Interaction of a Bacterial Endotoxin with Model Membranes and Other Surfaces Investigated by Fourier Transform Infrared Attenuated Total Reflection (FTIR ATR) Spectroscopy

G. Reiter*, M. Siam*, D. Falkenhagen‡, and U.P. Fringeli*
*Institute of Physical Chemistry, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria
‡Center of Biomedical Technology, Danube University Krems, Austria

Introduction

Lipopolysaccharides (LPSs, endotoxins) are complex lipid-linked carbohydrates which are found in the outer membranes of Gram-negative bacteria (Fig. 1 and 2). These negatively charged molecules are usually composed of a polymeric carbohydrate (O-antigen), a short oligosaccharide (R-core) and a fatty-acylated region (lipid A). Through its action on macrophages, LPS can trigger responses that are protective or injurious to the host[1].
In order to get more insight into the mechanisms of blood purification[2] and toxic action, FTIR ATR spectroscopy[3] was used to investigate the interactions of LPS from Pseudomonas aeruginosa with a hydrophilic and a hydrophobic surface, with a positively charged crosslinked polymer and a positively charged bilayer.

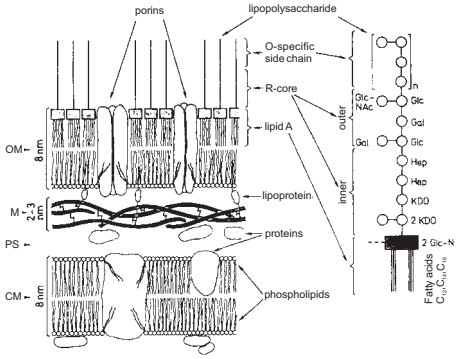

Fig. 1: Model of the cell wall of Gram-negative bacteria. *Left:* The Murein (M) layer adjacent to the cytoplasma membrane (CM) is covalently bound to the hydrophilic ends of lipoproteins. Their lipophilic ends are attached to the outer membrane (OM) consisting of phospholipids and lipid A of LPSs. The hydrophilic, O-specific heteropolysaccharide side chains of the LPSs are located on the surface of the bacterial cell. *Right:* Schematic representation of a LPS molecule consisting of lipid A, R-core and O-Antigen. Symbols: Glc-NAc = N-acetylglucosamine; gal = galactose; Hep = heptose; KDO = 2-keto-3-deoxyoctonate; PS = periplasmic space. (From H.G. Schlegel: *Allgemeine Mikrobiologie*, Thieme. Stuttgart, New York (1985): p. 50.)

Materials and Methods

In each FTIR ATR experiment, an aqueous buffer solution of LPS from *Pseudomonas aeruginosa* (serotype 10) (Sigma, L-7018) was pumped into a flowthrough cuvette and contacted with the surface of interest. Furthermore, a chopper behind the cuvette split the IR-beam and allowed one to measure sample and reference spectra quasi-simultaneously (single beam sample reference (SBSR)-technique)[3].
First, the interaction of LPS with a plasma-cleaned, hydrophilic germanium (Ge) surface was investigated. A trapezoidal Ge plate was always used as internal reflection element and represented the carrier for all other surfaces.
Second, with the help of a film balance, a monolayer consisting of dipalmitoyl phosphatidic acid (DPPA) (Fig. 2) was transferred to the Ge plate creating a hydrophobic surface (Fig. 3, step I).
Third, the interaction of LPS with a supported positively charged lipid bilayer was monitored. The bilayer consisting of DPPA as inner and a (1:1)-mixture of palmitoyl oleoyl phosphatidylcholine (POPC) and Hexadecylpyridinium (HDPyr) as outer leaflet was prepared using the Langmuir Blodgett (LB)/Vesicle Method[3] (schematically shown in Fig. 3, step I-III).
Finally, a positively charged crosslinked polymer consisting of γ-aminopropyltriethoxysilane (ATS) was prepared by polymerisation of ATS on Ge in toluene (Fig. 4)[4]. This method of silanization gives few adjacent monolayers of silane across the carrier surface.

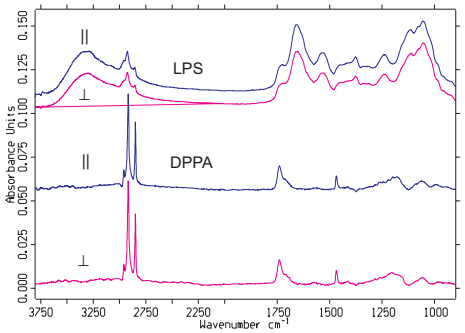

Fig. 2: Parallel (||) and vertical (⊥) polarized absorbance spectra of LPS and DPPA. *Top:* Polarized IR ATR absorbance spectra of LPS adsorbed on Ge; T, 25°C; Spectra were measured against air; angle of light incidence θ, 45°; number of active internal reflections N, 15. *Bottom:* DPPA transferred at 30 mN/m from aqueous subphase (10^{-4}M $CaCl_2$) to a germanium ATR plate. Spectra were measured against air. Surface concentration was calculated with the thin film approximation: surface concentration $\Gamma = 3.80 \cdot 10^{-10}$ mol cm^{-2}; dichroic ratio R ($A_{\parallel}/A_{\perp}$) = 0.93 (at 2850 cm^{-1}); angle of light incidence θ, 45°; number of active internal reflections N, 39.

