Lateral organization of bacterial model membranes

Jakub Kubiak
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MEMPHYS - Center for Biomembrane Physics
Department of Physics and Chemistry
University of Southern Denmark
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Lateral organization of bacterial model membranes

by

Jakub Kubiak

Supervised by:
Prof. Luis A. Bagatolli

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Preface

This thesis is submitted in partial fulfillment of the requirements for obtaining the Ph.D. Degree in physics at the University of Southern Denmark. It contains work in the field of membrane biophysics, with focus on the bacterial membrane models. This work has been conducted at MEMPHYS - Center for Biomembrane Physics, Department of Physics and Chemistry, University of Southern Denmark, in the period from January 2008 to December 2010. Part of the work was carried out in the Department of Cancer and Inflammation Research, University of Southern Denmark. The work was supervised by Professor Luis Bagatolli from the Membrane Biophysics and Biophotonics group at the Department of Biochemistry and Molecular Biology associated with MEMPHYS.

Part of this thesis is based on the following manuscript:

“Lipid lateral organization on giant unilamellar vesicles containing lipopolysaccharides”  
Jakub Kubiak, Jonathan Brewer, Søren Hansen, Luis A. Bagatolli.
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Abstract

Gram-negative bacteria are enveloped with a double membrane system. While the inner membrane is enriched in proteins (up to 70% of the membrane weight) and is similar in character to the mitochondrial inner membrane, the outer bacterial membrane has a very different structure. The special character of the bacterial outer membrane is partly due to its almost complete asymmetry and the presence of peculiar lipid species: lipopolysaccharide (LPS), in the outer leaflet [1]. The outer bacterial membrane is the barrier, which separates the bacterial cells from their environment and protects them against stress factors, e.g., antimicrobial agents. The outer membranes and LPS were studied in aspects of their transport/barrier functions [1], their structure and thermodynamic phases [2, 3], and their interactions with mammal immune system [4]. The characterization of the bacterial outer membrane and the development of the model systems are vital for studying the action of known therapeutic agents and developing new ones.

The focus of this thesis is the preparation of LPS-containing model membranes and the characterization of LPS phase and aggregation properties in these models. Within this work, a new protocol to generate giant unilamellar vesicles (GUVs) composed of mixtures of a single LPS chemotype and Escherichia coli polar lipid extracts was developed and characterized. The impact of the size of LPS polysaccharide group and the LPS concentration on membrane incorporation of LPS and lateral structure of the model membrane system was evaluated. The model membranes were characterized using LAURDAN GP 2-photon microscopy and FCS; the results show the demixing of LPS and E. coli lipids, and formation of LPS clusters, which size depends on the polysaccharide group. The studies suggest significant difference between smooth or Ra and deep rough LPS chemotypes, in the incorporation rates and the lateral structure. Moreover, in case of lipid A and deep rough chemotypes, the presence of the LPS enriched micrometer-size domains in a gel-like phase was observed. For smooth and Ra LPS chemotypes, containing the more bulky polar head group, the absence of micrometer sized domains was observed for all LPS concentration explored in the GUVs (up to ~15 mol%). However, fluorescence correlation spectroscopy (using fluorescently labeled LPS) and LAURDAN GP experiments in these microscopically homogeneous membranes suggest the presence of LPS clusters of a size below the optical resolution of our microscopy system. The
method to generate GUVs, composed of native inner bacterial membranes (inner bacterial membrane vesicles) was developed, but the full evaluation of the system has not been completed.

In addition, the selection of LPS and LPS-containing liposomes was chosen to characterize the binding of LPS by a surfactant protein D (SP-D), which is a part of the innate immune system. Different binding to various LPS chemotypes, both free and incorporated into the vesicles, was observed, in agreement with previously published results [5]. The LPS-containing vesicles are particularly useful models to study the binding efficiency, the aggregation potency, and the possible membrane permeabilization effects. The study presented in this thesis contributes to a better understanding of the behavior of the LPS in both, bacterial and model membranes. This thesis provides also a new model for studying LPS, and interactions of the immune systems components with LPS-containing membrane models.
Dansk resumé


