemical composition and colloidal state that influences the effectiveness of the antibacterials. Thus, this thesis aims to study the relationship between chemical structure of antibacterial agent with its antibacterial or bactericid properties.

Membrane affinity as a crucial property of the antibacterials can be characterized using model systems to estimate the interaction of the antibacterial agent with the cell membrane. The simplest and powerful lipid model, the Langmuir monolayer was used in the present work.

Two homologous series of antibacterial agents are planned to study extensively applying the Langmuir membrane model. Specified experimental monolayer model was introduced with composition characteristic to certain bacterial cell envelop. The membrane affinity and molecular interactions will be evaluated and compared to reveal the influence of chemical composition of antibacterial molecules.

4. Materials and methods

4.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phospholcholine (DPPC, M_w=734.01 g/mol) purity≥99 % (Avanti Polar Lipids Inc, USA)

Sphingomyelin (SM, M_w=703 g/mol), purity >98 % (Larodan AB, Sweden)

Dichloromethane analytical purity (VWR Chemicals, Hungary)

Methanol Reag. Ph. Eur. For HPLC (VWR Chemicals, Hungary)

Chloroform analytical purity (VWR Chemicals, Hungary)

Alkyl-4-hydroxybenzoates obtained from Sigma Aldrich Kft. Hungary. Their main properties are summarized in Table 3.

Paraben	M_w (g/mol)	S (g/L)	log$P_{oct/w}$
methyl	152.1	2.50	1.96
ethyl	166.2	0.885	2.47
propyl	180.2	0.500	3.04
butyl	194.2	0.207	3.57

Table 3: Main parameters of alkyl-4-hydroxybenzoates, molecular weight (M_w), aqueous solubility at 25°C (S) and partition coefficient between n-octanol and water (log$P_{oct/w}$)

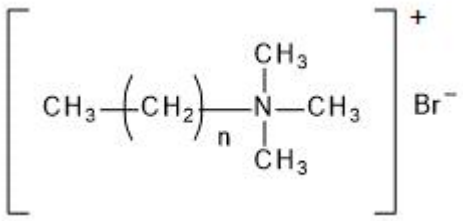


Figure 16: General structure of trimethyl ammonium surfactants

Trimethyl ammonium surfactant obtained from Sigma Aldrich Kft., Hungary. The general structure is as Figure 16 and their main properties are summarized in Table 4.

Cationic surfactant	M_w (g/mol)	CMC (M)
Octyl-trimethyl-ammonium-bromide (OTAB)	252.23	2.82×10^{-1}
Decyl-trimethyl-ammonium-bromide (DeTAB)	280.29	6.5×10^{-2}
Dodecyl-trimethyl-ammonium-bromide (DTAB)	308.34	1.42×10^{-2}
Tetradecyl-trimethyl-ammonium-bromide (TTAB)	336.39	3.69×10^{-3}
Hexadecyl-trimethyl-ammonium-bromide (HTAB)	364.45	8×10^{-4}

Table 4: Main parameters of alkyl-trimethyl-ammonium-bromide (purity ≥98 %), molecular weight (M_w), and critical micelle concentration (CMC) at 25°C

Double-distilled water was checked by its conductivity (<5 mS) and surface tension (>72.0 mN/m at 23±0.5°C) values.

4.2. Sample preparation

A solution of DPPC-SM in different molar ratio is produced by firstly preparing the stock solutions of DPPC and SM. 1mg of DPPC powder is weighed in a plasma-cleaned bottle and is dissolved in pure chloroform to produce a 1 mg/ml stock solution. The same amount is measured for SM to produce solution of the same concentration. Hamilton syringe was used to measure the given amount of the DPPC solution and it was mixed with SM solution to produce a DPPC-SM solution as shown in Table 5. The measured quantities were diluted to 1 ml and the final concentration of the mixed lipid solution was 0.2 mg/ml. Different ratios were prepared with different compositions presented in Table 5.

SM content (%) in the lipid mixture	Amount of SM (µL)	Amount of DPPC (µL)
5	9.6	190.4
10	19.2	180.8
20	38.4	161.6
30	58.2	141.8

Table 5: Amount of DPPC and SM solution added for each ratio

Paraben solutions of 200 µM concentration was prepared by mixing measured amount of paraben powder with double distilled water. The powder is ensured to fully

dissolve in the solution using an ultrasonic bath. The sample is stored in a refrigerator at 2-8°C.

4.3. Experimental setup

The preparation of Langmuir monolayers were achieved by the Langmuir technique. The Langmuir balance (KSV MiniMicro Finland) had a shallow through which was made of Teflon and was equipped with one electro balance and two polyoxymethylene (POM) barriers. Surface pressure was recorded with the tensiometric method with ±0.5 mN/m accuracy, employing previously purified filter papers (Whatman Chrl) as Wilhelmy plates. The system is very sensitive to contamination therefore, the Langmuir through was cleaned before each measurements. Dichloromethane was used for cleaning the trough while the barriers were wiped with methanol. Double distilled water was filled in the trough between the organic solvent cleaning to ensure the trough is free from any contamination. The experiment was started by filling of fresh double distilled water in the trough. In all cases, 50 µL of the lipid solution was spread drop by drop on the top of the water subphase using Hamilton syringe and left for 10 minutes to allow the evaporation of chloroform. Upon starting the experiment, the thermostat is adjusted so that the temperature of the subphase is set to be at 37°C±0.5°C. After the evaporation of chloroform, the monolayer was compressed by two movable barriers at a speed of 12 mm/min until the surface pressure reached 35 mN/m, then the barriers were expanded. During this compression/expansion cycle, the surface pressure of the lipid film was continuously detected by the electro balance resulting the surface pressure-area isotherms (π -A). Each measurement was repeated three times. The isotherms were compared in terms of hysteresis size as well as the shifting of the isotherms to smaller molecular area per lipid to determine the best ratio to be used for stability measurement. Recording the isotherms is essential before the stability and the penetration measurements. During the stability measurement, the changes of the surface pressure were recorded as a function of time at given surface pressure.

