Experimental Report

Proposal: **8-02-669** **Council:** 10/2012

Title: Biomimetic models of the outer membrane of Gram-negative bacteria andtheir interaction with two complementary antimicrobial peptides.

This proposal is a new proposal

Researh Area: Soft condensed matter

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Samples:
1-stearoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine C41H80NO8P
chloroform CHCl3
methanol CH4O
Lipopolysaccharides from Salmonella minnesota Re 595 (Re mutant not toxic)
peptide PTCDA1 sequence GVVTDLLNTAGGLLGNLVGSLSG-NH2
peptide PTCDA1-KF sequence GVVTDLLKTAGKLLGNLFGSLSG-NH2
buffer 100 mM PBS Na2HPO4/NaH2PO4 pH7 + 150 mM NaCl
1-stearoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) C42H80O10PNa
1',3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol C81H148O17P2Na2

Instrument	Req. Days	All. Days	From	To
D17	6	3	05/03/2013	08/03/2013

Abstract:

Resurgence of bacterial resistance to antibiotics has become a major public health issue. Antimicrobial peptides are very promising molecules for the development of new anti-infective therapies. However, their mechanisms of action on bacterial membranes are still poorly understood. My project aims to develop innovative models of membranes, imitating both the structure and the composition of the membranes of Gram-negative bacteria, and allowing the screening of potentially active antimicrobial peptides and the elucidation of their mechanism of action. Two new antimicrobial peptides belonging to the family of plasticins, one active and the other inactive, will be used as model drugs. Their interaction with pure symmetric PL/PL and asymmetrical LPS/PL floating and adsorbed bilayers will be studied by neutron reflectommetry in order to examine both the physical state and the symmetrical/asymmetrical organization of the membrane, as well as the influence of concentration and aggregation state of plasticins penetrating into it.

Experimental report of the proposal 8-02-669/Beamline D17

Biomimetic models of the outer membrane of Gram-negative bacteria and their interaction with two complementary antimicrobial peptides

Context and objectives of the project

Over the past twenty years, the increase of bacteria resistant to conventional antibiotics has urged the need for novel therapeutic sources. Antimicrobial peptides are very promising candidates for the development of new anti-infectious therapies. We know now that they mainly act by significantly disrupting the integrity of the whole bacterial membrane via interaction with membrane phospholipids and peptidoglycan layers, although the exact mechanism has not been elucidated yet [1]. In fact more than one mechanism seems to apply, and it might depend upon the chemical structure and conformation changes of a peptide when it is in interaction with the complex bacterial cell wall [2]. A better understanding of this mechanism of action would open the door to the selection and development of new therapeutically active peptides. To reach our goal, we build biomimetic models of the bacterial membrane in order to study the penetration of antimicrobial peptides (e.g plasticin) into the asymmetric outer membrane of the Gram negative bacteria cell wall.

The structure and proteo-lipidic composition of bacterial membranes differ depending on the bacterial type (Gram-negative or Gram-positive). The structure of Gram-negative bacteria cell wall is structurally complex and consists of two membranes separated by a thin layer (5-8 nm) of peptidoglycan, a polysaccharide consisting of polymer of N-acetyl muramic and N-acetyl glucosamine. The outer membrane is asymmetric, consisting of an external leaflet of lipopolysaccharide (LPS) and another internal one made of anionic phospholipids (PL) (phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL)), which is anchored in the peptidoglycan. The inner membrane is only composed of the precited phospholipids. In this project, we focused on the asymmetric outer membrane of Gram negative bacteria.

Methodology

Neutron reflectometry is a technique that has proved valuable in determining the structure and composition of model membrane systems at the solid/liquid interface [3-4]. Model bilayers were prepared by using the Langmuir-Blodgett (LB) and Langmuir-Schaeffer (LS) techniques in the Soft Matter Laboratory facility at the ILL.