Fokus af denne afhandling er forberedelsen af modelmembraner indeholdende LPS og karakteriseringen af fase- og aggregeringsegenskaber for LPS i disse modeller. Arbejdet indeholder udviklingen samt karakteriseringen af en ny protokol til at lave gigantiske unilamelle vesikler (GUVer) bestående af blandinger af en enkelt LPS kemotype og polære Escherichia coli lipidekstrakter. Betydningen af hhv. størrelsen af polysaccharidgruppen i LPS og LPS koncentrationen for inkorporeringen af LPS i membraner og for den laterale struktur af modelmembransystemet er vurderet. Modelmembranerne er karakteriseret ved brug af LAURDAN GP 2-foton mikroskopi og FCS; resultaterne viser adskillelse af LPS og E.coli-lipider samt dannelse af LPS-klynger, hvis størrelse afhænger af polysaccharidgruppen. Studierne foreslår en signifikant forskel i inkorporeringshastighed samt i lateral struktur mellem glat (smooth) eller Ra og dybt ru (deep rough) LPS kemotyper. I tilfælde med lipid A og dybt ru kemotyper blev tilstedevedrørelsen af LPS-berigede mikrometer domæner i en gel-lignende fase desuden observeret. For glat eller Ra LPS kemotyper, der indeholder mere pladskrævende polære hovedgrupper, blev fraværet af mikrometer domæner observeret for alle LPS koncentrationer undersøgt i GUVerne (optil ~15 mol%). Fluorescence korrelationsspektroskopi (ved brug af fluorescenceemærket LPS) and LAURDAN GP eksperimenter i disse mikroskopisk homogene membraner foreslår imidlertid tilstedevedrørelsen
af LPS-klynger mindre end den optiske opløsning af vores mikroskopisystem. En metode, til at generere GUVer betående af native indre bakteriemembraner, blev udviklet, men fuld evaluering af systemet er endnu ikke afsluttet.

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I. FUNDAMENTALS
Chapter 1. Introduction

1.1. Membranes

Membranes are vital structures of all living organisms. They are present in certain types of viruses, in prokaryotic and eukaryotic cells. In fact, there would not be any life without membranes. These structures separate not only all the cell content from the surrounding media, but also compartmentalize the cell. Membranes act as very selective barriers between external environment and components of the cellular metabolism [6], i.e., complex sets of chemical reactions catalyzed by different enzymes, which in turn requires proper environmental conditions like, different ion activities (e.g., $H^+$, $Ca^{2+}$), and presence of different substrates to function properly. Membranes also protect cells from unfavorable external conditions, e.g., toxic compounds or other chemical or physical stress factors [6].

Despite having a cell plasma membrane which separates them from their surroundings, eukaryotic cells contain a set of compartments delimited by membranes, i.e., the nucleus, mitochondria, lysosomes, etc. Each of those compartments, called organelles, are delimited with particular membranes, which separate unique conditions and content to allow simultaneous processes to occur in the same cell.

Membranes are not static barriers; they are dynamic supramolecular entities, optimized to perform particular functions during life processes. Membranes control the communication between the external medium and the inner environment. They are able to sense and respond to external signals, starting reaction cascades inside the cell. In certain conditions these phenomena can cause a global cellular response like apoptosis [7]. Membranes are important actors in the transport of small molecules like ions to maintain proper chemical and electrical gradients, which is crucial for, e.g., the energy transformation in mitochondria or nerve pulse conduction [8, 9]. Membranes can also fuse with other membranes, or form vesicles to facilitate the transport of larger molecules like lipids, sugars, or proteins [10].
1.1.1. Membrane composition

Biological membranes are supramolecular entities composed of a vast number of different components: lipids, proteins, and sugars (0-10 wt%). In the view of currently accepted models, i.e., Singer-Nicolson model (cf. section 1.2.6 and ref. [11]), lipids form bilayer, proteins are associated with the lipid bilayer in many ways, sugars are bound to lipids and proteins (cf. Fig. 1.1).

Fig. 1.1. Schematic drawing of typical plasma membrane of an eukaryotic cell. Note the presence of various lipid species marked with different colors, exhibiting some degree of non-random spacial distribution, membrane proteins with various degree of glycosylation, and underlying cytoskeleton structures. figure taken from http://www.scienceinyoureyes.com/

In different types of membranes, the proportion between those components can vary significantly, e.g., myelin, a specialized membrane existing in nerve cells, where proteins constitute 20 wt% of the total membrane, or the mitochondrial inner membrane where proteins constitute 77 wt% [12]. The membrane composition varies not only between membrane types but also depends on environmental conditions and the stage in the cell life cycle. Lipids, proteins, and sugars are synthesized, incorporated, and turned over as a response to different stimuli, e.g., chemical factors or physical parameters (such as temperature). This distinction of the membrane components into lipids and proteins has purely chemical character. Functional biological membranes are extremely diverse and complex systems in non-equilibrium and their functions require a constant cross-talk between membrane elements. Proteins and lipids modulate their action, their synthesis and turn-over [13]. In other words, biological membranes function as single supramolecular entities.
1.1.2. Membrane Proteins

