INTERACTION OF CATIONIC ANTISEPTICS WITH CARDIOLIPIN-CONTAINING MODEL BACTERIAL MEMBRANES

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Plasma membrane is one of the major targets for cationic antiseptics (CA). The study was aimed to assess molecular effects of CAs of different chemical classes on cardiolipin-containing regions of bacterial plasma membranes. The study was carried out using coarse-grained molecular modeling. Interaction of CAs, such as miramistin, chlorhexidine, picloxidine, and octenidine, with cardiolipin-containing bilayer was assessed based on the CA coarse-grained models. CAs reduced lipid lateral diffusion coefficients and increased the membrane area per lipid. All CAs, except miramistin, reduced the lipid fatty acid chain order parameters. Adding octenidine at a CA : lipid ratio of 1 : 4 resulted in cardiolipin clustering with subsequent pulling the neutral phosphatidylethanolamine molecules out of the model bilayer. It was found that CAs have the potential for sorption to lipid bilayer, causing clustering of negatively charged lipids. Antiseptic octenidine causes formation of cardiolipin microdomains. Abnormal lateral lipid distribution together with pulling out phosphatidylethanolamine molecules can result in increased lipid bilayer permeability. The most significant reduction of cardiolipin lateral diffusion coefficient by 2.8 ± 0.4 times was observed in the presence of CA chlorhexidine at an antiseptic : lipid ratio of 1 : 4.

Keywords: antiseptic, bacterial membrane, molecular modeling, miramistin, chlorhexidine, picloxidine, octenidine

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ВЗАИМОДЕЙСТВИЕ КАТИОННЫХ АНТИСЕПТИКОВ С КАРДИОЛИПИНСОДЕРЖАЩЕЙ МОДЕЛЬНОЙ БАКТЕРИАЛЬНОЙ МЕМБРАНОЙ

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Плазматическая мембрана является одной из главных мишеней действия катионных антисептиков (КА). Целью исследования было изучить на молекулярном уровне действие относящихся к разным химическим классам КА на кардиолипинсодержащие участки плазматической бактериальной мембраны. Исследование выполнено с применением крупнозернистого молекулярного моделирования. На основе созданных крупнозернистых молекулярных моделей КА, включая мирамистин, хлоргексидин, пиклоксидин и октенидин, изучено их взаимодействие с липидным кардиолипинсодержащим бислоем. КА снижали коэффициенты латеральной диффузии липидов и увеличивали площадь поверхности мембраны, приходящуюся на липид. Кроме мирамистина, все КА снижали параметры порядка жирнокислотных цепей липидов. Добавление октенидина в соотношении КА : липид как 1 : 4 приводило к кластеризации кардиолипина с последующим вырыванием из модельного бислоя нейтральных молекул фосфатилилэтаноламина. Выявлено, что КА обладают способностью сорбироваться на липидном бислое, вызывая кластеризацию отрицательно заряженных липидов. Антисептик октенидин вызывает образование кардиолипиновых микродоменов. Нарушение латерального распределения липидов и вырывание молекул фосфатидилэтаноламина может привести к повышению проницаемости липидного бислоя. Наиболее значимое уменьшение коэффициента латеральной диффузии липида кардиолипина в 2,8 ± 0,4 раза отмечено в присутствии КА хлоргексидина при соотношении антисептик : липид как 1 : 4.

Ключевые слова: антисептик, бактериальная мембрана, молекулярное моделирование, мирамистин, хлоргексидин, пиклоксидин, октенидин

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Antiseptics come from one of major groups of compounds extensively used to prevent and combat infectious diseases. Activity of antiseptics is associated with their ability to inhibit the growth (bacteriostatic activity) or inactivate microbial cells (bactericidal activity). Among all antiseptics, cationic compounds, which electrostatically bind to the negatively charged groups of bacterial cell wall components and displace the stabilizing divalent cations, are one of the most effective. Assessment of antiseptic antimicrobial activity revealed rupture of cell membrane with subsequent leakage of intracellular components [1], impairment of cellular metabolism [2, 3], enzyme inhibition, inhibition of electron transport and oxidative phosphorylation [4, 5]. In particular, electron microscopy showed specific ruptures in bacterial cell walls [6, 7].

