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Review

Antibiotic interactions using liposomes as model lipid membranes

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ABSTRACT

Lipidomics and proteomics have undergone a tremendous revolution, and the knowledge about drugs' mechanism of action in biological membranes has been deepened. Methods to study the interactions of drugs with biological membranes have opened new perspectives to rational drug design, based not only in the pharmacological target of the drugs but also on the interaction with biological membranes. These methods expand our ability to acquire the ADME-Tox profile of drugs, simplifying the complexity of biological membranes. Particularly, antibiotic resistance is considered one of the greatest threats to human health, being the prospects for replacing current antimicrobial drugs extremely scarce. With the decline of the discovery and the emergence of multidrug resistant pathogens to the existing arsenal, the objective in the development of new drugs to combat the resistance to antibiotics has been replaced by the modification of existing antibiotics. Therefore, drug-membrane interaction studies using membrane models of the eukaryotic and prokaryotic cell membranes, associated with a broad of complementary methods, may contribute to a deep picture concerning the effect of antibiotics upon their intake until their pharmacological target. This critical review will discuss the relevance of a range of different methods to study the interaction of antibiotic drugs using liposomes as biological membranes models. The advantages and the limitations of these methods will be discussed and future perspectives in this field will be proposed.

1. Introduction

Antibiotics have been intensively precribed in the last decades (Butler and Cooper, 2011; Lewis, 2013). With the decline of discovery and with the emergence of multidrug resistant pathogens to the existing arsenal (Ji and Lei, 2013), the objective in the development of new drugs to combat the resistance to antibiotics has been the modification of existing drugs (Moir et al., 2012). The antibiotics mode of action has been described since the early 1960s, being the classification based on their pharmacological target. In fact, the differences between bacterial and eukaryotic cells provide drug targets that have been exploited to design and develop antibiotics. Generally, antibiotics interfere with membrane integrity, cell wall synthesis, ribosomal function and nucleic acid synthesis (Neu and Gootz, 1996). Notwithstanding, the selective toxicity against bacteria while not harming host cells is only a theoretical concept not completely followed by antibiotics. In fact, the emergence of lipidomics and proteomics has contributed to a more comprehensive knowledge about drug-membrane interactions (Pignatello et al., 2011; Pinheiro, 2013). Immediatly after administration, antibiotics contact with different biological membranes. Thus, to exert their pharmacological effect, orally administrated antibiotics need to cross biological membranes, including, among others, gastrointestinal tract, plasma cell membranes, and bacteria cell membranes (Fig. 1) (Lewis, 2013).

Undeniably, the interaction of drugs with biological membranes strongly affects their therapeutic efficacy. The relevance of biological membranes to the antibiotics' efficacy are illustrated by one of the main mechanisms of antibiotic resistance that is the reduction of permeability in the outer membrane, hindering the diffusion of drugs (Gerrits et al., 2006). The complexity in the structure of bacterial, walls, outer membranes, plasma membranes and/or the presence of wax-like mycolic acids (MAs) creates impressive permeability barriers, accounting for the described intrinsic resistance to antibiotics (McDonnell and Russell, 1999). In fact, biological membranes are dynamic structures, being already described that bacteria are able to modify their biochemical composition and membranes' properties in order to survive (*i.e.* by adding positively charged amino acids to the negatively charged outer membrane) (Butler and Cooper, 2011; Lewis, 2013; Zhang and Rock, 2008) In this context, drug-membrane interaction studies using membrane models of prokaryotic and eukaryotic cell membranes, associated with biophysical methods will be useful to understand the ADME-Tox (absorption, distribution, metabolization, elimination and

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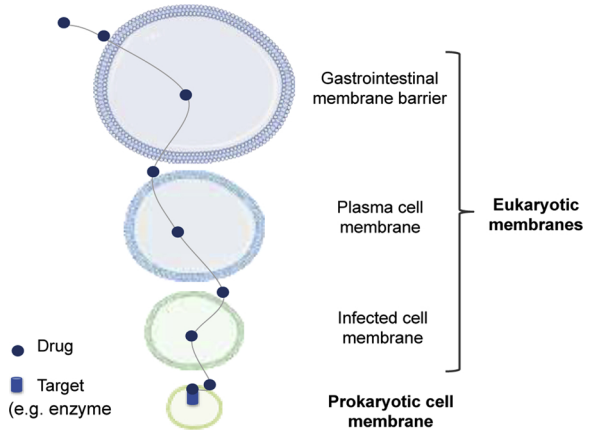

Fig. 1. Schematic representation of the antibiotics route after the oral administration in order to reach their pharmacological target.

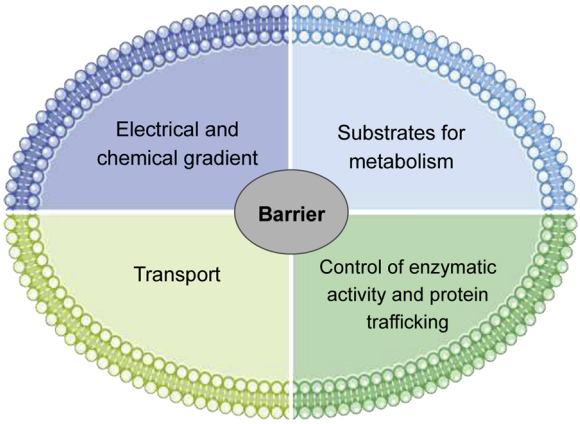

Fig. 2. Schematic representation of the membrane's functions.

toxicity) of antibiotics, and consequently their pharmacokinetic and pharmacological properties. Moreover, the study of drug-membrane interactions at molecular level may be useful to a more rational drug design, allowing the development of more effective antibiotics with less adverse effects (Pignatello et al., 2011). Additionally, interaction studies of drug-bacterial membrane may be a tool to unravel bacterial multidrug resistance (Davies and Davies, 2010; Deamer, 1987; Schmidtchen et al., 2014; Sessa and Weissmann, 1968).

This critical review will be focused on the use of liposomes as membrane model systems to assess the interactions between antibiotics and biological membranes. In addition, the relevance of drug-membrane interaction studies and their application on understanding the pharmacokinetics and pharmacological effects of the antibiotics will be discussed.

2. Antibiotics

Antibiotics can be defined as substances designed to treat infectious diseases in humans and animals (Lancet, 2009). Serendepism and traditional empirical assays of fermentation products lead to the "golden era" discovery of antibiotics (Moir et al., 2012). However, since 1990 a huge decline in the pharmaceutical industry in the antibiotic market has been observed, and in 2004 antibiotics represented less than 2% of drugs in clinical development (Moir et al., 2012).

Antibiotics can be classified according to their chemical structure, mechanism of action, and based on their pharmacological target, being the most common classes used in human therapy, the β-lactams, aminoglycosides, macrolides, quinolones, tetracyclines and rifamycins (Davies and Davies, 2010). A compilation of these classes of antibiotics, as well as their described mode of action are shown schematically in Table 1 and in Fig. 2.