Full asymmetric planar bilayers with a PE/PG/CL proximal leaflet and a LPS distal one was built in order to mimic the lipid matrix of the outer membrane. Pure mutant LPS Re 595 molecules from *Salmonella enterica* as well as the ternary PL mixture (SOPE/SOPG/CL) with the molar ratio (80/15/5) were used.
Full symmetric planar bilayers composed of two identical PE/PG/CL leaflets were also prepared in two ways: by LB/LS transfers of PL monolayers or by PL liposomes fusion.
Finally, we focused on a bilayer model introduced by Charitat et al. [3] involving a planar bilayer floating at 2–3 nm away from an adsorbed on a solid surface in contact with bulk water. This model has been used for surface scattering studies of a highly hydrated, accessible and fluctuating bilayer, in which the composition of each leaflet can be chosen separately. Since the second bilayer is only weakly bound, the preparation needs particular care. Such floating bilayers were formed on solid substrates by three successive monolayer depositions with the LB/LS technique [4].

Since bacterial lipids are mainly anionic and silicon wafers are negatively charged in aqueous medium (here PBS buffer), we first functionalized our silicon surfaces with the aminosilane APTES (aminotriethoxysilane) to make the surface positively charged. Sapphire surfaces are naturally positively charged. This surface charge issue is necessary for an effective transfer of anionic PL or LPS monolayers by the LB/LS technique. Neutron reflectometry (NR) measurements were thus performed on symmetric PL/PL, asymmetric PL/LPS and asymmetric PL/PL/PL/LPS floating bilayers formed on silicon or sapphire blocks in D_2O, H_2O, SMW or 4MW media. Experimental data were fitted with the Aurora fitting program (developed by Yuri Gerelli and Giovanna Fragneto) through a multi-slab model taking into account the different parts of the bilayer: substrate, silane layer on SiO_2, water layer, PL inner polar headgroups, aliphatic tails, and PL or LPS polar headgroups.

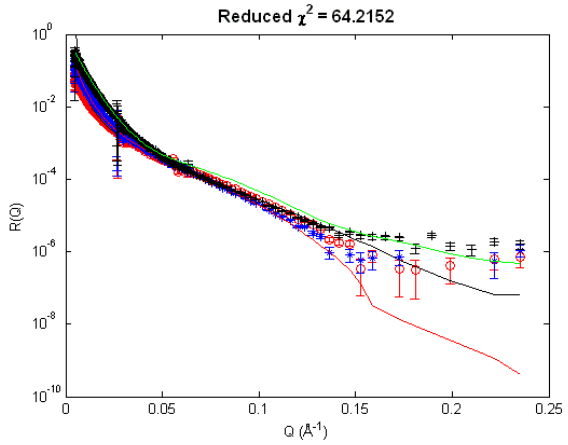
Results and discussion

-SiO_2-APTES and sapphire bare surfaces were first analysed by NR. The rugosity of sapphire surfaces was 5 ± 1 Å. SiO_2-APTES surfaces were fitted with a two slab model (SiO_2 and APTES layers). The SiO_2 layer was 8 ± 1 Å thick and 2 ± 1 Å rough. The aminosilane layer was thicker (15 ± 1 Å), rougher (9 ± 1 Å) and moderately hydrated (16 ± 5%) with a calculated SLD of 0.122 $x10^{-6}$ $Å^{-2}$.

-Symmetric planar PL/PL bilayers were formed on both SiO_2-APTES and sapphire surfaces by the LB/LS technique. On SiO_2-APTES surface, the inner PL heads layer was separated from the substrate with a 12 Å-thick water layer and appeared rougher than thick (8 ± 1 Å versus 5 ± 1Å) with a very high water content (70 ± 20%), indicating that the bilayer was incomplete. On sapphire, the results were better with reasonable values for the rugosity and water content of PL polar headgroups, but their thickness was higher than expected (15 ± 1 Å instead of 8 Å). In both systems, values associated to the PL hydrocarbon chains layer (taken all together) were reasonable when compared to the litterature: 25-30 Å in thickness with low water content (around 10%) and 8-9 Å in rugosity. Finally, liposomes rupture on sapphire did not give good results: incomplete bilayer was obtained with a too thin external leaflet (5 Å) and containing 75% water.