The human genome encodes approx. 10 000 proteins that were identified as a membrane associated [14]. The variety of membrane associated proteins in other organisms is similarly rich. The presence and number of particular protein types depends on the cell and membrane type and is continuously being adjusted to changes in the environmental conditions, to perform specific biological activities. This last fact gives great variety in membrane composition, physical and chemical properties, and function. Membrane proteins are associated with membranes in many ways. The association can be stable or transient; the protein interaction with or within the surrounding membrane is regulated by electrostatic interactions, hydrophobic effect, or combination of these. Some membrane proteins, called transmembrane proteins, span the entire bilayer, and connect two environments on both membrane sides. Other, peripheral membrane proteins are associated with one of membrane surfaces, which is achieved in various ways: electrostatic binding to the membrane surface, anchoring by lipid molecules (fatty-acids, prenyls, glycosylphosphatidylinositol) covalently bound to the protein, binding lipid tails extended from the membrane in hydrophobic cavities in the protein, or burying the hydrophobic protein domains in the membrane [15, 16]. Membrane proteins are involved in many cellular functions. They can assist transporting small molecules, like sugars, amino-acids water or different ions (H\(^+\), K\(^+\), Ca\(^{2+}\), Mg\(^{2+}\)), through the membrane after particular stimuli. These processes use chemical energy or ion gradients to transport molecules across the membrane. Last but not least membrane proteins can function as receptors binding signaling molecules, or enzymes modifying proteins or lipid composition in in the membrane [6].

1.1.3. Membrane lipids

Lipids constitute extremely broad chemical class, which contains several hundreds of different compounds. Lipids are group of organic compounds poorly soluble or insoluble in water, but soluble in organic solvents like chloroform or methanol. Their nature can be hydrophobic (combination of Greek words hydro, which stands for water, and phobos - fear; property of molecule to be repelled from mass of water), or amphiphilic (combination of Greek words amphis – both, and philia – love, friendship; combination of two spatially
separated hydrophobic and hydrophilic s within one molecule). Lipids are divided into many classes: fatty acids, glycerolipids, waxes, prenol lipids, polyketides, steroids, phospholipids, sphingolipids, saccharolipids [17].

Biological membranes consist mainly of phospholipids, steroids (ergosterol in fungi, sitosterol in plants, and cholesterol in other eukaryotes), and sphingolipids (Fig. 1.2) [15]. Others lipids, like lipopolysaccharides, are important constituents of bacterial membranes. All these lipids have amphiphilic nature, and can be imagined as two-part molecules having hydrophilic “head” and hydrophobic “tail” (cf. Fig. 1.2). Archeabacteria use ether phospholipids, where fatty alcohols are attached to glycerol backbone by ether bonds, while classic phospholipids have fatty acids attached to glycerol by ester bond. Other lipids are present in membranes in smaller number and can play various functions.

Fig. 1.2. Chemical structures of typical lipid representatives occurring in natural membranes: A. POPC, B. POPE, C. SM, D. GM3, E. cholesterol, F. LPS Re from E. coli. Amphiphatic properties of lipids together with division on hydrophilic headgroups and hydrophobic tails are marked by showing orientation of the molecules on the water-hydrophobic interface.

Lipids perform various functions in living organisms: they serve as energy source and energy reservoir, signaling molecules, hormones and vitamins. According to classical membrane models (e.g., Singer-Nicolson model, cf. 1.2.6) lipids provide the membrane framework (hydrophobic phase separating two aqueous phases), but situation may be more complex,
particularly in membranes of high proteins content. Lipid composition of membranes is linked to membranes’ activity [15].

1.1.4. Lipids and the hydrophobic effect

The amphiphilic properties of lipids give rise to a very peculiar feature: in presence of water the amphiphilic molecules start to form supramolecular structures. Water cannot form favorable dynamic hydrogen bonds with non-polar domains of lipids. Instead, water molecules form a solvation shell, the layer of water molecules around the lipid molecule, where their movement is restricted and the entropy decrease (or what is the same the free energy of the system increase). This is unfavorable. The free energy is also partially compensated by a decrease of the enthalpy due to an increase of strength of hydrogen bonding in the solvation shell.

\[ \Delta G^0 = \Delta H^0 - T\Delta S^0 \]  

where \( \Delta G^0 \) is the standard Gibbs free energy change of the system, \( \Delta H^0 \) is the change of standard enthalpy, \( \Delta S^0 \) the change of the standard entropy and \( T \) is the temperature.