The mode of action described for the above-mentioned antibiotics include the inhibition of enzymes involved in the synthesis of bacterial cell wall, nucleic acid, and proteins. For instance, β-lactams inhibit bacterial enzymes involved in the terminal stages of peptidoglycan biosynthesis, and consequently contribute to the inhibition of the biosynthesis of the bacterial cell wall (Kimura et al., 1980; Pucci and Bush, 2013; Satake et al., 1990; Yamaguchi et al., 1982). Quinolones exert their bactericidal action through inhibition of the DNA gyrase and topoisomerase IV enzymes (Pucci and Bush, 2013). Aminoglycosides have high affinities for certain portions of ribonucleic acid (RNA), especially for the prokaryotic rRNA, and thus inhibiting the protein synthesis (Pucci and Bush, 2013). Macrolides bind to different sites of the peptide exit tunnel of the 50S ribosome subunit and consequently, block the protein synthesis (Davies and Davies, 2010). Tetracyclines also inhibit the protein synthesis by hindering the attachment of the aminoacyl-tRNA to the ribosomal acceptor site (Chopra and Roberts, 2001; Pezeshk et al., 1993; Sigler et al., 2000). Rifamycins bind to the RNA polymerase β-subunit, inhibiting the RNA transcription, and thus the bacterial synthesis (Pucci and Bush, 2013).

Despite the specificity of several antibiotics to their pharmacological targets, the toxicity of antibiotics commonly occurs. Several antibiotics act in pharmacological targets that only exist in the bacterial cell or have a much more higher binding constant for the pharmacological targets than to the ones that exist in the eukaryotic cell, being however still toxic, which suggests that the toxicity and side effects are beyond directly related to the binding with the pharmacological target. In general, all the above-mentioned classes of antibiotiocs exhibit gastrointestinal disturbances as the most frequent side effects (Davies and Davies, 2010). Aminoglycosides antibiotics also exhibit severe side effects, such as nephrotoxicity and ototoxicity (Prayle et al., 2010). Tetracyclines, beyond the gastrointestinal disturbances can cause the discoloration of the dentition (Chopra and Roberts, 2001; Vennila et al., 2014). Rifamycins exhibit some adverse effects, such as, anemia, discoloration of the skin and body fluids (*e.g.*, tears, saliva, perspiration and urine), neutropenia, rash, uveitis, and rarely but clinically important, the injury of the liver function (Gisbert and Calvet, 2012). In this context, some of the exhibited side-effects are expected to be related with some unspecificity and beyond the binding to the

Table 1
Mechanism of action and side-effects described for β-lactams, aminoglycosides, rifamycins, tetracyclines and quinolones.

Class	Examples	Mechanism of Action	Side effects
β-Lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Bacterial cell wall biosynthesis	Gastrointestinal disturbances
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation inhibition	Nephrotoxicity Ototoxicity
Macrolides	Erythromycin, azithromicin	Translation inhibition	Gastrointestinal disturbances
Quinolones	Ciprofloxacin, enrofloxacin, moxifloxacin	DNA replication inhibition	Gastrointestinal disturbances Skin hypersensitivity and photosensitivity
Tetracyclines	Minocycline, tigecycline,	Translation inhibition	Dentition discoloration
Rifamycins	Rifampicin, rifabutin	Transcription inhibition	Discoloration of the skin and body fluids

Table 2
Antibiotics studied in terms of the biophysical approach of drug-membrane interactions.

Drug(s)	Antibiotic class	Reference
Imipenem	β-lactam	(Satake et al., 1990)
Meropenem	β-lactam	(Satake et al., 1990)
Cephalexin	β-lactam	(Kimura et al., 1980; Satake et al., 1990)
Cefaclor	β-lactam	(Satake et al., 1990)
Cephaloridine	β-lactam	(Kimura et al., 1980; Kobayashi et al., 1982; Satake et al., 1990; Yamaguchi et al., 1982)
Cefoperazone	β-lactam	(Satake et al., 1990)
Piperacillin	β-lactam	(Satake et al., 1990)
Ceftazidime	β-lactam	(Satake et al., 1990)
Cefsulodin	β-lactam	(Satake et al., 1990)
Aztreonam	β-lactam	(Satake et al., 1990)
Sulbactam	β-lactam	(Maurizi et al., 1993; Satake et al., 1990)
Ampicillin	β-lactam	(Kimura et al., 1980; Yamaguchi et al., 1982)
Cefazolin	β-lactam	(Kimura et al., 1980; Yamaguchi et al., 1982)
Cephaloridine	β-lactam	(Kobayashi et al., 1982)
Cephalothin	β-lactam	(Kimura et al., 1980; Kobayashi et al., 1982)
Amoxicillin	β-lactam	(Kimura et al., 1980)
Cephradine	β-lactam	(Kimura et al., 1980)
Ceftezol	β-lactam	(Kimura et al., 1980)
Amikacin	Aminoglycoside	(Au et al., 1986)
Gentamicin	Aminoglycoside	(Aramaki and Tsuchiya, 1989; Au et al., 1986; Kovacs et al., 2012; Trombetta et al., 2001)
Azithromycin	Macrolide	(Berquand et al., 2005; Fa et al., 2007)
Ciprofloxacin	Quinolone	(Bensikaddour et al., 2008)
Moxifloxacin free and metalloantibiotic	Quinolone	(Lopes et al., 2013; Ventura et al., 2008)
Enrofloxacin free and metalloantibiotic	Quinolone	(Ribeiro et al., 2011)
Tetracycline	Tetracycline	(Kuhn et al., 2011; Sigler et al., 2000)
2-tetracyclinonitrile	Tetracycline	(Sigler et al., 2000)
9-(*N,N*-dimethylglycyl amido)-6-demethyl-6-deoxytetracycline	Tetracycline	(Sigler et al., 2000)
Demeclocycline hydrochloride	Tetracycline	(Kuhn et al., 2011)
Meclocycline sulfosalicylate	Tetracycline	(Kuhn et al., 2011)
Minocycline hydrochloride	Tetracycline	(Kuhn et al., 2011)
Doxycycline hydrate	Tetracycline	(Kuhn et al., 2011)
Tetracycline	Tetracycline	(Pezeshk et al., 1993)
Tetracycline docusate	Tetracycline	(Alves et al., 2013)
Rifabutin	Ryfamicin	(Pinheiro et al., 2013c, d; Pinheiro et al., 2013e, f; Vostrikov et al., 2007)
N'acetyl-rifabutin	Ryfamicin	(Pinheiro et al., 2013a, d; Pinheiro et al., 2013f)
Rifampicin	Ryfamicin	(Rodrigues et al., 2003, 2001)

pharmacological target. This unspecificity may be a key to understand some of the above-mentioned side effects, being the methods to assess the drug-membrane interaction an important tool to get a deeper knowledge concerning the mode of action of antibiotics. For an in-depth discussion of antibiotics mechanism of action see Gottlieb and Shaw et al (Gottlieb and Shaw, 2013), and a comprehensive review on the antimicrobial resistance is given in the last WHO report from 2014 (WHO, 2014).