-Two asymmetric PL/LPS Re 595 planar bilayers were successfully formed on both SiO_2-APTES and sapphire surfaces. SLD values of the polar part of the LPS Re 595 molecule was estimated to 5.13 $x10^{-6}$ $Å^{-2}$ for the polar part [5], and was fitted around 1± 0.1 $x10^{-6}$ $Å^{-2}$ for the PL/LPS hydrocarbon chains part, a value fully consistent with recent literature [5-6]. An example of asymmetric PL/LPS Re 595 single bilayer is shown in figure 1.

(a)



(b)

Layer	Thickness (Å)	SLD $x10^{-6}$ ($Å^{-2}$)	Water content (%)	Roughness (Å)
water	7,3 ± 1	*-0,56*	100	11 ± 1
Inner PL heads	8 ± 1	*1,36*	70 ± 10	3 ± 1
All Tails	29 ± 1,2	0,99 ± 0,1	41 ± 5	8 ± 1
Outer LPS heads	16 ± 1	*5,13*	70 ± 10	10 ± 1

Figure 1: (a) NR spectra in D_2O, H_2O and 4MW media of an asymmetric PL/LPS Re 595 planar bilayer adsorbed onto sapphire surface. (b) Fitted values are presented in the table. Fixed parameters are in italic.

Values obtained for the PL part were in very good agreement with the ones expected. For the LPS leaflet, values are fully consistent with the ones found in litterature: in particular,

- The high water content of the LPS Re 595 headgroup (70 ± 10%) is close to the one obtained for the LPS Rc mutant of *E. coli* (74 ± 12% found by Lebrun [5]).
- The thickness of the LPS Re 595 polar headgroup was 16 ± 1 Å, a value similar to the one of the inner polar headgroup of the LPS Rc mutant of *E. coli* (14 ± 4 Å [5]).

-Two asymmetric PL/PL/PL/LPS Re 595 floating bilayers were formed on both SiO_2-APTES and sapphire surfaces. However, fitting these systems with a 9 or 11-slab model corresponding to the superposition of a symmetric PL/PL bilayer and an asymmetric PL/LPS bilayer did not provide satisfactory results. The fit of NR spectra in the 3 media was always better with a model corresponding to a single PL/PL bilayer adsorbed on the surface: we could not form a second bilayer composed of an external LPS leaflet, probably due to a too high electrostatic repulsion from the PL head of the external leaflet of the first bilayer. A zwitterionic lipid such as DMPC would be a better choice for the composition of this first bilayer.

-Note that we faced some difficulties to fit correctly some of the 4MV or SMV spectra with the expected SLD values (4 or 2.07 $x10^{-6}$ $Å^{-2}$), probably due to a bad mixing between H_2O and D_2O before injection (technical problem with the pump?).

-Interaction with peptides solubilized in D_2O buffer can be analyzed only with the full complete bilayers. Experimental data showed a striking modification of the shape of the spectra of the single asymmetric PL/LPS Re595 bilayers after incubation with the cationic active plasticin at 1 µM concentration, indicating a strong interaction between them. The fitting of these spectra is currently under progress.

References
[1] Y. Shaï, Biochimica Biophysica Acta-Biomembranes 1462, 55-70 (1999).
[2] Z. Oren, Y. Shaï, Biochemistry 36, 1826-1835 (1997).
[3] T. Charitat, E. Bellet-Amalric, G. Fragneto, F. Graner, Eur Phys J B 8:583–593 (1999).
[4] G. Fragneto, T. Charitat and J. Daillant, Eur Biophys. Journal 2012, 10.1007/s00249-012-0834-4.
[5] A.P. Le Brun, L. A. Clifton, C. E. Halbert, B. Lin, M. Meron, P. J. Holden, J. H. Lakey, and S. A. Holt, Biomacromolecules 14, 2014−2022 (2013).
[6] T. Abraham, S. Schooling, M.-P. Nieh, N. Kučerka, T.J. Beveridge and J. Katsaras, J. Phys. Chem. B 111, 2477-2483 (2007).