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Effects of antibiotics on bio-mimetic model membranes

Theses of Ph.D. dissertation

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1 Introduction and motivation

All living cells are surrounded by a cytoplasmic membrane, the structure of which is based on a quasi spherical shell-shaped lipid bilayer. The properties, stability and functioning of the membranes are defined by the interactions of membrane constituents: lipids with associated protein and carbohydrate molecules.

The cell of the Gram-negative bacterium is surrounded by a unique structure: apart from the inner membrane and the cell wall, the surface of the cell is covered by a second structure, the outer membrane. Both inner and outer membranes are bilayers, but they differ not only in their function, but also in their biochemical composition. The outer membrane possesses an asymmetric lipid and a simple protein composition. The outer leaflet of the outer membrane consists of a heat stabile product of bacteria: the lipopolysaccharide molecule or endotoxin.

Highly simplified lipid-water systems are prevalent in scientific research to model the complex biological membranes from a physico-chemical aspect and to analyze their properties. The shape of the lipid molecules and the nature of their headgroups determine their mixing properties and the structures which are spontaneously formed in the solution (Fig. 1).

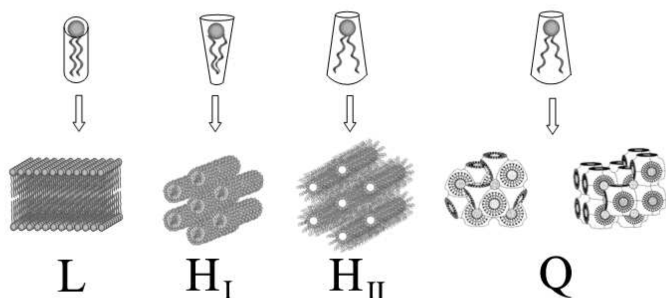

Fig. 1: Lipid polymorphism: Structures formed by lamellar and nonlamellar lipids.

Although only the lamellar arrangement of the lipids can ensure normal barrier function in biomembranes, there is some evidence that certain microorganisms regulate their membrane lipid composition to balance on the borderland of the lamellar – nonlamellar phase transition.

Foreign molecules, such as antimicrobial agents appear in nature and may lead to toxic effects in environmentally relevant organisms. We focused our attention on the effect of different antimicrobial agents, and since the lipid composition of biological membranes is highly varied, the characteristics accompanying the constituent changes of the model systems were to be examined together with the changes caused by the antibiotics. We chose sulfadiazine and some aminoglycosides as foreign molecules to reveal their physico-chemical effects on

lipid/water systems modelling the lipid bilayer of membranes.

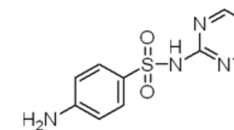

Fig. 2: Structural formula of the sulfadiazine.

The sulfadiazine (Fig. 2) is a synthetic antimicrobial agent and is active against many bacteria. It is rarely applied as human medication, but was once widely used by stock-breeders. Although EU has recently banned the use of this active substance in the form of feed additive, it got into the natural environment in high quantities, and together with several similar drugs did immense damage to both land and water ecosystems, and is even detrimental to microbes.

In clinical practice, the aminoglycosides are widely used antibiotics in the treatment of aerobic Gram-negative infections. The major problems associated with these antibiotics are the oto- and nephrotoxicity in humans and the drug-resistance of the pathogens. Different research groups have done extensive work on the lipid-antibiotic interactions to reveal the mechanism of uptake and action, the toxicity of the drug, and the drug-resistance methods of pathogens. The direct

effects of aminoglycosides on lipid model membranes were investigated extensively by different research groups, and several conflicting results have been published. These antibiotics destroy the membrane structure in an indirect way, which they achieve through misfolded proteins.

For further investigation, we chose three members (Fig. 3) of the aminoglycoside group that are special for different reasons.

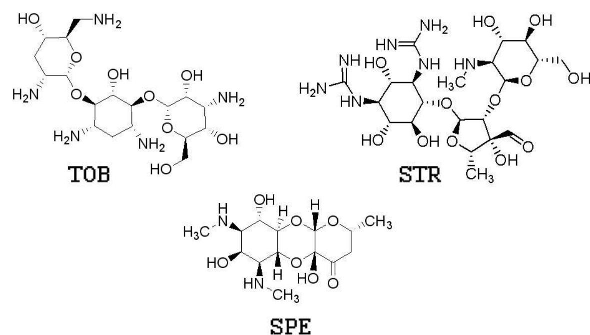

Fig. 3: Structural formula of the investigated aminoglycosides.