At room temperature, the entropic component largely dominates and drives the system to minimize the contact area between hydrophobic domains of lipids and water molecules. This is achieved by formation of the lipid assemblies, where the hydrophobic parts of lipids are screened from water by hydrophilic heads in case of amphiphiles, or a separation of the system into two phases to minimize the contact area in case of oils. The process described above is spontaneous, and is called hydrophobic effect [18]. This effect is crucial not only for membrane self-assembly but also for proteins folding.

1.1.5. Lipid assemblies

Lipids aggregates display polymorphic behavior. The morphology of the lipid assembly is determined by several factors, such as an overall shape of the lipid molecules, the balance between the the sizes of the hydrophobic and the hydrophilic molecular parts, and the solvent. Lipid molecules do not have a defined shape, the effective shape is an average volume shape occupied by molecules in the lipid aggregate. If the lipid molecule is characterized by the
hydrophobic volume \( (v) \), the molecule length \((l)\), and the head group area \((a)\), then the ability to form a particular structure will be reflected in the according value of a packing parameter (Fig. 1.3):

\[
P = \frac{v}{a \cdot l}
\]  

For \( \frac{v}{a \cdot l} \sim 1 \), the lipid effective shape is cylindrical and such lipid molecules will tend to form stable bilayers. \( P > 1 \) (small headgroup, bulky tail) corresponds to inverted cone shape and \( P < 1 \) (large headgroup, small tail) to normal cone. Large deviation from \( P = 1 \) will rise to formation of non-bilayer, or even non-lamellar structures. As \( P \) rises above 1, lipid molecules have tendency to form a cubic phase, an inverted hexagonal phase \( (H_{II}) \), or inverted micelles in the extreme. When \( P < 1 \), lipids favor a hexagonal phase \( (H_i) \), and forms micelles when \( P < 1/3 \) (Fig. 1.3A) [19].

The packing parameter is reflecting the molecule’s overall geometry and is dependent not only on the chemical structure of the molecule, but also on environmental conditions such as the degree of hydration, temperature, and pH. One could expect that membranes are formed mainly by lipids of cylindrical effective shape \( (P \sim 1) \), but lipids with other packing parameters are also present, e.g., cholesterol with very low \( P \) is very abundant in eukaryotic cells plasma membranes (up to 30-50 wt%).

Another feature related to the packing of lipid molecules in the bilayer (or some other phases) and to their packing parameters is the distribution of the lateral pressure (the lateral pressure profile) [21]. Uneven distribution of lateral pressure is caused by different and contrary forces acting on a different membrane depth. On the headgroups level, the pressure is positive (expanding the membrane) due to the repulsive interaction between them. The hydrophilic-hydrophobic interface is a site of a strong negative pressure (contracting the membrane), which is a result of the hydrophobic effect (cf. 1.2.3). In the hydrophobic core of the membrane, the lateral pressure becomes positive due to the entropic repulsion between fatty-acid chains (cf. Fig. 1.3 B). The magnitude of the lateral pressure in the membrane is to about 350 atm \((35.5 \cdot 10^6 \text{ Pa}) \) [15]. The presence of different and contrary forces acting on the lipid molecules (and proteins embedded in membranes) are a cause of stress which may lead to various bilayer curvatures, or can non-specifically modulate proteins activity [22]. The lateral...
pressure profile is in fact responsible for building a curvature stress and formation of non-lamellar phases.

![Lyotropic phases](image)

Fig. 1.3. A: Lyotropic phases (and their schematic structures) vs packing parameter and “molecular shape”. Note that single lipid molecules have no distinct shape, the tendency to form particular lyotropic phase (in given conditions: degree of hydration, pH, ion content) is a feature emerged from assembly of many molecules. figure redrawn after ref. [15]. B: The lateral pressure profile and its components. figure taken from ref. [20]

Change of one phase into the other due to the change in hydration upon isothermal conditions is called lyotropic mesomorphism. The non-lamellar phases as well as regions of highly curved membrane exist in living organisms, and play various functions, e.g., enhancement of membrane fusion, vesicles budding, action of some membrane proteins is proposed to be regulated by membrane curvature [23]. Many other lipids from biological membranes prefer rather cubic or inverted hexagonal than lamellar phase, e.g., 70 wt% of *E. coli* phospholipids are PE-lipids, which prefer a H$_{II}$ phase. Many natural membranes may be close to transition from a lamellar to non-lamellar structures [24]. Cells can modify their membrane properties (lateral pressure profile) by, e.g., synthesizing new lipids of an appropriate influence on the lateral pressure profile (or packing parameter). Another way to change the membrane
properties is binding of proteins or hormones, or modifying existing membrane lipids by enzymatic action of lipases: removal of one chain (by the enzyme called phospholipase A) or headgroup (phospholipase C or D) greatly changes the packing parameter of the lipid molecule [23].