Among all CAs, quaternary ammonium compounds (QACs) and biguanides are the largest groups of compounds [8]. The first owe their name to the presence of quaternary nitrogen atom covalently attached to hydrophobic substituent [8]. Miramistin (MIR), carrying single positive charge, is an example of nonheterocyclic QAC. Spatial structure of MIR adopts bent conformation, resembling the hook with its head group tilted back to the long-chain alkyl tail [9]. It is assumed that positively charged nitrogen of MIR interacts with negatively charged phospholipids, which results in abnormal membrane surface charge distribution and incorporation of hydrophobic tails into bacterial membranes, leading to the membrane physical and biological function impairment. Antiseptic octenidine (OCT) is an example of heterocyclic QAC. Here, two pyridinic nitrogen atoms linked via an alkyl bridge have alkylamine substituents in the para-position [10]. OCT, carrying a double positive charge, shows high affinity for lipids forming bacterial membranes, especially for negatively charged cardiolipin (CL). Biguanides are the compounds, in which the amidine group is bonded to the guanidine group to form the –C=N–C=N– conjugated system. Chlorhexidine (CHL) is the best studied representative of biguanides. The symmetric structure of CHL consists of two hydrophilic biguanide groups connected by a hydrophobic linker, each of them bound to chlorphenol ring. Spatial conformation in the form of the bracket is typical for CHL [11]. At physiological pH values, CHL molecule carries a double positive charge [10]. CHL has become widely used due to antimicrobial activity against many microorganisms, including a broad range of gram-positive and gram-negative bacteria, viruses and fungi. However, CHL possesses higher activity against gram-positive bacteria. Some gram-negative species, such as *Proteus mirabilis* (minimum inhibitory concentration (MIC) is 115 mg/L), *Providencia stuartii* (MIC is 102 mg/L), show high resistance to CHL [12].

Bacterial plasma membrane plays an important role in maintaining cell function and has multiple functions, such as regulation of substance transport and involvement in cell division. Lipids, forming the bacterial plasma membrane, differ in the number of fatty acids and their chain length, number and location of double bonds, structure and charge of hydrophilic part [13]. Neutral phosphatidylethanolamine (PE) and negatively charged phospholipids, phosphatidylglycerol (PG) and CL, which make up at least 15% of total content, are common for most bacteria [14]. In contrast to PE and PG, CL has a more massive structure due to the presence of two phosphate residues and four fatty acids.

Bacterial plasma membranes are characterized by heterogenic lipid distribution [15]. PE phospholipid is distributed evenly in the cells of a broad range of gram-negative bacteria (*Escherichia coli*, *Salmonella Typhimurium*, *Pseudomonas putida*, *Azotobacter vinelandii*, *Proteus vulgaris*), however, localization of those in septa was shown for cells of the Bacillus species [16]. Microdomain formation was shown for anionic lipids. In particular, there are microdomains formed of CL molecules in the plasma membranes at the cell poles of gram-negative bacteria. It is believed that CL localization at the poles in associated with CL involvement in the cell division processes, in particular, with interaction with cell division proteins DnaA, MinD, FtsA. DnaA is responsible for initiation of DNA replication, MinD, being a part of the MinCDE system, prevents divisome localization to the cell poles, FtsA is a bacterial actin, a protein linker for bacterial tubulin FtsZ, forming the Z ring in the center of the cell. These proteins interact mainly with anionic lipids of bacterial plasma membranes due to the presence of amphipathic motifs enriched in positively charged amino acids [17]. The other important cellular processes, involving CL due to interaction with proteins, are as follows: energy transfer, osmoadaptation, and protein translocation. X-ray diffraction analysis revealed the presence of CL in the structures of reactive center and cytochrome c oxidase of Rhodobacter sphaeroides, formate dehydrogenase and succinate dehydrogenase of *E. coli* [13]. Colocalization of CL with osmosensory transporter ProP [18], which responds to changes in osmolality by increased transport of organic osmolytes to cell, was found in *E. coli*; colocalization with Eps system, responsible for export of cholera toxin, was found in *Vibrio cholera* [19].

Regardless of their amount, experimental data on cationic antiseptic mechanisms of action cannot give a clear answer to the question, what is the root cause of the antiseptics' bactericidal action: membrane disintegration or cell metabolism inhibition. Thus, exact molecular mechanisms of action are poorly understood in this group of antimicrobial substances. Taking into account the earlier suggestion about the potential role of CL molecules as CA binding sites [6], the study was aimed to assess the effects of CAs on the CL-containing bacterial plasma membrane areas by molecular modeling.