To investigate the interactions between the drug and the lipid monolayer, penetration measurements were performed. The monolayer was compressed to a given surface pressure and the monolayer stability was studied in the first 10 minutes.

In case of adequate stability, the solution of the given drug was injected under the lipid monolayer by a syringe to achieve 2 μM final concentration in the trough. The changes of the surface pressure as a function of time were recorded by the electro balance until 60 minutes after the injection. The degree of penetration gives information of the membrane affinity. It was defined as the increase of surface pressure after 1 h long penetration.

5. Results and discussion

5.1. Lipid monolayers

Langmuir monolayers on the surface of water subphase were formed from DPPC, sphingomyelin and their mixture with different ratio of the components. Surface pressure – area isotherms were detected by compression and expansion of the monolayer at a constant speed. This process was repeated two times on the same film to get information on the compositional stability of the film. Figure 17 shows the pressure-area isotherm (π -A) of pure DPPC obtained at 25°C.

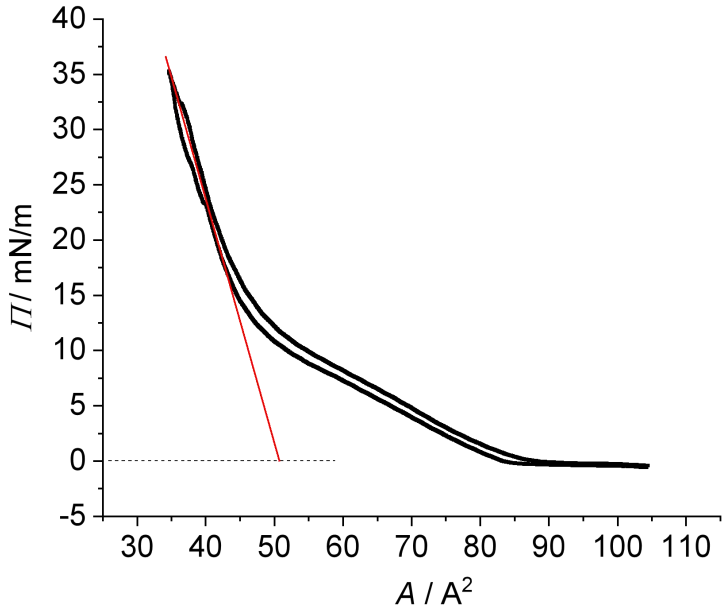


Figure 17: Surface pressure-area isotherm of DPPC layer at 25°C

During the compression the surface pressure increases gradually as the area available for the lipid molecules is decreased. The DPPC monolayer is observed to exhibit a phase transition corresponding to the change from liquid expanded (LE) to liquid condensed (LC) phase as discussed in the literature review. As the monolayer is compressed further, a steep change in pressure is observed with smaller change in the molecular area indicating the formation of close-packed structure. The average area occupied by each lipid molecule (A_o) can be determined by extrapolating the steepest region of the slope to zero surface pressure which corresponds to the maximum packing condition. From the graph, the molecular area occupied by one DPPC molecule is $A \approx 50 Å^2$

The increase of temperature from 25 to 37°C results in a significant change (Figure 18) in the structural behaviour of lipid film. The shape of the isotherm reflects the continuous increase of surface pressure with decreasing area. There is no sign of phase transition in this surface pressure range. That is due to the less tendency of self-assembly of lipid molecules at the higher temperature. The raise in temperature increases the thermal motion of molecules which leads to an increase in the area occupied by each DPPC molecule in the monolayer. Extrapolation from the graph determines that the area occupied by each DPPC molecule at 37°C is $A \approx 65 Å^2$.

There is a certain difference between the compression and expansion curves. This hysteresis is connected to the speed of the compression. The difference approaches zero when the speed of the barrier is low enough.

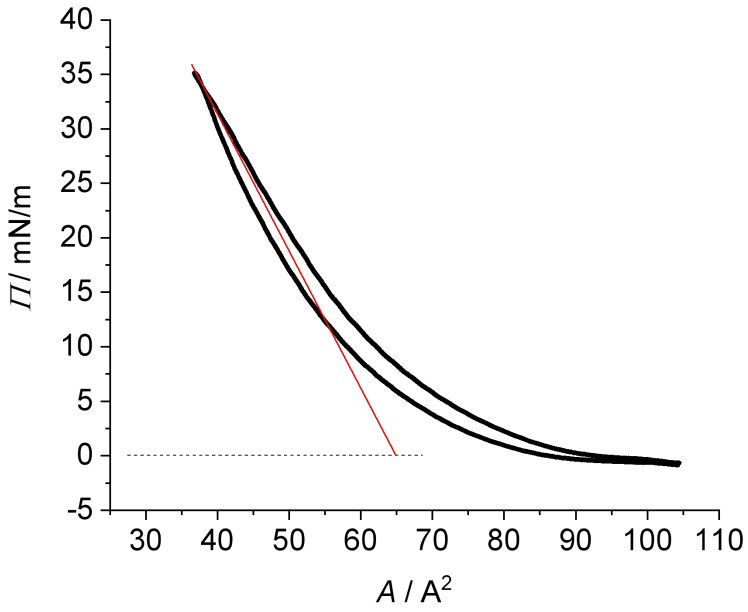


Figure 18: Surface pressure-area isotherm of DPPC layer at 37°C

The stability of the lipid layer can be characterized by repeating the compression-expansion cycle and compare the shape of the first, second and third surface pressure – area isotherms. This representation is displayed in Figure 19 for DPPC lipid film at 25°C. Comparing the shape of the isotherms there is no change in the state of the film, the compression and expansion seem to be reversible. The hysteresis loop is also very narrow showing that the molecular arrangement is a rather quick process. There is only a minor shift in the position of the isotherm towards smaller molecular area.