In summary, the present review aims to explore the information that can be gathered by the application of several methods to assess drug-membrane interactions to the antibiotics field, as well as to discuss the relevance of this interaction to the pharmacokinetics and therapeutic properties of antibiotics, and to better understand the side effects of these drugs.

Table 2 gatheres a list of the above-mentioned classes of antibiotics that were studied in terms of the biophysical approach to drug-membrane interactions.

3. Biological cell membranes

In 1925, Gorter and Grendel described the lipid bilayer as the key to the cell membrane architecture (Fuhrhop, 2002; Mouritsen, 2011; Seydel, 2002). In 1935, Danielli and Dawson added the proteins to a more complex overview of membranes structure and in 1966, Robertson's included the proteins in the membrane model unit (Fuhrhop, 2002). A more detailed description was proposed in 1972, by Singer and Nicholson, with the fluid mosaic model (Mouritsen, 2011), which reveals the membrane as a fluid lipid bilayer with proteins that diffuse freely within the plane of the cell membrane (Peetla et al., 2009). Later, the existence of large membrane domains (*e.g.*, apical, basal, and lateral membrane regions of endothelial, epithelial, and glandular cells) and lateral microdomain structures (*e.g.*, caveolae, coated pits, and lipid rafts) were reported (Mouritsen, 2011). Lipid rafts are referred to a domain, where specific lipids are associated with each other to form key platforms for membrane protein sorting and construction of signaling complexes (Nicolson, 2014; Peetla et al., 2009). In addition, the function of proteins and channels may be regulated by lipids through alterations in membranes' pressure profile, which supports the importance of the membranes' lipid bilayer (Seydel, 2002). Presently, membranes are viewed as a mosaic of different compartments or domains that are maintained by an active cytoskeleton network (Helms and Zurzolo, 2004; Nicolson, 2014).

The most basic and important function of biological membranes is to define a boundary, whether between or within cells and the internal compartments (Marsden et al., 2011). Despite this separation in different areas, cell-cell communication and tighly regulated transport are maintained, which is determinant for many celular processes (Couto et al., 2013; Marsden et al., 2011). Other important functions of the cell membranes include the existence of chemical and electrical gradients, the regulation of solute transport in the inner and outer compartments of cells, the control of membrane bound enzymes activity, the supply of substrates for metabolism, the promotion of signal transduction and protein trafficking (Brown, 2008). Commonly, cell membranes composition consists in a bilayer of phospholipids, proteins and also other macromolecules (Marsden et al., 2011; Pinheiro, 2013). However, the composition of biological membranes varies extraordinarily depending on the type of cell (*i.e.* eukaryotic or prokaryotic) and even within the cell type, being their complex and dynamic organization able to modulate and mediate conformational changes, trafficking, signaling, and recognition (Chan and Boxer, 2007; Chichili and Rodgers, 2009). The

differences between the composition of the eukaryotic and prokaryotic cell membranes will be discussed in the following sections.

3.1. Membrane lipid composition in eukaryotic cells

Eukaryotic cells display well-defined internal membranes surrounding the nucleus and the organelles (Mouritsen, 2011). The membrane composition of these internal membranes is usually different from the plasma membrane composition (Mouritsen, 2011). For instances, the presence of cholesterol (CHOL) in the endoplasmic reticulum membranes is much less representative (roughly 10%) than the amount of CHOL in eukaryotic plasma membranes (20–50% of total lipids) (Feigenson, 2006; Mouritsen, 2005). CHOL is involved in the regulation of the degree of fluidity-rigidity of the membrane, and is an essential constituent of lipid rafts (van der Meer-Janssen et al., 2010). Lipid rafts are phase-separated micro-domains, composed by CHOL, sphingolipids and proteins, which are involved in the rigid and relatively ordered state, generally defined as the liquid ordered (L_o) phase of membranes (Kulkarni, 2012; Quinn and Wolf, 2009). Additionaly, there is an asymmetry in the lipid composition of the two monolayers of the assymetric bilayers of the cell membrane (Lenoir et al., 2007). The more representative lipids of the outer monolayer are sphingomyelin (SM), phosphatidylcholine (PC), CHOL and glycolipids; whereas the phosphatidylinositol (PI), the phosphatidylserine (PSer), and phosphatidylethanolamine (PE) are the most frequent lipids of the inner monolayer (Lenoir et al., 2007; Pinheiro, 2013; van Meer, 2005). Membranes of the eukaryotic cells are predominantly composed of zwitterionic lipids. PC is a zwitterionic lipid that represents 50% of the standard membrane lipids (Lenoir et al., 2007; van Meer, 2005). The negatively charged lipids represent approximately 10% of total lipids and the positively charged lipids are usually absent (Lodish et al., 2000; Mouritsen, 2005; Yamaji-Hasegawa and Tsujimoto, 2006). Fig. 3 illustrates the main characteristics of the membranes that exist in eukaryotic cells.

3.2. Membrane lipid composition in prokaryotic cells

Prokaryotic cells display a plasma membrane and some internal membrane structures (Pinheiro, 2013). In the membranes of prokaryotic cells, the sterols (including CHOL) are commonly absent (Mouritsen, 2005). Remarkably, the composition of prokaryotic cells membrane is more varied than the composition of eukaryotic membranes. Thus, the membrane's composition constitutes an important parameter for species identification (Mouritsen, 2005).

Glycerophospholipids are the main constituints of the bacterial membranes, being the lipids phosphatidylglycerol (PG), cardiolipin (CL), PE and PI the most representative lipids in the membrane of prokaryotic cells (Geiger et al., 2010; Roy, 2009). In bacteria, lipid synthesis occurs due to the activity of enymes, mainly integral membrane proteins that display their catalytic domain in the cytoplasm (Huijbregts et al., 2000; Kol et al., 2002; Pinheiro, 2013). Fig. 3 lists the main characteristics of the membranes that exist in prokaryotic cells.

The knowledge about the composition and function of lipid domains in bacterial membranes is scarce. Contrastingly to the lipid composition of mammalian membranes, CHOL and sphingomyelin are usually absent in the lipidic domains composition. The bacterial lipidic domains seem to be composed by CL, PE and proteins (Epand and Epand, 2009).