METHODS

Coarse-grained molecular models of CAs were described earlier [20]. To assess the effects of CAs on the model bilayer, the following biguanides were selected: CHL, picloxidine (PIC), and QACs (MIR, OCT). All CAs, except MIR, carry double positive charges. CA chemical structures, partitioned to coarse grains using the MARTINI force field, are presented in Fig. 1. Particle type C1 was selected for description of hydrophobic CA fragments by analogy with lipid parameterization; SC2/SC3/SC4 were selected for aromatic fragments, and P5 were selected for fragments containing peptide bonds, by analogy with amino acid parameterization in the second version of MARTINI force field. Antiseptics were added to model bilayer in different ratios: CA : lipid 1 : 8 and 1 : 4, in accordance with the concentrations used in medical solutions.

Coarse-grained molecular model of bilayer was built using the CHARMM-GUI MARTINI Maker [21], developed by the research group of Professor Im at Lehigh University (USA), in the MARTINI force field [22]. Plasma membrane model, simulating lipid composition at the bacterial cell poles, consisted of lipids palmitoyl-oleoyl-PE (POPE), POPG and CL, carrying the charge –2 (CDL2), at a ratio 81 : 7 : 12 by mass. Coarse-grained molecular dynamics (MD) was calculated using the Gromacs 2019.4 software package (developed by Universities of Uppsala and Stockholm, together with the Royal Institute of Technology, Sweden) [23] during 5 μs for the systems CA : lipid 1 : 8, and during 35 μs for the systems CA : lipid 1 : 4. Modeling was performed in the NVT ensemble using V-rescale thermostat (T = 320 K; τt = 1 ps) and Parrinello–Rahman barostat (*pref* = 1 bar; τp = 12 ps) [23]. MD calculation was performed by adding polarized water [24], with dielectric constant εr = 2.5, and integration step 20 fs. Characteristics of model bilayers in the presence of CAs were calculated using the built-in utilities of the Gromacs 2019.4 software package. Area per lipid was calculated with the *gmx energy* tool, and lateral diffusion coefficients were calculated with the *gmx msd* tool. Density profiles of the molecular dynamic system components relative to the center of bilayer, radial distribution functions, amount of lipids outside the plane of the bilayer were assessed using our *Python* script with the use of MDAnalysis library functions. Model membrane thickness was defined based on the density

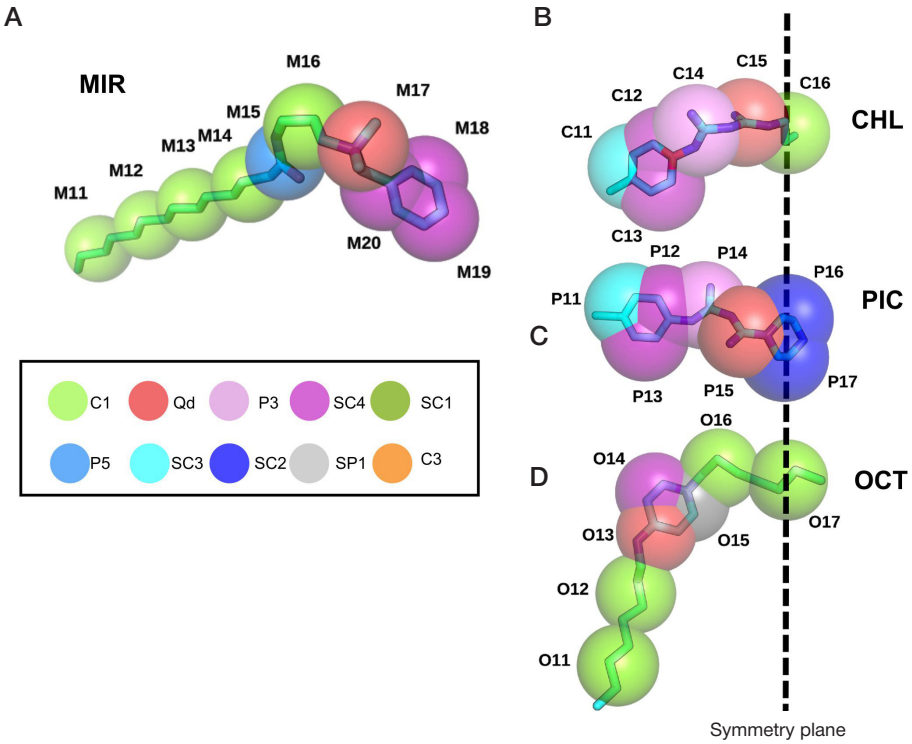

Fig. 1. CA chemical structures with overlapping coarse grains. **A**. Miramistin (MIR). **B**. Chlorhexidine (CHL). **C**. Picloxidine (PIC). **D**. Octenidine (OCT). Coarse grains are highlighted in different colors in accordance with the particle type selected in MARTINI force field (bottom part of A panel)