Tobramycin (TOB) is a typical aminoglycoside used in human therapy and an effective drug against *Pseudomonas* spp. Streptomycin (STR) is one of the most toxic aminoglycoside, and due to its strong side effects it has almost entirely disappeared from everyday therapy. Because spectinomycin

(SPE) has the same mechanism of action as aminoglycosides, it is usually classified as one, but actually, its chemical structure is entirely different.

We wished to examine whether the changes observed in lipid-water based systems are present in complex systems possessing some of the characteristics of biological and model systems at the same time. Thus, we have tried to insert different bacterial membrane components, proteins and endotoxins, with and without antibiotic treatment into lipid-water based model systems to be nearer to the real membranes and to arrive at a proper understanding of their functioning.

2 Experiments

We used fully hydrated 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)/water system for the mimicking of human membranes. The fully hydrated 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) – 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DPPG)/water systems with different lipid ratios were applied to mimic the bacterial cytoplasmic membrane. The lipid molar ratios of the systems were chosen based on their physico-chemical properties and on their biological relevance.

Differential scanning calorimetry (DSC) was used to investigate phase transitions of lipid aggregates. The small-angle X-ray scattering (SAXS) was applied to determinate the structures in the subnanometer scale. The scattering of the atomic level that occurs in the wide-angle regime was measured by wide-angle X-ray scattering (WAXS). The direct visualisation of the morphology of the samples was analysed by using freeze-fracture (FF) electron microscopy in the size range between 0.1 and 10 µm. We applied electrophoretic techniques to reveal the lipopolysaccharide and protein profiles of the *Pseudomonas fluorescens* and to determine the molecular masses of the inner and outer membrane proteins.

3 Results and discussion

The presence of the sulfadiazine (SD) causes loss in the layer correlation in the highly ordered arrangement of the investigated DPPE-DPPG vesicular system. The distortion of the lamellar structure of the lipids appears only in the regular vesicles of the systems with high DPPE amount (DPPG/DPPE+DPPG=0.05) as shown in Fig. 4. Together with the destruction of the regularity of the multilayers the interactions between the lipid layers also vanish. This phenomenon, known as “unbinding”, causes the disappearance of the membrane-membrane interactions in biological systems.

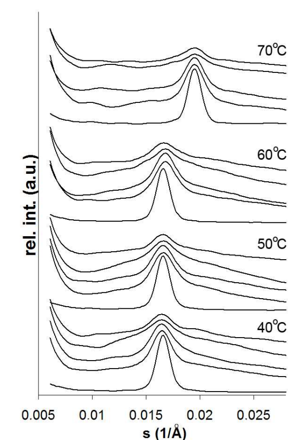

Fig. 4: The Bragg peaks of the SAXS patterns of the DPPE-DPPG/water system broaden under the influence of sulfadiazine (gel phase (40, 50, 60 ^{0}C), liquid crystalline phase (70 ^{0}C), DPPG/DPPE+DPPG=0.05, SD/lipid=0 (a), 10^{-3} (b),10^{-2} (c),10^{-1} (d) and 10^{0} (e)).

The changes of the lamellar structure are not accompanied by the perturbation of the subcell. In the gel phase the diffraction peak of the lipid packing in the bilayer does not shift, the sulfadiazine remains in the water shell region of the headgroups and does not distort the subcell of the carbon chains. The sulfadiazine is locally segregated and forms microdomains in the 0.1 – 1 SD/lipid molar ratio range, which is indicated by the additional diffraction peaks next to the peak featuring the chain packing of the lipids (Fig. 5).

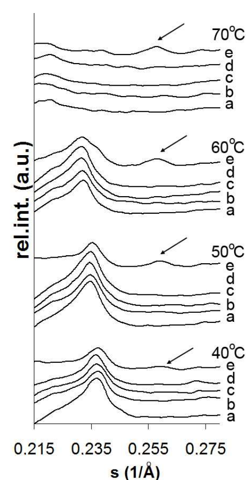

Fig. 5: WAXS patterns of the DPPE-DPPG/water system under the influence of sulfadiazine (gel phase (40, 50, 60 ^{0}C), liquid crystalline phase (70 ^{0}C), DPPG/DPPE+DPPG=0.05, SD/lipid=0 (a), 10^{-3} (b),10^{-2} (c),10^{-1} (d) and 10^{0} (e)). The peaks marked with arrows show the formation of microdomains containing a high concentration of sulfadiazine.

The presence of the aminoglycoside molecules induces the formation of weakly correlated lipid layers in the regular, multilayered DPPC/water system modelling the human membrane (which effect is similar to that of the sulfadiazine's on the 0.05 DPPG/DPPE+DPPG system).