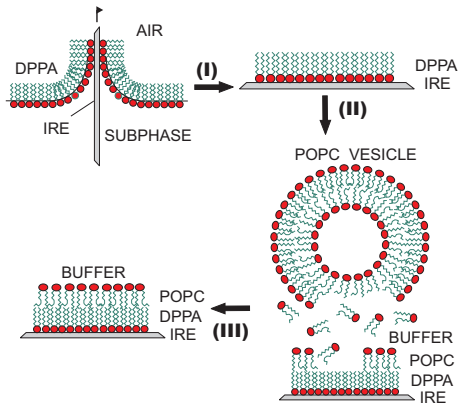

Fig. 3: Schematic description of preparing lipid model membranes attached to an IRE-plate. (I) Transfer of the inner IRE-attached DPPA monolayer from the air/water interface of a film balance to an internal reflection element (IRE) by the Langmuir-Blodgett (LB) technique; (II) spontaneous adsorption of POPC lipid molecules from vesicles energetically driven by the reduction of the unfavorable high energy of the hydrophobic surface of the DPPA monolayer in contact with the aqueous environment; (III) completed asymmetric DPPA/POPC bilayer.

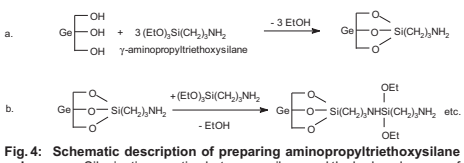

Fig. 4: Schematic description of preparing aminopropyltriethoxysilane polymers. a. Silanization reaction between a silane and the hydroxyl groups of a Ge surface. b. Polymerization reaction between a free silane and a silane already coupled with the Ge surface.

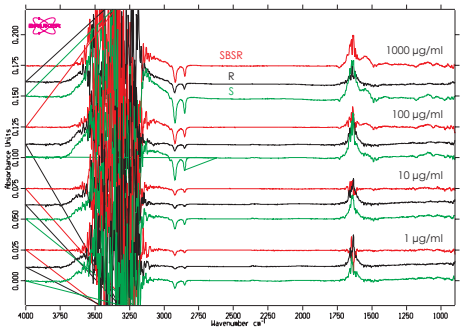

Fig. 5. IR ATR absorbance spectra of a DPPA/(POPC:HDPyr)(1:1) bilayer after treatment with different concentrations (1-1000 µg/ml) of LPS from Pseudomonas aeruginosa (serotype 10). ATR parallel polarized light. Buffer: 20 mM phosphate buffer pH 7, 100 mM NaCl. Angle of light incidence θ=45°; number of active internal reflections N = 33; germanium ATR plate; T = 25°C. *Red:* SBSR absorbance spectra (S-R). Quasi-simultaneous difference of the effect of LPS and the effect of buffer on the bilayer. *Black:* Reference part (R). Effect of buffer on the bilayer. *Green:* Sample part (S): Effect of LPS on the bilayer. The ν(CH) region shows the loss of lipid caused by simultaneous pumping LPS (S) and buffer (R) solution over the bilayer. At all concentrations, the loss of ν(CH) is greater in the case of LPS. This leads to negative absorbances in the corresponding SBSR spectra.

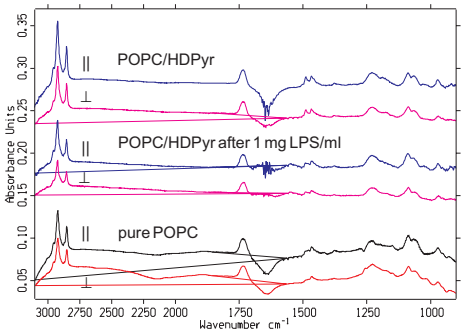

Fig. 6: Parallel and vertical polarized absorbance spectra of lipid bilayers. *Top*: DPPA/(POPC:HDPyr) bilayer; 20 mM phosphate buffer pH 7.0, 100 mM NaCl; T = 25°C; reference, DPPA monolayer in phosphate buffer; dichroic ratio R, 1.29 (at 2853 cm^{-1});surface concentration $\Gamma = 3.86 \cdot 10^{-10}$ mol cm^{-2}; angle of light incidence θ, 45°; number of active internal reflections N, 33. *Middle*: DPPA/(POPC:HDPyr) bilayer after contact with 1 mg LPS/ml. Dichroic ratio R, 1.59; surface concentration $\Gamma = 1.90 \cdot 10^{-10}$ mol cm^{-2}. *Bottom*: DPPA/POPC bilayer. Dichroic ratio R, 1.35; surface concentration $\Gamma = 2.50 \cdot 10^{-10}$ mol cm^{-2}.
The middle and bottom spectra are quite identical, representing pure POPC. LPS obviously removes only the positively charged HDPyr from the membrane.