profiles as the difference between the positions of phosphate density peaks relative to the center of bilayer. Characteristics of model bilayers for each system were calculated based on two last μs of MD trajectory.

RESULTS

Reduced lipid lateral diffusion coefficients (Fig. 2A), slightly decreased bilayer thickness (Fig. 2B), and increased area per lipid (Fig. 2C) were observed in the presence of all studied CAs. In the presence of all CA types, except MIR, there was a decrease in lipid fatty acid chain order parameters (no data reported), which could be explained by chemical nature of MIR substantially different from other CAs. Molecules of MIR, having longer hydrophobic regions, penetrated deeper into model bilayer, and their interaction with fatty acid resulted in lipid ordering in the model membrane.

Adding OCT in high concentrations contributed to formation of CL microdomain in the bilayer. Initially, some OCT

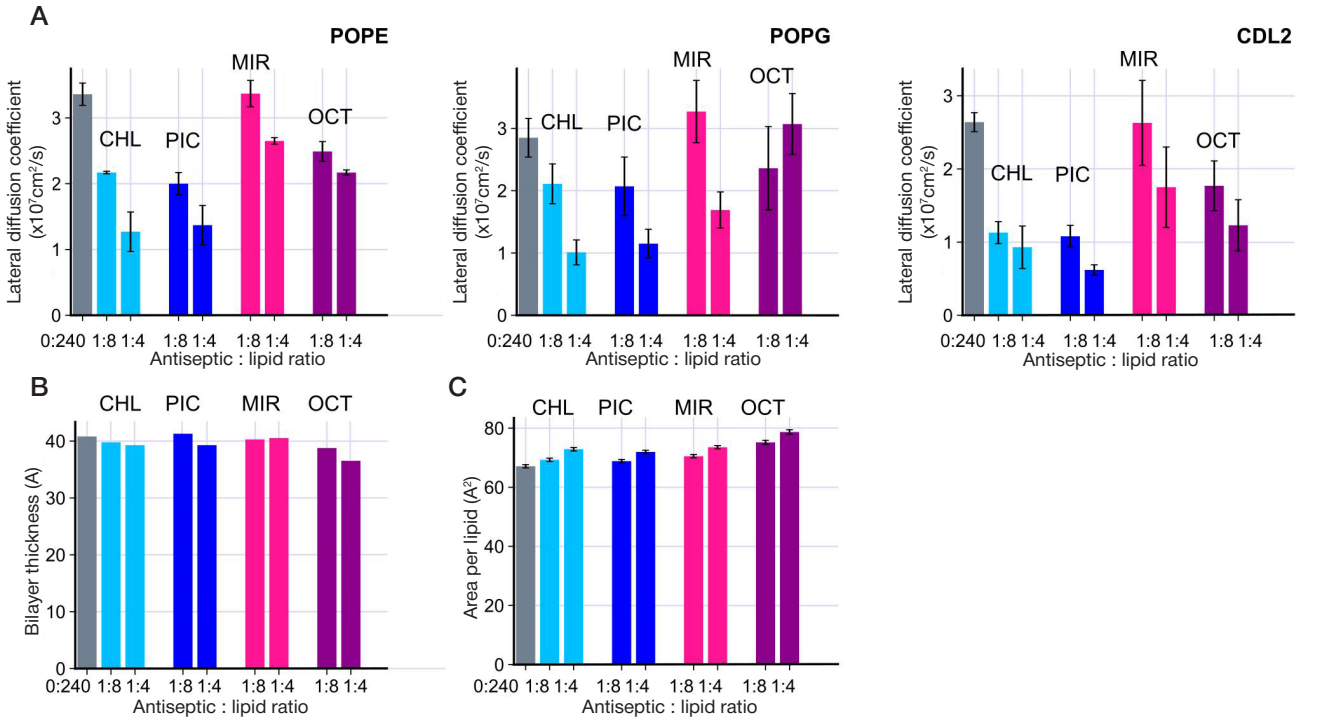

Fig. 2. Characteristics of model membrane comprising POPE:POPG:CDL2 in the presence of various CA concentrations. **A**. Lateral diffusion coefficients for the following lipids: POPE (*left*), POPG (*centre*) and CDL2 (*right*); **B**. Bilayer thickness; **C**. Area per lipid. (Parameter values for model membrane obtained without adding CAs are marked in *gray*)