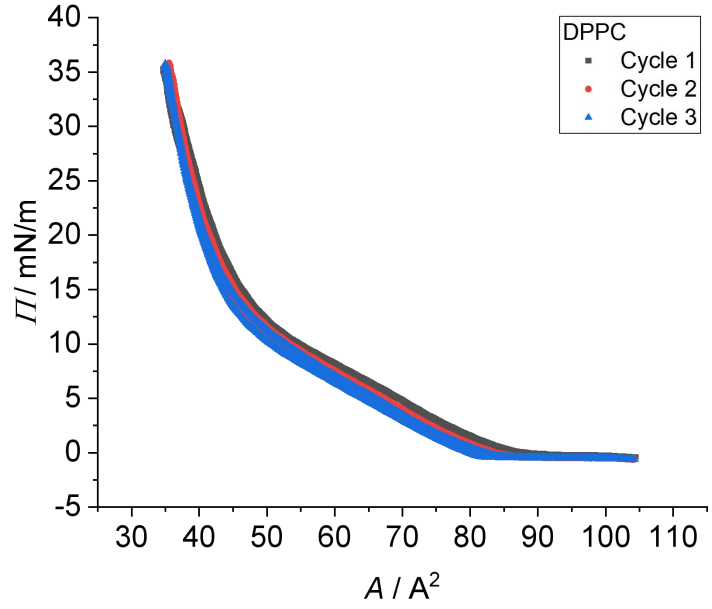


Figure 19: Surface pressure-area isotherms of DPPC layer at 25°C

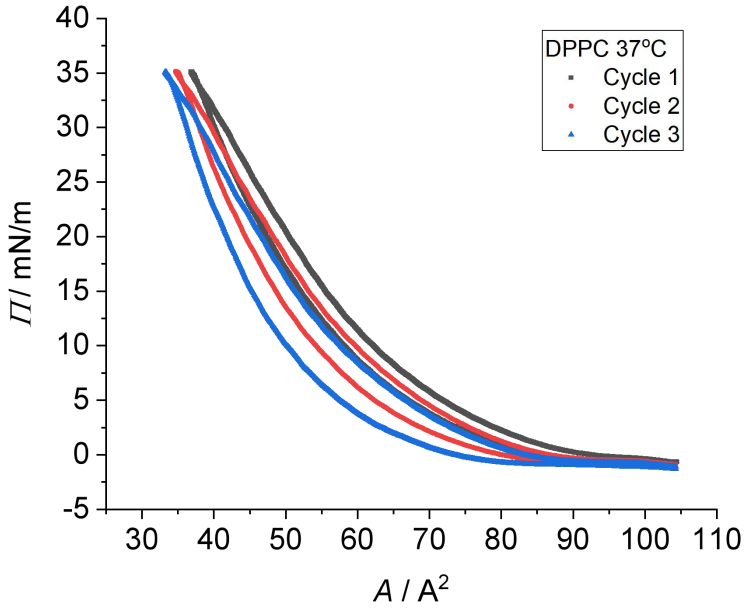


Figure 20: Surface pressure-area isotherms of DPPC layer at 37°C

Three surface pressure isotherms of DPPC at 37°C detected consecutively are shown in Figure 20. The shape of the isotherm is the typical gradual increase with decreasing area, but there is a significant shift of isotherm observed repeating the compression, expansion cycle. That indicates that the structure of the film is not identical during the first and the following compressions. The shift found at the compressed state at 35 mN/m is about 5 A^2/molecule during the three cycles which

can be originated from a small loss of molecules from the surface monolayer. The type of these molecules is not possible to define from this measurement. That can be the lipid itself or some contamination with similar surface activity as the lipid present in a small amount in the surface layer. That is noteworthy, that this effect is exhibited only at the higher temperature of the measurement.

The other type of lipid used in the model experiment was sphingomyelin. Figure 21 shows the surface pressure-area isotherm (π -*A*) for pure sphingomyelin at 37°C. The shape of the curve is similar to the corresponding curve of DPPC without any special feature. The surface pressure increases at the area ≈ 80Å^2/molecule and does not have significant plateau region. In previous studies, it was found that a phase transition to a condensed state should occur at about π = 10-18 mN/m even though it is not observable from the graph [58]. Supplementary measurements are needed such as fluorescence microscopy or Brewster angle microscopy (BAM) to further examine if the changes occurred. A possible explanation for that difference in the film forming properties can be that there is a structural variation in the sphingomyelin depending on the source of the compound [59,60].

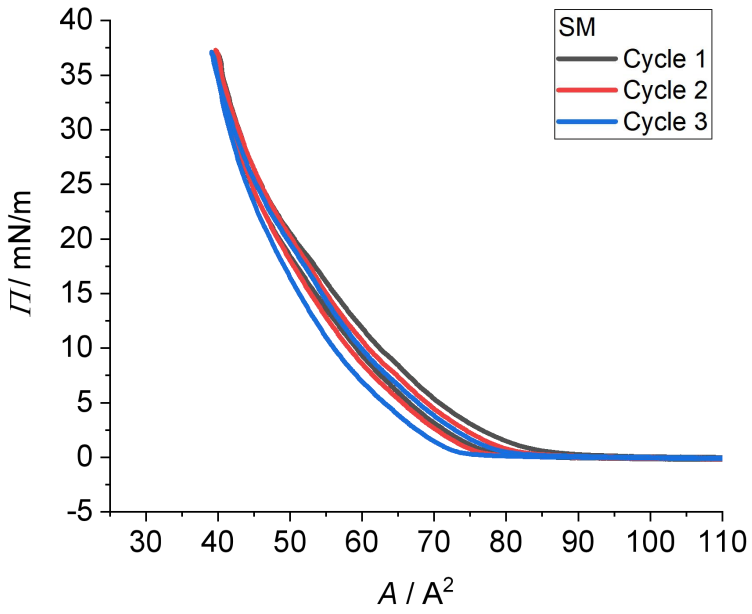


Figure 21: Surface pressure-area isotherms for pure sphingomyelin at 37°C

The isotherms detected consecutively on sphingomyelin layer show similar shape and practically no shift in the compressed state. That means the composition of the layer, the surface density of the molecules is quite stable. The hysteresis loop is

also rather narrow, so we can deduce that the orientation of molecules occurs easily. The only small difference found between the three isotherms is related to the less compressed state. This “memory” effect is known and that is the reason for recording not only one (the first) isotherm to characterize the surface film of oriented molecules.