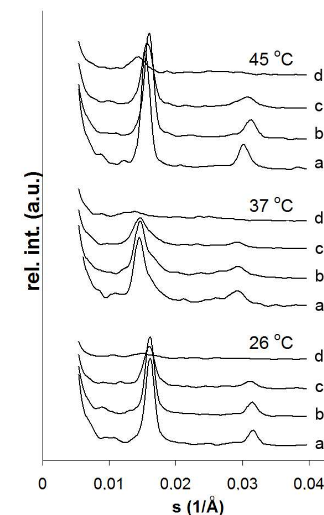

Fig. 6: The changes of the SAXS curves of the DPPC/water system under the influence of spectinomycin (gel (26 ^{0}C), rippled gel (37 ^{0}C) and liquid crystalline (45 ^{0}C) phases, SPE/lipid=0 (a), 10^{-2} (b), 10^{-1} (c) and 10^{0} (d)).

The aminoglycosides with different chemical characters trigger divergent alterations. They cause a different degree of loss of the layer correlation in the DPPC/water system.

Spectinomycin (SPE) causes the most intensive unbinding effect (Fig. 6).

In the DPPE-DPPG vesicles, especially at 0.2 DPPG/DPPE+DPPG molar fraction the presence of aminoglycosides results in reverse effect as compared to that of the sulfadiazine and the weakly correlated layer arrangement of the system becomes highly ordered.

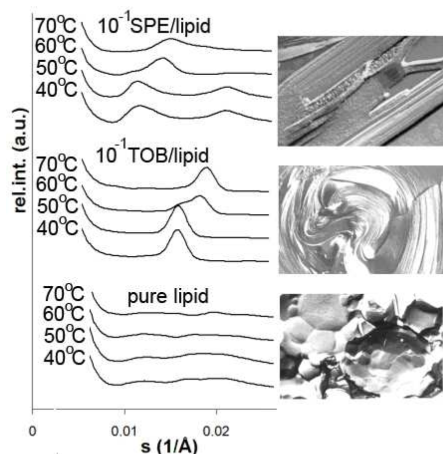

Fig. 7: Changes of the SAXS curves and FF electronmicrographs of the DPPE-DPPG/water system under the influence of aminoglycosides (AG), (DPPG/DPPE+DPPG=0.2, AG/lipid=10^{-1}, spectinomycin (SPE), tobramycin (TOB)).

In the presence of tobramycin regular lamellar structures are formed, while spectinomycin induces the

formation of hexagonal arrangements as it is shown in the SAXS patterns and freeze fractured morphologies of the systems (Fig. 7).

During the investigation of the inner and outer membrane proteins (IMPs and OMPs), and lipopolysaccharide (LPS) profiles of the *Pseudomonas fluorescens* bacteria, we set down as a fact that under the influence of TOB new, misfolded proteins with high molecular masses are formed and appear in the inner membrane, as it is also mentioned in the literature. At the same time, we concluded that TOB does not have a strong influence on the OMP and LPS constituent of the bacteria. The separation of the MPs was incomplete; therefore, we can assume that it was not the type of the protein mixtures (IMP or OMP), but the kind of the model membrane that determined the properties of the protein-lipid mixtures. In the DPPE-DPPG/water based system doped with untreated or treated proteins so called lipid rafts, that is domains with two different lateral lipid packing appear (Fig. 8). The well correlated layer structures of the DPPC based multilayered system are distorted in the presence of the protein molecules. At shorter antibiotic treating time, the proteins induce the formation of uncorrelated lipid layers; while at longer treating time a new, complex character of the model system structure was observed.

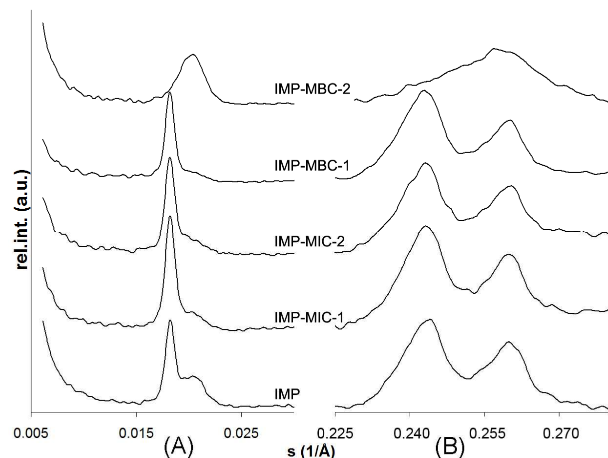

Fig. 8: SAXS (A) and WAXS (B) curves of the inner membrane proteins (IMP)/DPPE-DPPG/water system (DPPG/DPPE+DPPG=0.05, untreated reference system (IMP), tobramycin treated systems: MIC dose, 1-hour treating time (IMP-MIC-1), MIC dose, 2-hour treating time (IMP-MIC-2), MBC dose, 1-hour treating time (IMP-MBC-1), MBC dose, 2-hour treating time (IMP-MBC-2)).