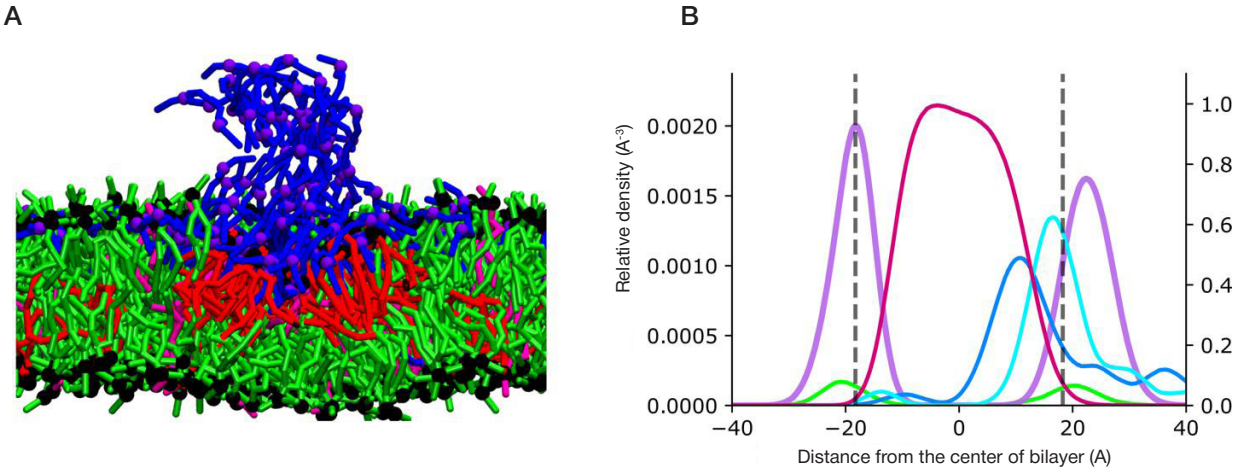

Fig. 3. Effect of CDL2 clustering in the presence of antiseptic OCT at the CA : lipid ratio of 1 : 4. **A**. Image of MD calculation at time 15 μs. POPE lipids are marked in *green*, POPG in *pink*, CDL2 in *red*, and OCT in *blue*. Phosphate residues are marked in *black,* and charged particles of OCT molecules are marked in *purple*. **B**. Density profiles for various components of model membrane. Density profile for lipid fatty acid chains is marked in *pink*, charged particles of OCT are marked in *cyan*, terminal OCT particles are marked in *light blue*, NH3 particle (ethanol) of POPE lipid is marked in *purple*, GL0 particle (glycerol) of POPG lipid is marked in *green*. Position of phosphates is represented by the dotted lines going through the centers of corresponding peaks. Density profiles for fatty acid chains normalized to the maximum peak value are shown on the second Y axis (*right*)

molecules integrated itself into the bilayer with subsequent clustering of negatively charged lipids CL and PG. Some OCT molecules, which remained in the "solution", formed a single micellar aggregate quite fast. Such behavior of molecules was due to large number of hydrophobic regions in the molecule of OCT (in addition to terminal end regions, there was a long hydrophobic linker between pyridine fragments). Micellar aggregate sorbed to OCT molecules found on the formed CL microdomain (Fig. 3A), and remained in this state for a few microseconds. In this case, the symmetry of POPE molecules in outer and inner monolayers was disturbed. The latter was confirmed by displacement of POPE lipid polar head peaks in