5.2 Mixed lipid monolayers

Lipid mixture of DPPC and sphingomyelin were also measured using different molecular ratios in determining the best lipid composition with optimum stability under compression and expansion. Mixtures of DPPC-SM with molar ratio of 90:10%, 80:20% and 70:30% are measured and compared as shown in Figure 22. Each isotherm curve represents the average of three measurements compressing the mixed lipid layer at 37°C.

It is observed that the addition of sphingomyelin to DPPC affects the position of the surface pressure – area curve. As the concentration of SM is increased from 10% to 30% molecular ratio, the curve appears at a higher molecular area, which indicates an increase in the average area per molecule. An inference to the shifting could be due to the larger size of SM head group.

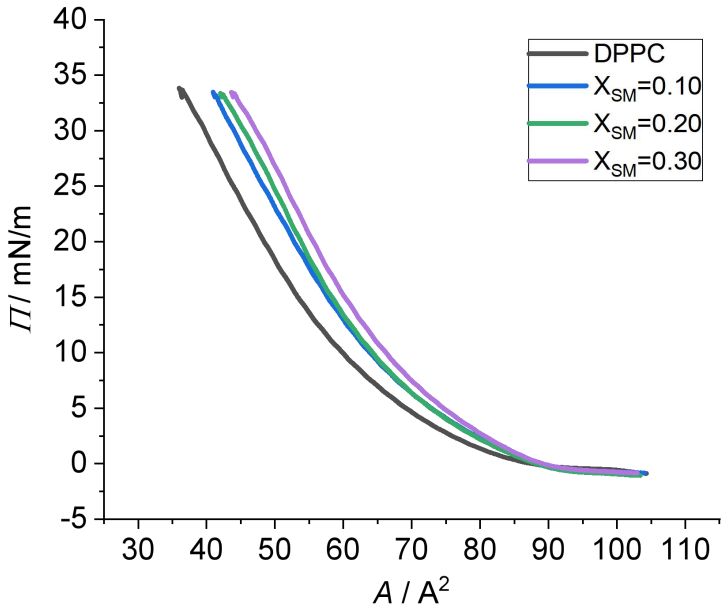


Figure 22: Surface pressure-area isotherms for DPPC+sphingomyelin lipid mixtures at 37ºC. Compression curves for mixtures with 0.1, 0.2 and 0.3 sphingomyelin molar ratio are plotted and compared for pure DPPC.

The stability of the mixed lipid layers were characterized by repeating the compression-expansion cycle and compare the shape of the pressure – area isotherms. This representation is displayed for DPPC+sphingomyelin (9:1) in Figure 23 as an example of mixed layers with different molar ratios. The shape of the isotherm shows the typical gradual increase with decreasing area, but there is a significant shift of isotherm observed repeating the compression, expansion cycle. This behaviour resembles the properties of DPPC monolayer. The results obtained show that the compositional stability of mixed films (this and the two other compositions of mixed film: 8:2, 7:3) is governed by the main component, DPPC.

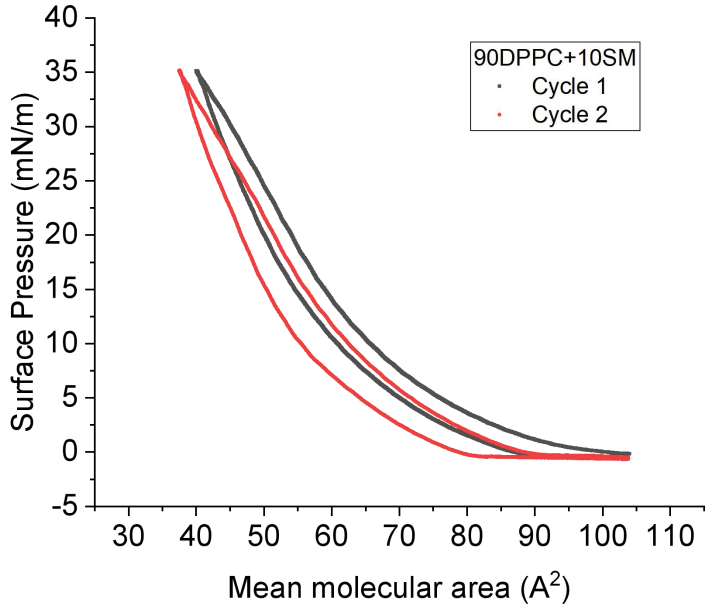


Figure 23: Surface pressure-area isotherms for DPPC+sphingomyelin (9: 1) lipid mixtures at 37°C

5.3 Lipid monolayer stability in time

It is necessary to know the stability of lipid monolayer in time for the evaluation of penetration experiments. Penetration of dissolved component into the lipid monolayer is followed for one hour, so the stability experiments were performed also for one hour. The stability of DPPC monolayer is shown in Figure 24. After compression of the film to the "target" surface pressure (20mN/m in our case), the barrier is stopped to keep the area constant, and the change of surface pressure is detected. There is usually a decrease in the surface pressure and later the change is smaller approaching a steady state value within the time interval studied here. That is considered a dynamic effect which is greatly decreasing when slower compression is applied. The stability of the monolayer represented by this curve is the reference calculating the membrane affinity of dissolved components in the penetration experiments.