Gram-positive bacteria, Gram-negative bacteria and Mycobacteria are different regarding the structure and chemical composition of their membranes (Fig. 4). Commonly, the membranes of Gram-positive bacteria are composed by anionic lipids, whereas the membranes of Gram-negative bacteria contain both anionic and zwitterionic phospholipids (Epand et al., 2009). Thus, Gram-negative bacteria have a highly permeable outer membrane, well-known for its asymmetric placement of lipopolysaccharides (LPS) and phospolipids, being composed by LPS on its outer leaflet, and by zwitterionic phospholipids (*i.e.* such as PE) in the inner leaflet (Marx et al., 2019). On the other hand, the membrane of Gram-positive bacteria contains PG and CL, and has lipoteichoic acid adhered to the cell surface membrane (Epand and Epand, 2009). The peptidoglycan layer, present outside of both Gram-negative and Gram-positive bacteria cell membranes, is thicker in Gram-positive bacteria. In mycobacteria, the cell wall contains a thick waxy mixture of lipids and polysaccharides, including a high content of MAs. MAs are α-alkyl-β-hydroxyl high molecular weight fatty acids, consisting on long hydrophobic saturated 2-alkyl branches and a hydrophilic head group that include the groups −COOH and −OH (Zhang et al., 2010b). The cell wall of *Mycobacterium tuberculosis* contains variable mixtures of different classes of MAs, namely α-MA, methoxy-MA and keto-MA (Villeneuve et al., 2007, 2010). The glycolipid trehalose 6,6′-dimycolate (TDM, cord factor), the main MAs-contaning molecule, has a fundamental role in the mycobacteria physiopathology (Indrigo et al., 2003). The plama membrane of mycobacteria is similar to a common bacterial membrane, and its wall is similar to a Gram-positive bacteria wall (Trifiro et al., 1990). However, the Gram stain classification of mycobacteria is controversial (Fu and Fu-Liu, 2002; Hernick, 2013). In fact, when a Gram stain is done on *Mycobacterium* samples, the staining for Gram-positive is very weak or totally absent (cells referred as "ghosts"), being the Ziehl-Neelsen stain the selected method for the identification of mycobacteria species (Trifiro et al., 1990). Due to the lack of an outer cell membrane on mycobacteria species, they are commonly classified as acid-fast Gram-positive bacteria (Hernick, 2013; McCulloch et al., 2012). Notwithstanding,

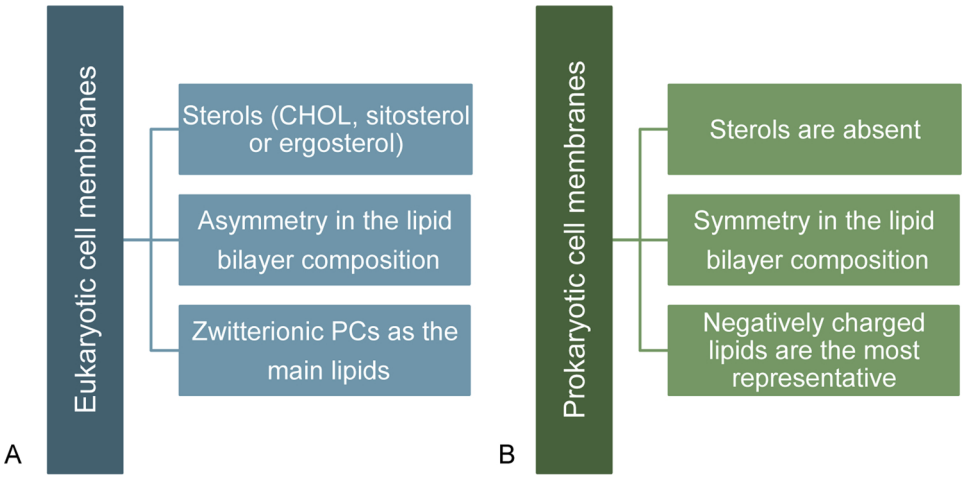

Fig. 3. Schematic representation of the main differences between eukaryotic and prokaryotic cell membranes.

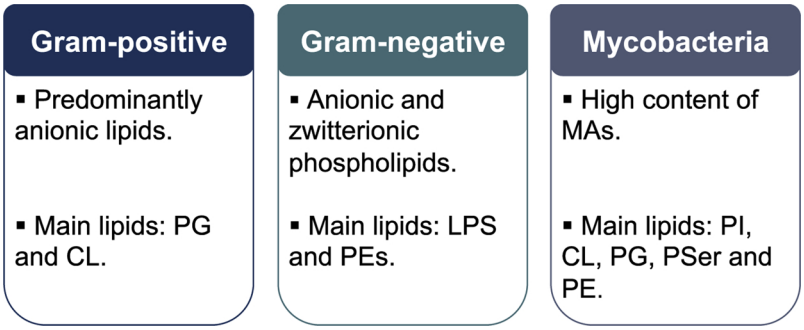

Fig. 4. Schematic representation of the differences between Gram-positive, Gram-negative and Mycobacteria cell membranes.

the phenotypic phylogenetic of the mycobacteria species is controversial since their cell wall has characteristics of both Gram-positive and Gram-negative bacteria (Fu and Fu-Liu, 2002). Moreover, the most common phospholipids in the mycobacterial plasma bilayer include PI, CL, PG, PSer and PE (Geisel et al., 2005; Haites et al., 2005; Jackson et al., 2000). The PI, a precursor for more complex glycolipids, such as the PI mannosides (PIMs) and lipoarabinomannan (LAM) is a phospholipid with important roles in the structure and physiology of these bacteria and also throughout the host infection (Haites et al., 2005; Jackson et al., 2000).

3.3. Membrane model systems

Over the last few years the increase in the knowledge about cell membranes has been remarkable in part due to the development of membrane model studies (Durr et al., 2012; Mozafari, 2005). The design of a representative cell biological model is difficult to achieve due to the complex system involved in cell membranes, where every layer is connected with each other (Fenz and Sengupta, 2012). The most common drawbacks of using cell membrane models include the lack of asymmetry and the absence of specific proteins that are commonly present in the plasma cell membrane (Cebecauer, 2012). To surpass these limitations, studies have included the insertion of proteins in the design of membrane model systems (Zagnoni, 2012). In biophysical studies of drug-membrane interactions, the development of suitable and simple membrane model systems to assess the interactions of the drug at the molecular level is more important than the reproduction of the complex nature of cell membranes (Pinheiro, 2013). This approach is focused on the diffusion and distribution of the drugs through the lipids of the membrane, to asses their interaction with membranes and predict their potential toxicity (Mouritsen, 2011). More recently, membrane models have also been used to understand the mechanisms of interaction of peptides and nanocarriers with cell membranes, which can be useful in the design and development of more efficient drug delivery systems (Tan et al., 2008). Membrane models have also been used to perform studies under conditions that the cells *in vitro* may not be able to withstand and still persist viable. Other application of membrane models studies include drug-membrane lipid bilayer interactions with specific lipid regions (*i.e.* with lipids head groups, with hydrophobic alkyl chains, and with both head groups and hydrophobic acyl chains) (Mozafari, 2005). To reach their target and elicit a pharmacological effect, drugs have to interact with membranes independently of the target location (intracellular or within membranes) (Mouritsen, 2011). Thus, drug-membrane lipid interaction studies are necessary.