### 1.1.6. Thermotropic phases

Lipid assemblies can exist at distinct level of organization in their lateral plane. When the system reach equilibrium these structures are called phases and can be solid, liquid or gas types. Different degrees of freedom come from different structural features of the material. The translational (positional) degree of freedom is linked to position of molecules in space, i.e., order, density, and later diffusion among other molecules. According to the translational degree of freedom, crystalline solid, amorphous solid, liquid, and gas phase can be distinguished.

Another degree of freedom comes from possibility to organize molecules in different directions within the assembly. This is orientational (configurational) degree of freedom, and it gives rise to so-called meso-phases, which are in between the transition from solid crystalline to liquid. Smectic phase is characterized by orientational order and some degree in translational order, i.e., distances between parallel planes are fixed like in solid crystal but are free to move within each plane, forming two-dimensional liquid. Nematic phase has no translational order as in liquid, but they have some orientational order. According to currently accepted models (i.e., Singer-Nicolson model), biological membranes have features of smectic liquid crystalline-phase, however the situation, particularly in case of membranes with high protein content or in environment of low water activity, e.g., in inner mitochondrial membrane, is not that clear. Each material have defined range of conditions such a temperature, pressure, hydration, ionic strength, where given phase can exist. The transitions between phases can be achieved by changing one or more of these conditions, e.g., phase transition induced by temperature change is called a thermotropic transition.

Main lipid structural components of membranes, e.i., phospholipids and sphingolipids, have yet another degree of freedom, which is linked to their internal structure. Phospho- and sphingolipid tails are in fact flexible fatty-acid chains (of different length and degree of
saturation), and this flexibility gives rise to internal (conformational) degrees of freedom. The transition entropy between two new phases depends on how many microstates of the system (chains conformations) are involved in this transition. Each state can be linked to a rotation of the C-C bond in the fatty-acid chain, e.i., free energy reaches minimum when so-called dihedral angle is $180^\circ$ (trans), and local minima in the gauche conformation. A chain is ordered when the distribution of these dihedral angles is shifted towards all-trans. When the gauche conformation dominates, the chain is in disordered state. The thermotropic transition between ordered and disordered phase is called main phase transition. Changes in conformational degree of freedom (ordered-disordered phase transition) during main phase transition are accompanied by changes in translational freedom of molecules. In membrane below main phase transition temperature ($T_m$), in so-called solid-ordered state, lipids are organized in a regular structure, as in solid crystalline phase, and chains are fairly ordered. The intermolecular interactions (van der Waals interactions between chains, intermolecular hydrogen bonding, and electrostatic interactions between charged lipid groups) dominate over the chains conformational freedom. As a consequence the diffusion of molecules in the membrane is largely reduced. When membrane is above $T_m$, the entropy change caused for an increase of the chains conformational freedom dominates. Molecules are free to move within the membrane, and chains have more conformational freedom. As a consequence the membrane is in liquid-disorder phase [25].

Membrane main phase transition is a cooperative process, which means that the change of state of each molecule is influenced by others. The cooperativity of this process can occur to a different extent, and depends on membrane composition (one or more compounds) and aggregate type, e.g., multilamellar vesicular aggregates show higher melting cooperativity than unilamellar structures. Biological membranes are composed of many different lipid species (of different $T_m$) and proteins, which contribute to $T_m$ in many ways (shifting and broadening of the transition). This fact makes the phase transition much more complex and much less cooperative.

Membranes composed of different lipids have different $T_m$. The transition temperature depends on the length and degree of saturation of fatty acid chains, type of the headgroup (charges and size), ionic strength and pressure. When more types of lipids of different $T_m$ are present in a membrane, it results in more complex behavior. There is no longer a single
transition, with one defined $T_{m}$, like in case of a bilayer composed of a single compound, but a range of temperatures, where the system can separates in many phases. The existence of more than one phase is linked to demixing of lipids, and a formation of domains of distinct composition and phase within the membrane. Although this phenomenon can be easily seen in simple binary or ternary lipid mixtures, which exhibit macroscopic phase separation, the situation in biological membranes is a way more complex due to the richness of molecular compounds (both protein and lipids) and the non equilibrium conditions. Macroscopic separation of domains is rarely seen in cellular membranes [14, 20, 26], although some specialized membranes like lung surfactant or skin lipids may, at particular conditions, show this phenomenon [27, 28].