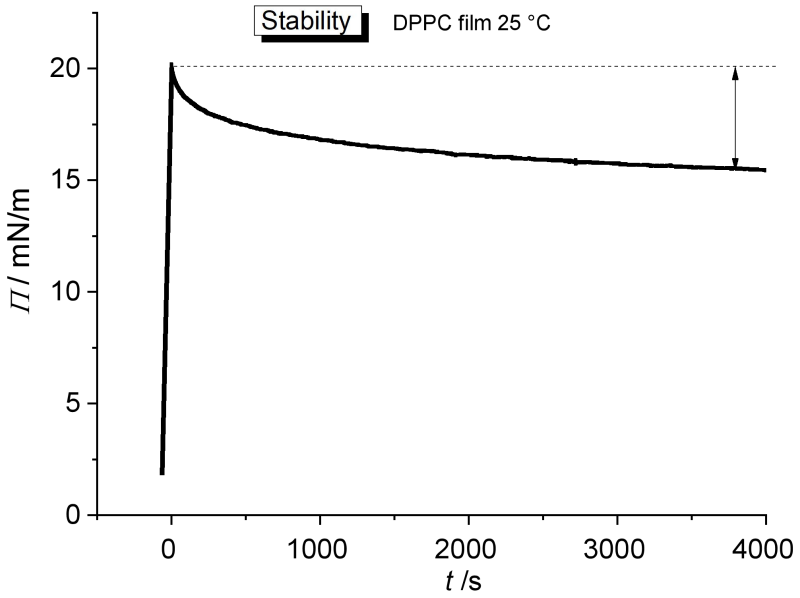


Figure 24: Monolayer stability, changing of surface pressure of DPPC film at constant area at 25°C

The corresponding stability curves for DPPC-SM mixed monolayers are displayed in Figure 25. The alteration of surface pressure with time at fixed area are plotted for three mixtures of DPPC+sphingomyelin (9:1, 8:2, 7:3).

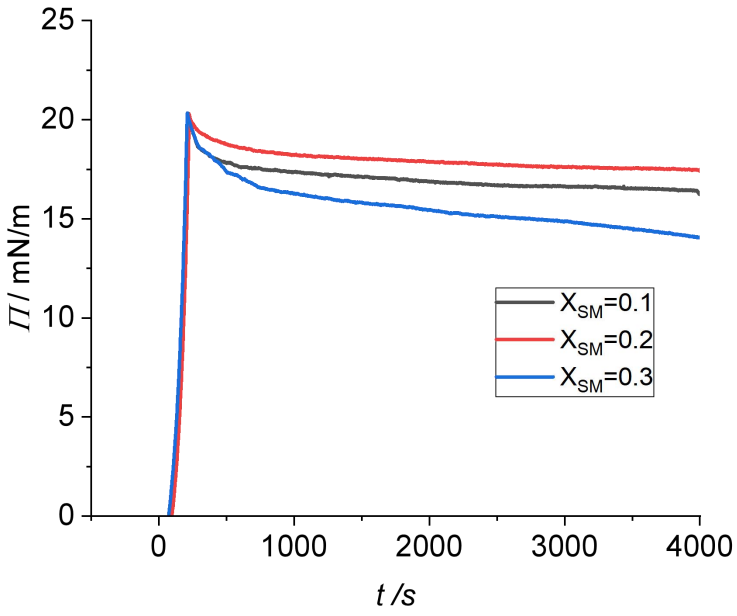


Figure 25: Monolayer stability, changing of surface pressure of DPPC+SM films at constant area

SM content (%) in the lipid mixture	Initial surface pressure (mN/m)	Surface pressure after 1 hour (mN/m)	Difference in surface pressure, $\Delta\pi$ (mN/m)
10	20.30	15.9	4.4
20	19.8	17.5	2.3
30	20.3	13.7	6.6

Table 6: Stability of mixed DPPC-SM monolayers compressed to 20mN/m denoted by change in surface pressure

Based on Figure 25 , it is observed that the DPPC+SM lipid mixture with 80:20 molar ratio exhibits the least fluctuation in surface pressure for the duration of one hour, with $\Delta\pi$ of only 2.3 mN/m, indicating very good stability. Mixture with 10% SM is shown to exhibit slightly more change in surface pressure, followed by a gradual drop for 30% SM. The drop in surface pressure may occur due to different reasons such as reorganization of lipid molecules on monolayer surface or desorption of monolayer lipid molecules into the bulk aqueous subphase. Thus, the stability of the monolayers can be concluded to decrease in the following sequence: $X_{SM=0.2} > X_{SM=0.1} > X_{SM=0.3}$. DPPC+SM lipid mixture with 80:20 composition is proved to be the most stable monolayer under compression which agrees well with the natural composition in a biological membrane [61].

5.4 Penetration experiments

Two sets of molecules were selected for the study of their penetration ability to lipid monolayers. The first one was the antibacterial parabens with various alkyl chain length. In the usual penetration experiment the dissolved component is applied in the micromolar concentration. That concentration is generally enough to detect the affinity of the molecules to the lipid monolayer within a reasonable time and does not require such amount of material which is not available in several cases. Therefore 2 micromolar concentration was chosen for the penetration experiment. To achieve this concentration in the trough a 100 times more concentrated solution is needed to prepare as a stock solution.

In the paraben homologous series (Table 3) enhanced membrane affinity was expected with increasing alkyl chain length. Penetration of propylparaben is shown in Figure 26. Stability of lipid monolayer is also displayed as a comparison. Within the experimental error there is no sign of lipid affinity of propyl paraben applied. The

same behaviour can be observed for the two more hydrophilic parabens, methyl- and ethyl-paraben.