The two main types of conventional membrane models are three dimensional membrane models that use an intact vesicle platform, and two dimensional membrane models that involve a planar lipid arrangement (Bagatolli and Mouritsen, 2013; Jackman and Cho, 2012). Lipid vesicles (or liposomes), Langmuir monolayers, micelles and supported lipid bilayers are the most common conventional membrane model systems (Peetla et al., 2009; Sezgin and Schwille, 2012). These models, also known as artificial membrane models, offer the possibility of manipulating the lipid content, pH, salt concentration and other factors to mimic the composition, curvature, electrostatic potential or permeability properties of a specific biological membrane (Le et al., 2011). In alternative, miniaturized lab-on-a-chip architectures and sensing platforms consisting in cell-derived membranes can be used on membrane studies. These models are considered more sophisticated and are commonly used to study membranes that contain, for example, specific receptors (Jackman and Cho, 2012; Zagnoni, 2012).

Three-dimensional models (*i.e.* liposomes) are the most common models used to mimic prokaryotic and eukaryotic cell membranes. Nevertheless, the two-dimensional models (*i.e.* monolayers) are beyond the scope of this review due to the specificity of the biophysical techniques applied to drug-membrane that generally uses these membrane model systems.

3.3.1. Liposomes

Liposomes discovery took place in 1965, when Bangham and coworkers showed evidence of the phospholipids self-assembly, with the phospholipid tails orienting towards each other to form one or more bilayers, entrapping an aqueous pool when in an aqueous compartment (Bangham et al., 1965; Owen et al., 2005; Pinheiro, 2013). This discovery confirmed previous studies that claimed that all intracellular and plasma membranes are based on phospholipid bilayers. Thus, the use of liposomes as the main model system to study the physicochemical and other properties of biological membranes was encouraged (Schroeder et al., 2009; Svetina and Zeks, 2002). Liposomes are closed vesicles that are formed by hydration of a dry phospholipid film above the main phase transition temperature (T_m) (Pinheiro, 2013). Liposomes can integrate in their composition proteins produced by the reconstitution of membrane-bound enzymes and transport proteins with the lipid bilayer, being in this case referred as proteoliposomes (Banerjee and Datta, 1983).

Multilamellar vesicles (MLVs) consist of many concentric bilayers in a single particle that can be sonicated or extruded through a filter to form liposomes with a single membrane bilayer (Moghimi et al., 2005; Schroeder et al., 2009). These unilamellar vesicles (ULVs) can be further classified into small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) depending on their size (Zhang et al., 2010a).

Liposomes are usually classified based on their size and number of lipid bilayers. The diameter size of liposomes ranges from 20 nm to several hundreds of nanometers, whereas the thickness of the phospholipid bilayer membrane is roughly 4–7 nm (Moghimi et al., 2005; Schroeder et al., 2009). MLVs contain several lipid bilayers separated by aqueous spaces. Their diameter ranges between a few hundred to thousands of nanometers. On the other hand, SUVs have a diameter inferior to 100 nm and LUVs a diameter superior to 100 nm (Moghimi et al., 2005; Mozafari, 2005). Alternatively to the thin lipid film hydration method, polyol dilution method (Kikuchi et al., 1994) and the bubble method (Sanvicens and Marco, 2008) can be used to prepare liposomes. These innovative techniques offer several advantages

including the possibility to prepare lipid vesicles without using detergents or volatile organic solvents (Mozafari, 2005; Talsma et al., 1994).

3.3.1.1. Prokaryotic membrane lipid models. Prokaryotic cell membranes are frequently mimicked using negatively charged phospholipids (Som and Tew, 2008). Generally PGs are used to form the liposomes, namely dipalmitoylphosphatidylglycerol (DPPG), palmitoyloleoylphosphatidylcglycerol (POPG), dioleoylphosphatidylglycerol (DOPG), and dimyristoylphosphatidylglycerol (DMPG), and even PGs with other fatty acids. Other negatively charged lipids, such as PI and dicetylphosphate (DSP) are also used. In the literature, it is also described lipid mixtures of the former negatively charged lipids with CL and/or PE to simulate the bacterial lipid domains. In addition, lipids extracted from bacteria and specially from *Escherichia coli* are also used to synthesize the liposomes.

Several authors take in account the major chemical differences between Gram-positive bacteria, Gram-negative bacteria and Mycobacteria to synthetize the cell membrane models. Assimetric LUVs with an outer leaflet enriched in LPS and an inner leaflet composed of a mixture of palmitoyloleoyl-phosphatidylethanolamine (POPE) and POPG are used to mimic the outer membrane of Gram-negative bacteria, whereas the inner membrane is mimicked using a mixture of POPE, POPG and tetraoleoyl-cardiolipin (TOCL) (Marx et al., 2019). Moreover, some authors go further, simulating the bacterial cell membrane of a specific specie, using the main lipid representants of each specie. To develop model systems that mimic one of the main representant of pathogenic Gram-positive bacteria *Staphylococcus aureus*, the two main lipids found in its membrane are commonly used, namely mixtures of PG and CL that are commonly described with variable proportions (Domenech et al., 2010; Epand et al., 2008; Lee et al., 2009; Sun et al., 2015; Vooturi et al., 2011; Wen et al., 2013; Xiong et al., 2005). To develop membrane model systems that mimic *Pseudomonas aeruginosa* that is one of the most relevant human pathogens, mixtures of PE, PG and CL are commonly used since the membrane of this bacteria is rich in these lipids (Ouberai et al., 2011). *Mycobacterium tuberculosis* is the most concerning pathogen of the Mycobacteria strains and is mimicked by using CHOL and PC (Kondo and Kanai, 1976; Kondo et al., 1985; Moura and Mariano, 1997). Furthermore, proteoliposomes are also produced to understand the interactions of the antibiotics with membranes that possess in their composition multidrug-efflux transporters. To produce proteoliposomes, the solubilized liposomes suspensions are mixed with the efflux pumps mainly expressed by each bacteria. Proteoliposomes are referred as bacterial membrane models of bacteria with a high prevalence and importance among human multidrug resistance infections. In this context, proteoliposomes with NorA to mimic the bacterial membrane of *S. aureus* (Simeonov et al., 2013), MexAB for *P. aeruginosa* (Verchere et al., 2014) and TBsmr as an efflux pump expressed by *Mycobacterium tuberculosis* were reported (Basting et al., 2008).

Tables 3A and 3B list the examples found in the literature, regarding different composition of liposomes used to study the interactions between antibiotics and eukaryotic/prokaryotic cell membranes.