At low LPS concentration the addition of LPS molecules, prepared from untreated *P. fluorescens*, to the fully hydrated DPPC/water model membrane system results in the distortion of the lamellar structure. In the higher concentration regime of the LPS, where it is rather a host than a guest molecule, complex, temperature independent structures are formed which are the mixture of the cubic and lamellar phases preferred by the LPS and phospholipid molecules, respectively.

4 Theses

1. In the fully hydrated 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) - 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DPPG)/water systems sulfadiazine (SD) causes the cessation of the layer arrangement. The distortion in the lamellar structure depends on the SD concentration and is more expressed at a high DPPE ratio (DPPG/DPPE+DPPG=0.05). The changes are accompanied by complex morphological features and domain formations, and depend on the DPPE/DPPG ratio. (Paper 1, 2, 3, 6)

2. I established that the intercalation of the SD molecules into the lipid bilayer is quantitatively limited: the 1/10 SD/lipid molar ratio can be considered as the maximum value. Above this SD/lipid ratio the formation of new domains rich in SD was observed with wide-angle diffraction method. (Paper 6)

3. The addition of aminoglycosides (AGs), especially that of spectinomycin (SPE) results in the loss of layer correlation of the highly ordered multilamellar arrangement of the fully hydrated 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) vesicles. The random increase of the layer distances is known as unbinding. (Paper 4)

4. The presence of AGs transform the badly correlated layer arrangement of the DPPE-DPPG/water system (DPPG/DPPE+DPPG=0.2) to more ordered, periodical layer structures (structure building effect). SPE (SPE/lipid=0.1) transforms the lamellar arrangement into hexagonal structures (Paper 5)

5. The effect of tobramycin (TOB) on bacterial protein synthesis was detected on complex model membranes prepared from synthetic lipid and treated bacterial membrane protein components. In lateral direction of the bilayer of the inner membrane protein (IMP)/DPPE-DPPG/water system two types of lipid packing was revealed. I proved that in the outer membrane protein (OMP)/DPPC/water system the correlation of the layers decreases. (manuscript in process)

6. At lower lipopolysaccharide (LPS) concentration (LPS/lipid=10^{-3}, 10^{-2}) the LPS prepared from *P. fluorescens* distorts the lamellar arrangement of the DPPC/water system. In the higher concentration regime of the LPS (LPS/lipid=10^{-1}, 10^{0}) temperature-independent, complex structures form. (manuscript in process)

5 Biological relevance

The investigated drugs are cyclic compounds which do not dissolve well in water and apolar solutions. Their intercalation into membranes is expected to be similar, but our experiments show that sulfadiazine and aminoglycosides behave differently. As we found, their common feature is that they cause drastic changes to the lipid/water systems only at higher drug/lipid ratios. Thus sulfadiazine is expected to influence the lipid bilayer only in the case of local accumulation of the drug molecules. The high amount of aminoglycoside applied in the model membrane investigations represent strong biological relevance since the once-daily dosing of these drugs is widespread in human therapy.

The examination of the interaction of lipopolysaccharide molecules and lipid/water based model systems help to reveal the dose-dependent toxicity of the endotoxin, the effect of which starts with the intercalation of the lipopolysaccharides into the membrane of the human host cells. Protein/lipid/water systems help us better understand the processes occurring in complex biological systems.

6 Publications

Papers

1. Oszlánczi, Á.; Bóta, A.; Varga, Z.; Goerigk, G. Effects of the sulfadiazine on the DPPE/DPPG/water vesicles. *Annual Report 2005 of HASYLAB at Deutsches Elektronensynchroton (DESY, Hamburg)* **2005** *Part I/Environmental Science* 841-842.