Unfortunately, the limited aqueous solubility of the butylparaben did not allow to perform a penetration experiment with this compound.

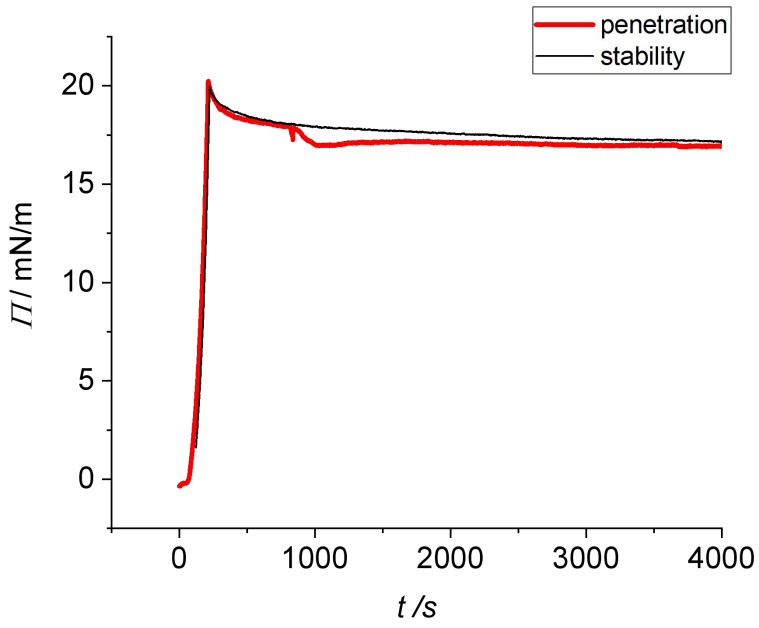


Figure 26: Penetration of propylparaben into DPPC monolayer

In the other set of penetration experiments cationic surfactants were studied. A homologous series of alkyltrimethylammonium bromide was applied from octyl to hexadecyl chain length. The DPPC lipid monolayer was compressed to reach a surface pressure of $\pi \approx$ 8mN/m and was held at the stated surface pressure for approximately 10 minutes, followed by injection of the concentrated aqueous solution of cationic surfactant. The change of surface pressure was followed for one hour. The penetration curve of HTAB at 8mN/m surface pressure is presented in Figure 27.

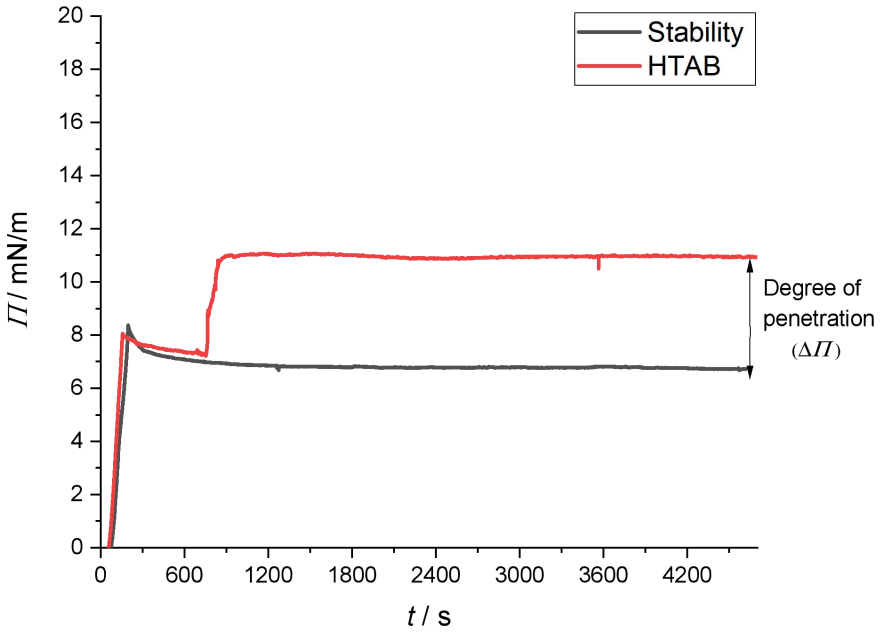


Figure 27: The penetration curve of HTAB comparing to DPPC stability at 8mN/m surface pressure

An abrupt increase of surface pressure was observed comparing to the original DPPC monolayer stability as the antibacterial agent was injected into the subphase followed by stagnant trend until the end of one hour. From the rise in surface pressure, we can conclude that there is an instant interaction between the HTAB and the DPPC monolayer. It is suggested that the increase is due to the adsorption of HTAB to the lipid monolayer and subsequent penetration [6].

The penetration curves for octyl-, decyl-, dodecyl-, tertadecyl- and hexadecyl –trimethylammonium bromide are shown together in Figure 28.

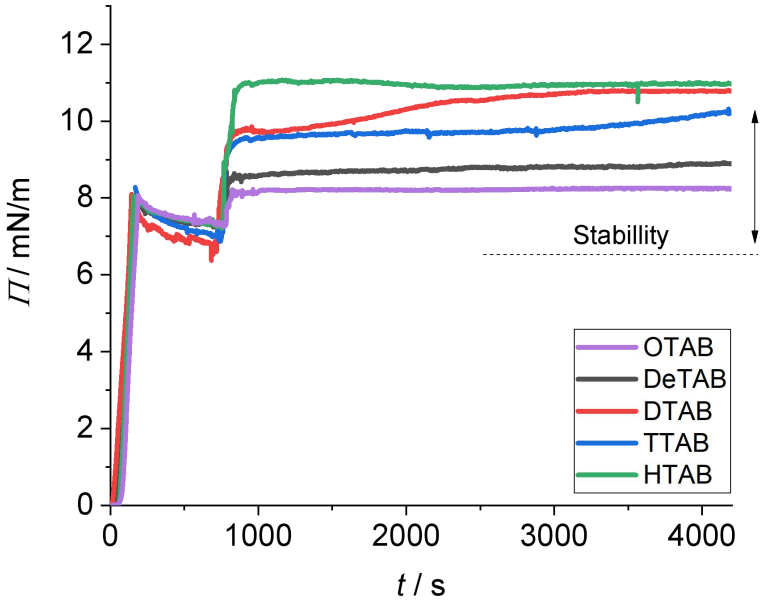


Figure 28: Penetration of cationic surfactants with different alkyl chain length: 8, 10, 12, 14, and 16 for OTAB, DeTAB, DTAB, TTAB, and HTAB, respectively.