3.3.1.2. Eukaryotic membrane lipid models. Eukaryotic cell membranes are frequently mimicked using zwitterionic phospholipids, being PCs commonly used as simple models of biological membranes. The main used PCs are dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), egg phosphatidylcholine (EPC) and dimiristoyphosphatidylcholine (DMPC). However, different research groups used different PCs or different ratios of several PCs (Table 3). Different PCs have different magnitude of interactions with evaluated drug. In order to use a more complex and reliable cell membrane, lipid mixtures are also used, being the CHOL commonly used in association with the PCs. PI, CL, and PE are the most common phospholipids that are frequently associated with PCs.

4. Biophysical techniques for the study of antibiotic-membrane interactions

The transport of antibiotics across the cell membranes is a biological process, often misunderstood because of its complex and dynamic nature (Peetla et al., 2009). Thus, model lipid membranes that mimic many aspects of the cell-membrane lipids, allow to account the passive diffusion across the bilayers, which is the most important process of the drug's permeation, especially in the case of drugs with high lipophilicity, which constitute the majority of antibiotics (Pinheiro, 2013; Pinheiro et al., 2013c). Actually, drug-lipid membrane interactions allow to predict pharmacokinetic properties, including absorption, distribution, transport, accumulation, metabolization, elimination and hence efficacy (Giaginis and Tsantili-Kakoulidou, 2008; Paloncyova et al., 2013; Peetla et al., 2009; Vourvahis et al., 2012). Indeed, drug-membrane interaction studies may be used as a retrospective approach to complete the pieces of the puzzle concerning the therapeutic and/or toxic effects of some drugs that are well-document, however misunderstood. On the other hand, these interaction studies can be of particular interest when applied to potential novel molecules, to predict their pharmacokinetic properties and to understand if they have physicochemical properties that enable them to reach clinical trials and even further, the market (Peetla et al., 2009).

To assess the interaction between drugs and membrane models, several biophysical techniques can be used. The drug-membrane interaction studies are used to assess the partition of the drug to the membrane models, the location of the drug within the lipid bilayers and the influence of the drug on the biophysical parameters of the chosen membrane models, which have implications in the pharmacokinetics and pharmacodynamic properties of antibiotics (Fig. 5).

In this review, the main biophysical techniques explored to assess antibiotics-three dimensional membrane models interactions were considered (Table 4).

4.1. Membrane affinity

Lipophilicity is a multifaceted conception since it covers both non-polar and polar interactions (Lin and Lu, 1997). The lipophilicity of a drug is generally expressed in terms of the logarithm of octanol–water partition, coefficient log *P*, or in the case of ionised molecular species throught the distribution coefficient log *D*. Lipophilicity reveals important information about the drugs' pharmacokinetics properties. Indeed, the absorption, distribution, metabolization and elimination of a drug is influenced by its lipophilicity, which ultimately influence its pharmacodynamic and toxicological properties (Lipinski et al., 2001). To reach the market, an appropriate drug should have a log *P* comprised in a specific range (de Castro et al., 2001). Low lipophilicity is associated with a limited interaction with biological membranes and therefore, low absorption and distribution (de Castro et al., 2001). On the other hand, a high lipophilicity leads to an extensive and unpredictable metabolism, high plasma protein binding and tissue accumulation (de Castro et al., 2001).

Several systems including oil-water, heptane-water and octanol-water methods have been used to evaluate drugs' lipophilicity since the 20th century (Giaginis and Tsantili-Kakoulidou, 2008; Meyer, 1899; Overton, 1901). The octanol–water system is the reference system to evaluate the lipophilicity of a drug, being the log *P* a constant characteristic for a chemical substance (Lin and Lu, 1997). However, in the last years, the octanol–water system has received a lot of criticism due to its limitation to encode all the interactions involved in biological membranes (de Castro et al., 2001). Indeed, the octanol–water system involves an hydrophobic chain with a polar head group (hydroxyl), and thus it is only possible to evaluate the hydrophobic and hydrogen bonds interactions (van Balen et al., 2004). The partition coefficient (*Kp*) is related with the neutral monomer species. The distribution coefficient is the result of the ratio of the sum of the concentration of all species in

Table 3
Molecular composition used to mimic human (A) and bacterial (B) membranes using liposomes as membrane model system.

A) Eukaryotic cell membrane models	Reference
PC:PI ranging from 1:1 to 15:1 (molar ratio)	(Au et al., 1986)
PC	(Trombetta et al., 2001; Vostrikov et al., 2007)
PC (88% total weight):POPG (10% total weight): DSPE-PEG_{2000} (2% total weight):CHOL (0, 10, 20 or 30 molar%)	(Kuhn et al., 2011)
DOPC	(Bensikaddour et al., 2008; Berquand et al., 2005; Fa et al., 2006)
DPPC:DOPC 1:1 (molar ratio)	(Bensikaddour et al., 2008; Berquand et al., 2005)
DPPE:DOPC 1:1 (molar ratio)	(Berquand et al., 2005)
SM:DOPC 1:1 (molar ratio)	(Berquand et al., 2005)
SM:Chol:DOPC 1:1:1 (molar ratio)	(Berquand et al., 2005)
DPPC	(Bensikaddour et al., 2008; Maurizi et al., 1993; Ventura et al., 2008)
EPC:CHOL:DSP 80:20:5 (molar ratio)	(Kimura et al., 1980)
EPC:CHOL	(Pezeshk et al., 1993)
EPC	(Alves et al., 2013)
EPC:Chol 4:1 (molar ratio)	(Pinheiro et al., 2013f)
DMPC	(Kovacs et al., 2012; Lopes et al., 2013; Pinheiro et al., 2013a, c; Pinheiro et al., 2013d, e; Ribeiro et al., 2011; Rodrigues et al., 2003, 2001)
DMPC:CHOL	(Pezeshk et al., 1993)
DMPC:CL 9:1 (molar ratio)	(Kovacs et al., 2012)
B) Prokaryotic cell membrane models	Reference
DPPG	(Bensikaddour et al., 2008)
DOPC:DOPG 1:1	(Bensikaddour et al., 2008)
POPE:POPG 0.75:0.25	(Lopes et al., 2013; Ribeiro et al., 2011)
POPE:POPG:CL 0.67:0.23:0.1	(Ribeiro et al., 2011)
DMPG	(Pinheiro et al., 2013a, c; Pinheiro et al., 2013d, e; Rodrigues et al., 2003, 2001)
PE:PG:CL 80:15:5	(Sigler et al., 2000)
PI	(Aramaki and Tsuchiya, 1989)
PC:DSP 97:3	(Satake et al., 1990)
DPPE:DPPG 8:2	(Pinheiro et al., 2013e)
Escherichia coli lipid extract	(Lopes et al., 2013; Ribeiro et al., 2011)
Phospholipids extracted from *Escherichia coli*:CL	(Yamaguchi et al., 1982)
Phospholipids extracted from *Escherichia coli* :LPS: porins	(Kobayashi et al., 1982)