The presence of cholesterol in lipid bilayers gives rise to a new, distinct phase. Cholesterol, being a rigid and hydrophobic molecule, with only small hydrophilic -OH group, preferably interacts with ordered fatty-acid chains of membrane lipids. But presence of this atypical structure of cholesterol fits better to a loose environment of disordered chains. This behavior results in formation of a new, so-called liquid-ordered phase, which is characterized by high lateral mobility and positional disorder (translational freedom), and relatively high conformational order of lipid chains [15, 29].

### 1.1.7. Membrane models

The currently most accepted model is so-called **Fluid-Mosaic Model** by Singer and Nicolson [11]. This model is seeing biological membranes as lipid bilayers (layers of lipids, two-molecules thick) with proteins associated in various ways, with sugars bound to both proteins and lipids. The Singer-Nicolson model postulates that biological membranes are fluid lipid bilayers (two-dimensional liquid), with two types of protein associated: integral membrane proteins, which traverse the membrane and is bound to it due to hydrophobic forces, and peripheral membrane proteins, interacting with bilayer trough electrostatic interactions or hydrogen bonding. In this model proteins are mobile, and can diffuse trough the bilayer in order to perform their functions (Fig. 1.4).
The Singer-Nicolson model is very general and does not provide much information of how the membranes function, except stressing the fluidity, and so, the dynamics in a membrane structure. It recreates well the situation in model membranes, such as liposomes (proteoliposomes, giant vesicles), black lipid membranes, or supported membranes, but it misses the complexity of natural membranes (proteins crowding, water activity, curvature).

The Fluid-Mosaic Model has been refined to include more biological membrane features. Jain and White proposed a model with a clustering of membrane lipids into domains, to incorporate findings from calorimetric experiments done on both biological and synthetic membranes [30]. A three-dimensional structure of membrane (curvature, thickness variations), a pore formation, an adjustment of proteins and lipids to each other, leading to membrane heterogeneity was incorporated in the Israelachvili model [31]. Another refinement of the Singer-Nicolson model, done by Sackmann, stressed an importance of structures underlying membranes, e.g., cytoskeleton and extracellular matrix [13].

Membranes, being dynamic structures, are not randomly organized. There are evidences that lipids and proteins can be organized in clusters or domains of different size and lifetimes [32]. A lipid sorting into different phases followed by a sorting of proteins was a key concept of a raft hypothesis introduced by Simons and van Meer [33]. The raft model postulates the existence of lipid/protein domains, so-called lipid rafts, composed of sphingolipids, saturated
phospholipids, cholesterol and GPI anchor proteins. Rafts are claimed (but not probed) to be in liquid-ordered phase, in contrast to liquid-disordered surrounding, and carry various functions: sorting molecules for transport, gathering proteins together and facilitating their interactions, etc. [34]. The segregation of lipids was suggested to be driven by difference in lipids $T_{m}$, as in model systems [26]. Details of the raft model, e.g., size, lifetime, and mechanism of their functions, are under vivid debate, especially because most of experimental evidences are indirect, and some of them are questioned, like detergent lipid extraction [20, 35]. An alternative mechanism of integral membrane proteins and lipids clustering was proposed to be facilitated by hydrophobic mismatch. According to so-called *Mattress Model*, the proteins of particular length of hydrophobic segments prefer the region of membrane of appropriate thickness, which may lead to proteins and lipids sorting [36]. Another source in membrane lateral heterogeneity can be assign to interactions with extracellular matrix or cytoskeleton, membrane curvature, binding proteins or signaling molecules [35, 37, 38].

To summarize, the biological membranes are very diverse and complex systems, and they work as single supramolecular entities. The cross-talk between membrane components is crucial for their functions. Membranes may display organization on many length- and timescales. Lateral heterogeneities (domains) can be induced by membrane composition, interaction with cytoskeleton, etc. and facilitate membrane activity due to segregation/grouping of lipids and proteins.
1.2. Bacterial membranes

Bacteria are a group of single-celled, prokaryotic organisms. Bacteria, unlike eukaryotic cells, have no nucleus, or other organelles. The only membrane present in bacterial cells is a plasma membrane (can be single or double). While lacking membrane based organelles, e.g., nucleus, mitochondria, chloroplasts, Golgi apparatus, and endoplasmic reticulum, bacteria have intracellular organization based on protein structures [39, 40]. Reactions important for energy generation such as an electron transport during oxidative phosphorylation or photosynthesis are localized on plasma membrane, which can be highly folded, occupying much of cell volume [40]. Considering a cell membrane structure, bacteria can be divided into two groups: Gram-positive and Gram-negative, according to results of so-called Gram staining [41].