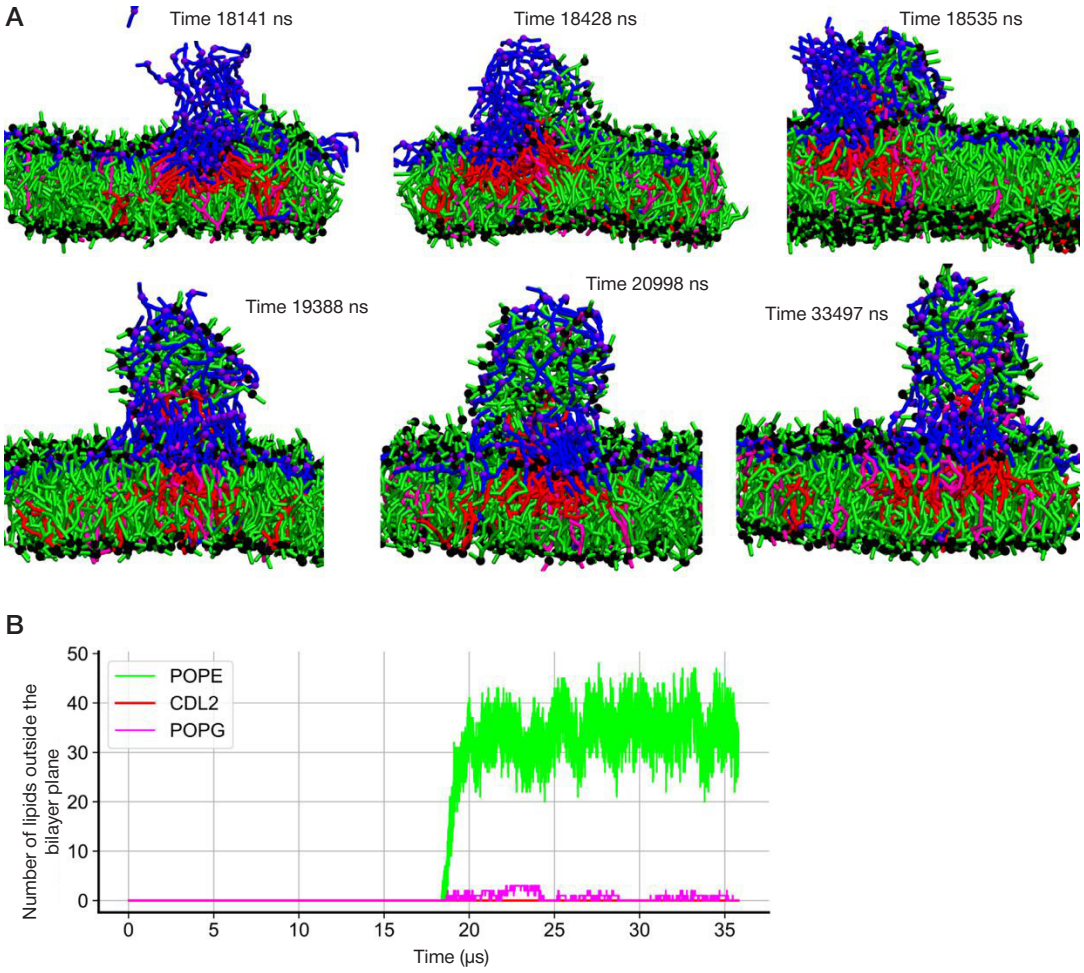

Fig. 4. Effect of pulling lipids out of the model bilayer by OCT molecules. CA : lipid ratio of 1 : 4. **A**. Consecutive images of MD calculation obtained at different times. POPE lipids are marked in green, POPG in pink, CDL2 in red, and OCT in blue. Phosphate residues are marked in black, and charged particles of OCT molecules are marked in purple. **B**. Number of lipids pulled out of the bilayer as a function on MD calculation time

Table. Ratios of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL) in plasma membranes of some species of gram-negative (–) and gram-positive (+) bacteria [13, 14]

Species	PE, %	PG, %	CL, %
Escherichia coli (–)	80	15	5
Yersinia kristensenii (–)	60	20	20
Proteus mirabilis (–)	80	10	5
Klebsiella pneumoniae (–)	82	5	6
Pseudomonas aeruginosa (–)	60	21	11
Caulobacter crescentus (–)	0	78	9
Staphylococcus aureus (+)	0	58	42
Streptococcus pneumoniae (+)	0	50	50
Bacillus cereus (+)	43	40	17
Bacillus polymyxa (+)	60	3	8

the outer monolayer relative to central position of phosphates in the relative density profiles of system components (Fig. 3B). This was due to the fact that phosphates of POPE lipid polar heads located close to CL domain were attracted to micellar aggregate formed by OCT molecules. From the moment after about 18 μs of MD calculation, POPE molecules located close to CL domain were pulled out gradually (Fig. 4A). The pulling out process lasted approximately 2 μs (Fig. 4B).