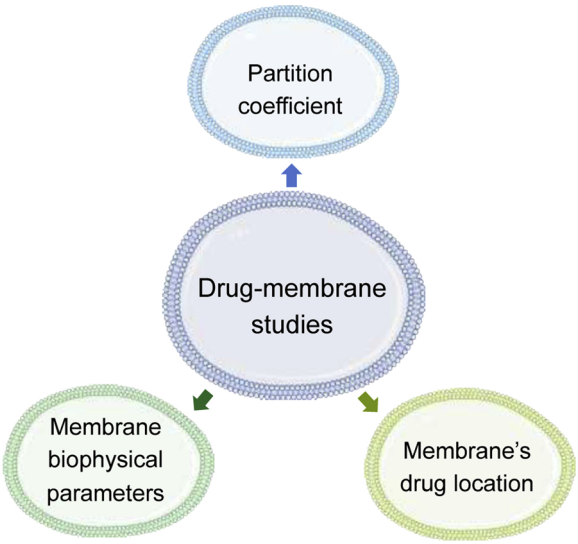

Fig. 5. Schematic representation of drug-membrane studies.

octanol to the corresponding concentration sum in water (Lin and Lu, 1997). Consequently, alternatives including partitioning into liposomes are gaining support due to their advantages (Ferreira et al., 2003, 2005). Liposomes constitute a realistic analytical system and can closely mimic the cell membranes, providing additional information to that obtained with the octanol–water system, which does not allow to account the electrostatic interactions between drugs and the apolar phase (Manuel and Martins, 2008; Matos et al., 2012; Nunes et al., 2013; Pinheiro et al., 2013b).

The lipophilicity of several antibiotics has been determined by diverse techniques, such as derivative spectrophotometry (Alves et al., 2013; Maurizi et al., 1993; Pinheiro et al., 2013a, c; Rodrigues et al., 2001) and fluorescence spectroscopy (Kovacs et al., 2012; Kuhn et al., 2011; Lopes et al., 2013; Ribeiro et al., 2011; Vostrikov et al., 2007). The majority of these studies are retrospective, using marketed drugs to study their differential affinity to the bacterial versus human cell membranes (Kovacs et al., 2012; Pinheiro et al., 2013c; Rodrigues et al., 2001). Other studies are focused on the interaction of the drugs with only human cell membrane models (Maurizi et al., 1993; Vostrikov et al., 2007) to understand some of their toxic effects, and others on the bacterial membane model with the purpose to study the drug´s diffusion through the bacterial cell membrane (Lopes et al., 2013; Ribeiro et al., 2011). Moreover, the lipophilicity of novel antibiotic molecules is frequently tested in the early stage of drug development to predict if they have desirable pharmacokinetic properties to reach the market (Alves et al., 2013; Kuhn et al., 2011; Pinheiro et al., 2013a).

4.2. Membrane location

The knowledge regarding the drug's location within the lipid bilayers give useful information about the process of drug diffusion across the lipid bilayers (Makriyannis et al., 2005). In addition, drug's location in the membrane may comprise the integrity of the biological membranes, promoting a membrane disturbance or even disruption (Giaginis and Tsantili-Kakoulidou, 2008). In fact, it is well-known that lipid bilayers are characterized by a fluidity gradient at both gel and fluid phases, *i.e.* the deeper region of the hydrocarbon chains near the centre of the bilayer is more "fluid" and disordered (C10-Cterminal) than the outer region of the chains near the phospholipids polar head group (C1-C9) (Brittes et al., 2010). Therefore, the location of a drug nearer the ordered region of the bilayer (*i.e.* C1-C9) has more consequences on the membrane biophysical properties and might comprise the integrity of the lipid bilayer (Brittes et al., 2010). On the other hand, a deeper location within the lipid bilayer does not generally affect the membrane's biophysics (*e.g.*, fluidity) (Brittes et al., 2010; Pereira-Leite et al., 2012).

Table 4
Methods applied to the study of antibiotics-membrane interactions using liposomes as membrane model system.

Biophysical Technique	Objective	Reference
Derivative spectrophotometry	Partition coeficient determination	(Alves et al., 2013; Maurizi et al., 1993; Pinheiro et al., 2013a, c; Rodrigues et al., 2001)
Fluorescence spectroscopy	Partition coeficient determination	(Kovacs et al., 2012; Kuhn et al., 2011; Ribeiro et al., 2011; Vostrikov et al., 2007)
X-ray	Membrane's drug location	(Pinheiro et al., 2013d)
Fluorescence Quenching	Membrane's drug location	(Ferreira et al., 2005; Pinheiro et al., 2013a, c; Rodrigues et al., 2003)
Fluorescence Polarization	Influence of the drug on the membrane's biophysical parameters	(Au et al., 1986; Bensikaddour et al., 2008; Berquand et al., 2005; Kovacs et al., 2012; Pinheiro et al., 2013a, c; Rodrigues et al., 2003)
Small and wide angle X-ray scattering	Influence of the drug on the membrane's biophysical parameters	(Pinheiro et al., 2013d, e; Pinheiro et al., 2013f)
Carboxyfluorescein studies	Influence of the drug on the membrane's biophysical parameters	(Trombetta et al., 2001)
Differential scanning calorimetry	Influence of the drug on the membrane's biophysical parameters	(Pinheiro et al., 2013a)
Nuclear Magnetic Resonance	Influence of the drug on the membrane's biophysical parameters	(Bensikaddour et al., 2008; Berquand et al., 2005; Rodrigues et al., 2003)
Calcein-entrapped liposomes	Influence of the drug on the membrane's biophysical parameters	(Berquand et al., 2005)
Differential scanning calorimetry	Influence of the drug on the membrane's biophysical parameters	(Ventura et al., 2008)
Computer modeling	Computacional prevision of drug-membrane interactions	(Aramaki and Tsuchiya, 1989; Fa et al., 2007)

The antibiotic drug's location within the lipid bilayer may be assessed by direct or indirect techniques. Direct techniques, such as X-ray and nuclear magnetic resonance (NMR) have been employed to study the location of the antibiotics within lipid bilayer (Bensikaddour et al., 2008; Pinheiro et al., 2013d). Indirect techniques generally require the use of fluorescent probes with a well known location within the lipid bilayers. Fluorescence quenching (*i.e.* deactivation of the probe's fluorescence induced by the drug) has been a commonly used method to study the antibiotics' relative location within the lipid bilayer (Ferreira et al., 2005; Pinheiro et al., 2013a, c; Rodrigues et al., 2003). The overall results suggest that cationic antibiotics, such as rifabutin and rifampicin possess a different location according to the cell membrane models (Pinheiro et al., 2013c, e; Rodrigues et al., 2003). In fact, the mentioned drugs have a deeper location within the lipid bilayer of the human cell membranes and a more superficial location in the bacterial cell membrane, mediated by ionic bonds and established between the drugs and the negatively charged phospholipids head groups (Pinheiro et al., 2013c; Rodrigues et al., 2003). This differential location is resposible for a more perturbing effect on the bacterial cell membrane biophysical parameters and may be resposible for a phase separation in the bacterial cell membranes (Rodrigues et al., 2003).