1.2.1. Gram-positive bacteria membrane

Gram-positive bacteria cells are enclosed in a membranous system, consisting of a cytoplasmic membrane, made of lipids, proteins, and lipoteichoic acid, and a thick (20-80 nm) layer of peptidoglycan facing out the cell. Lipoteichoic acid is a branched polyol phosphate polymer, with either ribitol or glycerol linked by phosphodiester bonds (teichoic acid), anchored in the outer leaflet of the plasma membrane via PG, and covalently bound to peptidoglycan [42]. Peptidoglycan (murein) is a polymer composed of two alternating sugars (N-acetylglucosamine and N-acetylmuramic acid) and short amino acid chains (4-5 residues, including D-amino acids) attached to N-acetylmuramic acid. Sugars form long chains linked in between by peptides. Peptidoglycan forms a dense mesh enclosing bacterial cell, and only small (up to approx. 2 nm) molecules can pass trough it [43]. Peptidoglycan provides a mechanical protection against various stress factors, such as osmotic stress. In specific cases, such as rod-shape bacteria, peptidoglycan determines their cells shape. Peptidoglycan and lipoteichoic acid are responsible for cation homeostasis (ion traps), trafficking of ions, nutrients, proteins, and antibiotics, regulation of autolysins, and presentation of envelope proteins [42]. Gram-positive bacteria, as well as Gram-negative, can have additional layer of peptides enveloping the cell. The S-layer (surface layer) is a planar, crystaline layer composed of identical proteins or glycoproteins. S-layer functions include acting as protective coats, molecular sieves, ion traps and structures involved in cell surface interactions [40, 44].
1.2.2. Gram-negative bacteria membranes

Gram-negative bacteria cells have, unlike Gram-positive, a double membrane. The inner (cytoplasmic) membrane (IM) is similar to the cell membrane of Gram-positive bacteria; both membranes contain proteins and lipids in approx. 3-to-1 weight ratio, which is close to the mitochondrial IM. Integral membrane proteins have most often various number of α-helical transmembrane domains, lipids are mainly principal phospholipids (PE, PG, cardiolipin). Bacterial membranes, with few exceptions, do not contain steroids because bacteria lack the enzymes required for steroid biosynthesis. Gram-negative bacterial membranes have no lipoteichoic acid. Both Gram-positive bacteria membrane and Gram-negative IM perform transport functions, similar to the mitochondrial IM, and some biosynthetic functions, characteristic for other eukaryotic organelles. These membranes are sites for active transport, respiratory chain components, energy transduction system, proton pumps, lipids (phospholipids, peptidoglycan, LPS) biosynthesis [45].

Fig. 1.5. Molecular structures of phospholipids of Gram-negative bacteria: A. PE, B. PG, C. Cardiolipin (predominant species in *E. coli* lipid extract according to Avanti Polar Lipids). The composition of these compounds varies depending on growth conditions: 81/17/2 mol% at 17°C, 79/15/6 mol% at 27°C, and 79/17/4 mol % at 37°C, for PE, PG, and CA, respectively [46].

Inner and outer bacterial membranes are separated by a space called a periplasm (periplasmic space). Periplasm contain a layer of peptidoglycan, looser and much thinner (only 7-8 nm) than in Gram-positive bacteria. Peptidoglycan is non-covalently linked to the outer membrane.
via Braun's lipoprotein. Besides peptidoglycan, periplasm contains binding proteins for sugars, amino acid, vitamins, and inorganic ions, which facilitate acquiring from the environment and membrane transport of those compounds. Periplasm holds also hydrolitic enzymes, including β-lactamases (degrading, e.g., penicillin) [45].

![Diagram of Gram-negative bacterial membrane](image)

Fig. 1.6. Drawing represents the structure of Gram-negative bacterial membrane. e.g. E. coli. Important components, such as IM and OM, LPS (exclusively in the outer leaflet of IM, facing external medium), porins (in IM), and peptidoglycan in periplasmic space, are marked on the scheme. *figure adopted from ref.* [47]