DISCUSSION

Plasma membranes of gram-negative and gram-positive bacteria have a different composition. Table presents ratios of three predominant lipids for best studied model species. These data show that PE lipid is the most abundant in the membranes of the majority of species of gram-negative cells compared to gram-positive cells. Usually, CL accounts for no more than 20% of the total amount, with the exception of species, containing no PE. The contents of CL in plasma membranes of such species can reach 50%.

Coarse-grained MD calculations showed that all studied CAs were incorporated into the model lipid bilayer. All studied CAs reduced lateral diffusion coefficients both in neutral POPE and in negatively charged lipids POPG and CDL2 (see Fig. 2A). Lipid mobility in fluid mosaic biological membranes [25] plays a vital part in maintaining activity of membrane proteins involved in all cellular processes, such as cell growth and differentiation, transport of substances, and cellular respiration. Lipid mobility is a measure of how easily these biomolecules can move along the plane of bilayer, it is assessed based on lateral diffusion coefficients [26], which could be obtained from the molecular dynamics results [27]. Reduction of lateral diffusion coefficients to 20% of baseline is observed with the antimicrobial substance : lipid concentration ratio of 1 : 5 [28, 29], which can adversely affect the membrane function.

The most significant reduction of lateral diffusion was observed with respect to CDL2 lipid, having a larger negative charge (–2 compared to –1 in POPG) and a more massive structure. MIR showed the slightest effect of lateral diffusion reduction. This could be due to the fact that, unlike other studied CAs, molecules of MIR carry a single negative charge and therefore are unable to bind several lipids and form long regions relative to immobilized lipids. Pronounced lateral lipid diffusion slowdown in the presence of biguanides CHL and PIC can be also attributed to their chemical nature. CHL and PIC, having the +2 charge and the short linker between the charged particles, contributed to formation of semi-rigid frame, linking the lipids to form the structured areas of the membrane.

When adding all studied CAs, the average thickness of model membrane declined slightly, and the area per lipid molecule increased (see Fig. 2). All antiseptics, except MIR, disrupted packing of lipid fatty acids due to pushing apart acyl chains by their terminal ends embedded in the membrane. Long tail of the MIR molecule is chemically similar to fatty acids of lipids. That is why adding MIR resulted in lipid ordering in the model membrane. The detected changes in the model bilayer may explain the disruptive effect of antiseptics on the bacterial cell plasma membrane function and barrier properties.

Antiseptic OCT contributed to formation of CL microdomain in the bilayer. Molecules of antiseptic sorbed to this microdomain in the form of micellar aggregate, pulling the adjacent neutral POPE lipids out of the bilayer. Such effects may result in the increased permeability of vesicles after adding OCT, observed during the experiment. The following molecular mechanism of the OCT bactericidal activity was proposed based on the obtained experimental results [30]. Initially, OCT binds to the outer bacterial membrane, causing the surface charge neutralization. Hydrophobic regions of OCT interact with acyl chains of lipid A, which results in hydrophobic mismatch, together with disrupted membrane structure and integrity. Likewise, OCT molecules affect plasma membrane, causing membrane depolarization, together with fluidity and phospholipid acyl chain packing impairment. As a result of this nonspecific action, both membranes of cell wall become disrupted, and intracellular fluid flows out of the cell. Our molecular modeling data on the OCT sorption to lipid bilayer, as well as reduced lipid lateral diffusion coefficients and acyl chain order parameters in the presence of OCT support the reported [30] mechanism of this antiseptic bactericidal activity.

CONCLUSIONS

Interaction of CAs belonging to biguanides (CHL, PIC) and QACs (MIR, OCT) with the CL-containing model bilayer, simulating the plasma membrane at the cell poles of bacilliform bacteria, was studied based on the constructed coarse-grain models of these substances. MD modeling results revealed both similarities and differences between the effects of various CAs on the model bilayer. Adding all studied CAs resulted in reduced lipid lateral diffusion coefficients, slightly reduced average membrane thickness and increased area per lipid.

High concentrations of OCT contributed to CL microdomain formation with subsequent pulling the POPE lipids out of the model plasma membrane. Studying the CA interaction with the model plasma membrane using computer modeling made itpossible to confirm the experimental findings at the molecular level. Comparison of chemically different CAs may contribute to development of effective new medications and enable rational use of antiseptics.

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