A quick increase in surface pressure is observed as the cationic surfactant is injected into the subphase, indicating an instant interaction with the floating monolayer. The detection of the change in surface pressure ($\Delta \pi$), summarized in Table 7 allows the characterization of membrane affinity and penetration ability of molecules into the Langmuir membrane. The increase in surface pressure ($\Delta \pi$) is a common parameter used in characterizing the membrane affinity/penetration ability for molecules of different hydrophobicities. The injection of surfactants with different chain length resulted in a varying membrane affinity as can be observed in Figure 28. It is observed that there is a monotonous increase in membrane affinity with the hydrophobicity of the molecules.

Type of cationic surfactant	Number of C in the alkyl chain	Initial surface pressure (mN/m)	Surface pressure after 1 hour (mN/m)	Difference in surface pressure, $\Delta\pi$ (mN/m)
OTAB	8	7.9	8.2	1.7
DeTAB	10	7.9	8.8	2.3
DTAB	12	7.8	10.0	3.5
TTAB	14	8.2	10.8	4.3
HTAB	16	7.9	11.0	4.5

Table 7: The change of DPPC monolayer surface pressure due to interaction with cationic surfactants with different alkyl chain length

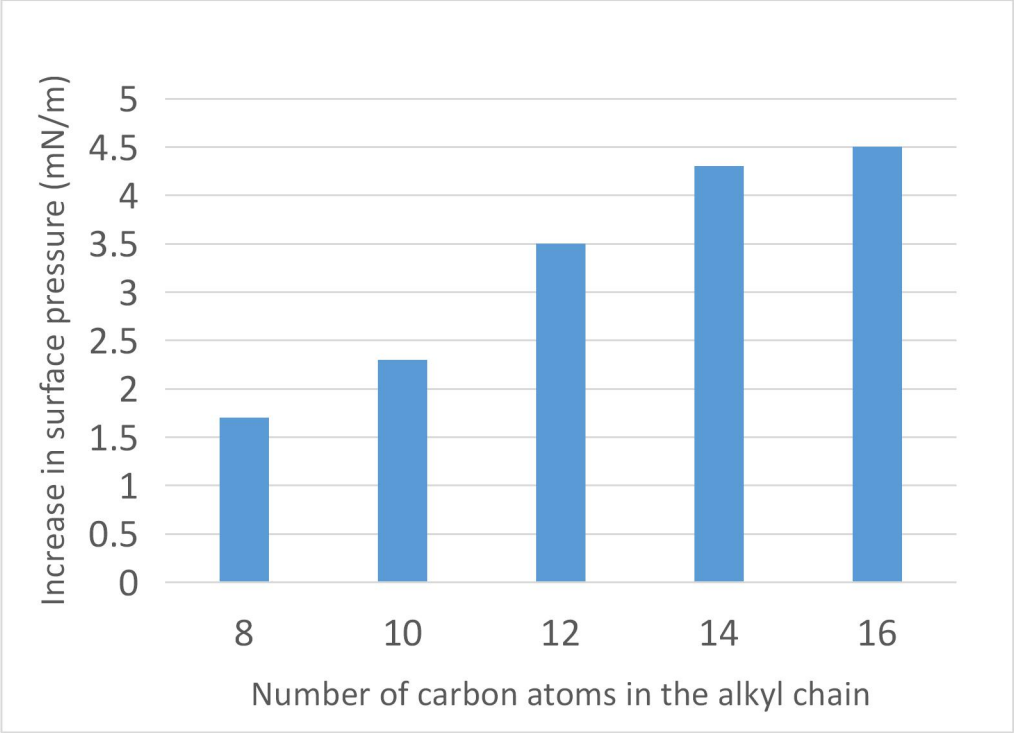


Figure 29: Increase in surface pressure of DPPC monolayer after injection of cationic surfactant solutions

As stated in the literature review, the chain length of a QAC group significantly affects the critical micelle concentration and the hydrophobicity of the molecule. An increase in the chain length of QAC causes it to be more hydrophobic while decreasing the chain length makes it less hydrophobic and has higher tendency to stay in the aqueous media. Based on Figure 29, it can be observed that an increase in the alkyl chain length of the QAC group increases the change in surface pressure of the

DPPC monolayer. Thus, the higher the hydrophobicity of the molecule, the less tendency it is to stay in aqueous media and will result in an increased affinity to the membrane monolayer. The antimicrobial activity of QACs relates significantly to the N-alkyl chain length which affect the lipophilicity properties. Chain length of 12-14 alkyls present the best antibacterial activity against Gram-positive bacteria and yeast while 14-16 alkyls shows the best activity against Gram-negative bacteria. QACs with <4 and >18 N-alkyl chain length are basically inactive [4]. The alkyl chain length relates significantly to the critical micelle concentration (CMC), where QAC group with the shortest alkyl chain length produces the highest CMC while groups with the longest chain length exhibit the lowest CMC. The effect of alkyl chain length on CMC indicates that CMC strongly correlates to the hydrophobicity of QACs [29].