4.3. Membrane modifications

In general, drugs interact with proteins and/or phospholipids, which directly or indirectly influence biophysical properties of biological membranes, such as their fluidity (Pabst et al., 2012; Pignatello et al., 2011). Fluidity is a fundamental parameter linked to the membrane structure. In fact, the fluidity interferes with the activity of the membrane proteins, modulating the membrane's permeability (Monteiro et al., 2013). Variations in the membrane fluidity can affect the receptor/enzyme activity and influence the drugs' ability to pass through the membrane, which in turn can affect drugs' efficacy. Furthermore, the fluidity is closely dependent of the biophysical parameters T_m, phospholipids packing order and cooperativity of the membranes (Michel et al., 2006).

Several biophysical techniques have been used to assess the influence of the antibiotics on the membrane dynamics and thermodynamics, including fluorescence polarization (Au et al., 1986; Bensikaddour et al., 2008; Berquand et al., 2005; Kovacs et al., 2012; Pinheiro et al., 2013a, c; Rodrigues et al., 2003), small and wide x-ray scattering (SAXS and WAXS) (Pinheiro et al., 2013d, e; Pinheiro et al., 2013f), carboxyfluorescein studies (Trombetta et al., 2001), differential scanning calorimetry (DSC) (Fa et al., 2006; Ventura et al., 2008), calcein studies (Berquand et al., 2005) and dynamic light scattering (DLS) (Pinheiro et al., 2013a). Several reports have shown that the antibiotics interact differentially with the bacterial and human membrane models. These studies have also demonstrated that the biophysical properties of the zwitterionic charged membrane models remain almost inalterable (Bensikaddour et al., 2008; Pinheiro et al., 2013a, c; Pinheiro et al., 2013d, e). Contrastingly, in the presence of the studied antibiotics, the negatively charged membranes suffer pronounced alterations in biophysical parameters, such as the fluidity or even in the lipids' order and cooperativity (Bensikaddour et al., 2008; Pinheiro et al., 2013a, c; Pinheiro et al., 2013d, e). In addition, some of these studies reported a lipid phase separation induced by antibiotics, including rifabutin (Pinheiro et al., 2013d, e; Pinheiro et al., 2013f) and aminoglycosides such as amikacin and gentamicin (Au et al., 1986).

4.4. Proteoliposomes as a tool to study drug resistance

The proteins inserted in the lipid bilayer are generally efflux transporters, being the proteoliposomes interesting membrane models to study the drug resistance mechanism (Banerjee and Datta, 1983; Beales et al., 2017) In this context, Verchère et al developed a method to produce proteoliposomes with MexAB as a membrane model of the bacteria *Pseudomonas aeruginosa*. A proteoliposome as a membrane model of *Mycobactreium tuberculosis* was developed by Basting et al using TBsmr as a efflux pump (Basting et al., 2008). Simeonov incorporated the multidrug resistance transporter NorA in liposomes as a membrane model of *Staphylococcus aureus*. In all of these studies, the developed protocol can be translated to mimic these bacteria and others with similar mechanisms of resistance, using all of the above-mentioned biophysical parameters to study the changes in the membrane induced by antibiotics (Verchere et al., 2014).

4.5. Computer simulations

Computer modelling studies may be integrated into the scientific method to test ideas before the execution of the experiments or in other hand, to corroborate the experimental results. Therefore, the computer simulations may be used as a powerful tool to study the interactions between drugs and membranes (Summers, 1998). Although computer simulations are commonly used to study drug-membrane interactions,

only two works were found concerning the antibiotics-membrane studies (Aramaki and Tsuchiya, 1989; Fa et al., 2007). Both studies performed computer simulations in order to confirm the experimental results obtained.

5. Conclusions

The study of the interactions of antibiotics with biological membranes is often underestimated. This contributes for a lack of knowledge regarding some mechanisms of action and drug's reported side effects. In this context, a reflective perspective beyond the biophysical studies that evaluate drug-membrane interactions may be a key to fully understand these mechanisms and probably would lead to the development of more effective and safer antibiotics (Pinheiro, 2013; Pinheiro et al., 2013c).

The design and development of drugs based on only a specific target is misconstrued and often leads to the screening of molecules that, despite an optimal binding to the target (*e.g.,* receptor, enzyme), will never reach the market due to the absence of an efficient pharmacokinetic profile to be used in the clinical practice. In addition, the passage of drugs through biological membranes is commonly unquestionable. However, the mechanisms involved and the effect of drugs in different biological membranes continues to be a challenge to solve (Pinheiro et al., 2013a, c). The contacts that a drug establish with different biological membranes go along its destiny after administration. In the particular case of antibiotics, from the complex absorption barriers until reach the target sites in the bacteria, their pharmacokinetics properties are influenced by the interactions that they establish with the different biological membranes. Besides, the study of the phenomena that occurs on the human and bacterial plasma membranes, as well as their different interaction with drugs under physiological conditions, is imperative to exploit the molecular basis of infectious diseases and to identify new potential therapeutic strategies (Pignatello et al., 2011). In fact, prokaryotes have the ability to modify their membrane's biochemical composition and properties to survive the hostilities imposed by the environment. The addition of positively charged amino acids to the negatively charged membrane is one of the most common mechanisms of resistance to the cationic antibiotics (Roy, 2009). Moreover, the mechanism of resistance may be related with the efflux pumps and biophysical parameters can be used to understand if the diffusion, location and permeability, among other parameters are affected in the presence of antibiotics. In this context, the majority of the antibiotic drugs-membrane interaction studies support differential interactions between the eukaryotic and prokaryotic membrane models. The mechanism beyond this seems to include electrostatic interactions between drugs and the negatively charged phospholipids, especially present in the bacterial membranes (Bensikaddour et al., 2008; Pinheiro et al., 2013a, c; Pinheiro et al., 2013d, e). Some of these studies have also shown that some antibiotics induce a lipid phase separation in the bacterial membrane (Au et al., 1986; Pinheiro et al., 2013d, e; Pinheiro et al., 2013f), which might compromise the integrity of the membrane (Marsden et al., 2011) and the efficacy of antibiotics. In addition, drug-membrane biophysical studies may be useful to establish the relationship between the drug's molecules chemical structures and their effects in the membranes, which is particular important for the design and development of novel drug molecules (Pinheiro et al., 2013f). The study of the influence of different structural interdependencies between the antibiotics chemical composition and the drug compound's expected effects is fundamental to the rational development of more effective drugs, with desirable pharmacokinetics properties and fewer toxic effects.

In summary, drug-membrane interactions studies contribute to a deeper understanding beyond the antibiotics' therapeutic and toxic effects, which may be useful in the design of new drugs with a more limited range of toxicity.

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