2. Oszlánczi, Á.; Novák, C.; Klumpp, E. Effect of sulfadiazine on biological model membranes. *Journal of Thermal Analysis and Calorimetry* **2005** *82* 457-462. IF: 1.630

3. Oszlánczi, Á.; Bóta, A.; Klumpp, E. Layer formations in the bacteria-membrane mimetic DPPE-DPPG/water system induced by sulfadiazine. *Biophysical Chemistry* **2007** *125* 334-340. IF: 2.362

4. Oszlánczi, Á.; Bóta, A.; Czabai, G.; Klumpp, E. Structural and calorimetrical studies of the effect of different aminoglycosides on DPPC liposomes. *Colloids and Surfaces B: Biointerfaces* **2009** 69 116–121. IF: 2.593

5. Oszlánczi, Á.; Bóta, A.; Klumpp, E. Influence of aminoglycoside antibiotics on the thermal behaviour and structural features of DPPE-DPPG model membranes. (2009) Accepted for publication by *Colloids and Surfaces B: Biointerfaces* IF: 2.593

6. Oszlánczi, Á.; Bóta, A.; Berényi, Sz.; Klumpp, E. Structural and morphological changes in the bacteria-membrane mimetic DPPE-DPPG/water system induced by sulfadiazine. (2009) Submitted to *Colloids and Surfaces B: Biointerfaces*

Presentations-Oral

7. Oszlánczi, Á.; Bóta, A.; Kocsis, B. *Biológiai membránok szerkezetvizsgálata* (Structural studies on biological model membranes) Hungarian Biophysical Society, Membrane Section, **2003** November; HAS, SzBK Club, Szeged, Hungary

8. Oszlánczi, Á.; Bóta, A.; Klumpp, E. *Szulfadiazin hatása modellmembránokra* (The effect of sulfadiazine on model membranes) PhD Conference, **2004** November; Budapest University of Technology and Economics, Budapest, Hungary

9. Bóta, A.; Csiszár, Á.; Oszlánczi, Á.; Urbán, E.; Fetter, G.; Drucker, T.; Varga, Z.; Goerigk, G. *Lyotropic liquid crystalline structures and their characterization (X-ray scattering, calorimetry, freeze-fracture)* Electronic-Liquid Communications, **2007** June; Liquid Crystal Institute, Kent State University, Kent, USA

Presentations-Poster

10. Oszlánczi, Á.; Bóta, A.; Szegedi, K.; Urbán, E.; Klumpp, E. *Effects of sulfadiazine on biological model membranes* Interfaces Against Pollution Conference, **2004** May; Forschungszentrum, Jülich, Germany

11. Oszlánczi, Á.; Bóta, A.; Klumpp, E. *Effects of sulfadiazine on biological model membranes* International Conference on Physiological Biophysics, **2004** November; Shanghai, China

12. Oszlánczi, Á.; Bóta, A.; Klumpp, E. *Effects of sulfadiazine on biological model membranes* 19th Conference of the European Colloid and Interface Society, **2005** September; Geilo, Norway

Others

13. Oszlánczi, Á.; Somfai, B. *Kémiailag módosított faminták felületének jellemzése* (Examination of chemically modified surface of trees) – oral presentation, II. Price at the Student Science Conference, Physical Chemical Section, **2001** November; BUTE, Budapest, Hungary

14. Oszlánczi, Á.; Somfai, B. *Kémiailag módosított faminták felületének jellemzése* (Examination of chemically modified surface of trees) – oral presentation, ELU XXVI. OTDK Conference, Chemical Section, **2003** April; Budapest, Hungary

15. Mohammed-Ziegler, I.; Oszlánczi, Á.; Somfai, B.; Hórvölgyi, Z.; Pászli, I.; Holmgren, A.; Forsling, W. Surface tension of natural and surface modified tropical and European wood species. *Journal of Adhesion Science and Technology* **2004** *18*(6) 687-713. IF: 0.869

16. Oszlánczi, Á.; Adányi, N. K.; Kiss, A.; Tóth, Sz. B.; Bóka, B.; Kiss, A.; Csutorás, Cs. *Biosensor and HPLC analysis of biogenic amine content in food samples* – poster presentation, XIV. Euroanalysis Conference, **2007** September; Antwerp, Belgium

17. Bóka, B.; Adányiné, N. K.; Szamos, J.; Oszlánczi, Á.; Csutorás, Cs.; Kiss, A. *Az enzimkémia szerepe az élelmiszeranalitikában a putreszcin példáján* (The role of the enzyme chemistry in the food analytics on the example of the putrescine) – poster presentation, VIII. Környezetvédelmi Analitikai és Technológiai Konferencia, **2007** October; Eger, Hungary

18. Oszlánczi, Á.; Adányi, N. *Analysis of biogenic amines by comparing biosensor and HPLC-MS methods* – oral presentation, Science Day Conference, Food safety research at EGERFOOD Regional Knowledge Centre of Eszterházy Károly College, Food analytical instrument and method developments Section, **2007** November; Eger, Hungary