That is actually in accordance with the higher antibacterial effect of the cationic surfactant with longer alkyl chain length. The HTAB is usually a reference antibacterial substance to evaluate the MIC values of various compounds [62,63,64].

6. Conclusion

Lipid monolayer formed in Langmuir balance is a wide-spread model system for characterization of membrane affinity of molecularly dispersed materials. To approach the composition of cell membranes besides the mostly used DPPC lipid layer, more complex films can be designed and introduced. The composition, compatibility of the various lipid components and knowledge of the behaviour of such mixed lipid system at body temperature are crucially important. Therefore thorough stability characterization was performed and suggested to characterize the membrane model composed from DPPC and SM. Such information could help to perform reliable penetration experiments.

Two sets of homologous series of antibacterial compounds were selected for the penetration experiments. The parabens from methyl- to butyl-parabens could be tested but there was no detectable interaction with model membrane for the more hydrophilic parabens even for propyl one. On the other hand, the limited solubility of the more hydrophobic ones does not allow their studying under the given experimental conditions.

The series of cationic trimethylammonium bromide surfactants was proved to be suitable compounds to evaluate their membrane affinity in the Langmuir monolayer penetration experiments. All the five members of the series (from octyl to hexadecyl alkyl chain) presented well defined increase in surface pressure. The structure-function relationship considering the hydrophobicity of the molecule and the membrane affinity was clearly demonstrated in the dependence of penetration ability on the alkyl chain length. It is remarkable, that the micelle formation is the phenomena which could provide the possibility of the measurement for the molecules with long alkyl chain.

Unfortunately, I had no possibility to investigate the developed mixed membrane models in penetration experiments within this thesis work. That might be the subject of future works.

7. Summary

The aim of the present work was to review the applications of different antibacterial agents and to study the relationship between the chemical structure and antibacterial properties of the selected molecules.

Ordered lipid monolayers were formed employing the Langmuir technique to prepare different membrane models. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine-sphingomyelin (DPPC-SM) mixed lipid monolayer surface pressure-area isotherms were characterized at different molar ratios and temperatures. In case of DPPC monolayer a phase transition from liquid expanded to liquid condensed phase was observed at 25°C, while there was no observable phase transition at higher temperature. Isotherms of mixtures of DPPC-SM with molar ratio of 90:10%, 80:20% and 70:30% were measured and compared. It was found that the compositional stability of mixed films was governed by the main component, DPPC. The selected DPPC-SM molar ratios were used to investigate the stability of monolayers for further penetration results. The 80:20% composition was proved to be the most stable monolayer.

Penetration measurements were performed to investigate the membrane affinity and molecular interactions of antibacterial agents with the above mentioned membrane models. For this purpose, homologous paraben (methyl-, ethyl-, propyl-, butyl-paraben) and alkyl-trimethyl ammonium bromide (OTAB, DeTAB, DTAB, TTAB, HTAB) series were used. According to the results, the methyl-, ethyl-, and propyl-parabens had no significant membrane affinity to the DPPC lipid monolayer. In contrast, there were remarkable interactions between the alkyl-trimethyl ammonium bromides and DPPC monolayer. The membrane affinity gradually increased as a function of alkyl chain lengths, which is in accordance with their antibacterial activity.

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Abbreviations

AMP	Antimicrobial peptide
CMC	Critical micelle concentration
DDA	*N,N*-dimethyldodecylammonium
DeTAB	Decyl-trimethyl-ammonium-bromide
DPPC	Dipalmitoylphosphatidylcholine
DPPG	Dipalmitoylphosphatidylglycerol
DTAB	Dodecyl-trimethyl-ammonium-bromide
E.coli	*Escherichia coli*
HTAB	Hexadecyl-trimethyl-ammonium-bromide
ICP	Intrinsically conducting polymer
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
Mtb	*Mycobacterium tuberculosis*
OTAB	Octyl-trimethyl-ammonium-bromide
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEI	Poly(ethyleneimine)
PG	Peptidoglycan
POM	Polyoxymethylene
POX	Poly(alkyloxazoline)
PPy	Polypyrrole
QAC	Quaternary ammonium compound
QAS	Quaternary ammonium salt
QPS	Quaternary phosphonium salt
RBC	Red blood cell
ROS	Reactive oxidation species
SM	Sphingomyelin
SPION	Superparamagnetic ironoxide
TB	Tuberculosis
TTAB	Tetradecyl-trimethyl-ammonium-bromide

NYILATKOZAT

Név: Nurul Izzah Binti Ahamed Kameel

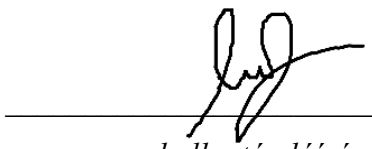
ELTE Természettudományi Kar, szak: MSc Materials Science

NEPTUN azonosító: UI5DJ5

Szakdolgozat címe: Development of Membrane Models for Study of Drug Interactions

A **szakdolgozat** szerzőjeként fegyelmi felelősségem tudatában kijelentem, hogy a dolgozatom önálló szellemi alkotásom, abban a hivatkozások és idézések standard szabályait következetesen alkalmaztam, mások által írt részeket a megfelelő idézés nélkül nem használtam fel.

Budapest, 2020. May 25



a hallgató aláírása

STATEMENT

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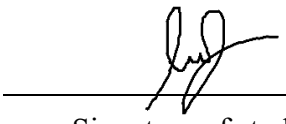
Neptun code: UI5DJ5

Topic of thesis: Development of Membrane Models for Study of Drug Interactions

As the author of the dissertation, I declare that I am fully aware of my disciplinary responsibility, that my dissertation is my own intellectual work. I have consistently applied the standard rules of references and citations and I have not used the parts written by others without proper citation.

Budapest, 2020. May 25



Signature of student