Results and Discussion

Whereas the hydrophilic LPS exhibits only weak adsorption to the Ge plate and the DPPA monolayer, it binds strongly to the ATS polymer (Fig. 7). However, in each case considerable and similar amounts of the amide I and II peaks are found. This can be explained by contamination of the LPS with protein which is able to bind quite unspecifically to any surface. After washing with buffer, only the hydrophilic Ge and, to a greater extent, the positively charged ATS-polymer are able to retain some LPS molecules which possess phosphate and carboxylic groups for electrostatic interaction. The main effect of the endotoxin on DPPA is the solubilization of DPPA molecules. Moreover, LPS prevents the adsorption of POPC vesicles to a DPPA monolayer exhibiting a strong detergent-like effect (data not shown).

Furthermore, the endotoxin removes predominantly the positively charged HDPyr from the DPPA/(POPC:HDPyr) bilayer. With the help of the SBSR-technique, this effect can be seen even at the lowest concentration of LPS (1 µg/ml) (Fig. 5). After reaching our highest concentration (1000 µg/ml), almost no amount of the positively charged HDPyr is found in the membrane that is now consisting of pure POPC. Fig. 6 shows that the spectra of the remaining bilayer resemble closely the spectra of pure POPC. The absorbance of the CO ester peak at 1738 cm^{-1} characteristic for POPC remains constant before and after treatment with LPS. Moreover, the calculated surface concentrations indicate that the loss after endotoxin incubation is about 50% corresponding with the solubilization of all HDPyr molecules. Finally, the dichroic ratios show that at the beginning, the order of the DPPA/(POPC:HDPyr) membrane is higher than that of a pure DPPA/POPC membrane caused by the fully saturated chains of HDPyr. However, after LPS treatment, the order of the remaining membrane is lower than that of a pure DPPA/POPC bilayer which is more densely packed than the DPPA/POPC membrane formed by removal of HDPyr. As can also be derived from the spectra of Fig. 6, the dichroic ratio of the removed HDPyr (data not presented) represents approximately the same high order like a densely packed DPPA monolayer (Fig. 2).

To sum up, it can be said that these experiments show a strong interaction of the negatively charged LPS with positively charged surfaces. The sensitivity of FTIR ATR spectroscopy makes it a powerful tool for elucidating mechanisms of endotoxin action upon lipidic or polymeric structures, even in a low LPS concentration range. Therefore, a first precondition for carring out further investigations of structure-function-relationships of LPS in other environments of interest is met.

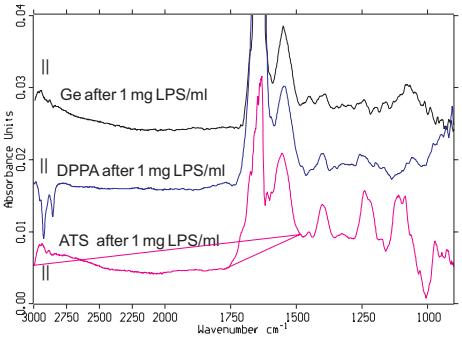

Fig.7: IR ATR absorbance spectra of LPS after contact with 1 mg LPS/ml . ATR parallel polarized light; 20 mM phosphate buffer pH 7, 100 mM NaCl. Angle of light incidence θ=45°; number of active internal reflections N=33; germanium ATR plate; temperature T=25°C.
Effect of LPS on a hydrophilic Ge Plate (*top*), on a hydrophobic DPPA monolayer (*middle*) and a positively charged crosslinked ATS-polymer (*bottom*). All surfaces have been washed with buffer before recording the spectra. In each case a great signal of amide I (at 1640 cm^{-1}) and amide II (at 1550 cm^{-1}) is seen whereas the absorbance of the phosphate and carbonyl groups of the endotoxin sugars (1000-1300 cm^{-1}) exhibits the following order: ATS > Ge >DPPA. DPPA is even destabilized by LPS, as can be seen by the decrease of the ν(CH) vibrations (2800-3000 cm^{-1}).

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Correspondence: Gerald Reiter (gerald.reiter @univie.ac.at)