The outer membrane (OM) is asymmetric, i.e., the inner leaflet contains only phospholipids (PE/PG/cardiolipin), and the outer leaflet of the bilayer consists almost exclusively LPS [48]. The degree of asymmetry varies between bacterial species [1]. Besides lipid components, the outer membrane contains proteins, mainly so-called porins. Porins have transmembrane domain of peculiar, β-barrel fold, which forms channel allowing the small (up to 600-700 Da), hydrophilic molecules, e.g., nutrients to pass freely through the OM. Porins are generally non-specific, some of them exhibit only small specificity, e.g., for different sizes, or anions over cations. There is also set of channels, specific for maltose (and other sugars), nucleosides, and other compounds vital for bacteria metabolism. There is no energy-traducing system in the OM, all processes, which active transport, like peptides or proteins secretion, use large multi-component protein complexes, connecting OM with IM, and being driven by energy from the IM. The main function of the OM is being a selective permeation barrier. The barrier function is achieved by exclusive presence of LPS in outer leaflet of the OM. LPS forms hydrophilic envelope, which prevents lipophilic compounds to pass through the membrane. Due to the bridging effect of multivalent cations on the negatively charged LPS inner core and the presence many (5-7, cf. 1.3.1) fatty-acid chains per LPS molecule, the layer of LPS molecules
is speculated to be in gel–like phase. All together, these features make LPS membrane an excellent permeability barrier [1,49]. Porins are another part of this system, they allow nutrients and other small molecules to pass, but larger hydrophilic molecules, which could be toxic, cannot enter the cell [1].
1.3. Lipopolysaccharide

LPS is a very distinctive molecule, characteristic for outer leaflet of OM of Gram-negative bacteria. It is an amphiphilic molecule, anchored in membrane by its lipophilic part, called lipid A, and presenting to the environment a poly- or an oligosaccharide group.

1.3.1. Lipid A

Lipid A from *E. coli* has β-1',6-linked glucosamine disaccharide backbone substituted in 2, 2' (amide link), 3, and 3' (ester link) positions by (R)-3-hydroxymyristic acid residues, and those which are in 2' and 3' positions are further acylated on their hydroxyl groups, also by (R)-3-hydroxy acid, forming acyloxyacyl structures. Positions 1 and 4' of glucosamine are phosphorylated, and polysaccharide group is attached in 6' position (cf. Fig. 1.7). The number and length of fatty-acid chains substituting glucosamine backbone can vary, and it depends on bacteria species and growth conditions, e.g., pH, temperature, Mg$^{2+}$ concentration, presence of cationic peptides. Heterogeneity in the glucosamine acylation within one bacterial species may be also a result of underacylation, caused by defects in fatty-acid-transferases, or post-synthesis degradation [50].

![Fig. 1.7. Molecular structure of LPS Re from *E. coli*. The length of fatty-acids and numbering scheme of glucosamine backbone are marked. The polysaccharide chain can be attached to 5th position of first Kdo unit (upper left sugar unit) by 1→5 bond.](image)
Although the glucosamine disaccharide backbone is most spread among bacterial species, some of bacteria have lipid A containing other saccharides, e.g., 2,3-diamino-2,3-dideoxy-D-glucose (Campylobacter)[51]. The most common number of fatty-acid in lipid A is 5 to 6, and their length is usually C\textsubscript{10}-C\textsubscript{16} (E. coli lipid A: hexaacylated by C\textsubscript{14} and C\textsubscript{12}, Salmonella lipid A: heptaacylated by C\textsubscript{14} and C\textsubscript{16}). Longer fatty-acid chains exist in other bacterial species, e.g., C\textsubscript{18} (Helicobacter pylori), C\textsubscript{18} and C\textsubscript{21} (Chlamydia trachomatis), or C\textsubscript{28} with β-hydroxybutyrate substituent at C-27 position (Rhizobium eli). Oxo-fatty-acids were also found in few species (Rhodobacter capsulatus), as well as unsaturated fatty-acids (Rhodopseudomonas), or methyl-branched fatty-acids (Porphyromonas) [50]. The number and the length of fatty-acids have effect on lipid A toxicity: 6 fatty-acids are optimal for toxicity; C\textsubscript{12}, C\textsubscript{12}OH, C\textsubscript{14}, and C\textsubscript{14}OH fatty-acids are found to be present in the most toxic lipid A [52]. Lipid A from E. coli is phosphorylated at 1 and 4' positions. This is most common modification of bacterial lipid A, but other modifications also occur (pyrophosphate, neutral or negatively charged sugar units). Similar modifications of saccharide unit occur within the inner core region of LPS (cf. next ) [50]. The accumulation of acidic chemical groups within the LPS molecule and presence of divalent cations (Mg\textsuperscript{2+}, Ca\textsuperscript{2+}) stabilizes the membrane structure due to a bridging effect, which greatly impairs the diffusion of (both hydrophilic and hydrophobic) molecules through the OM [1]. In some cases (Salmonella minnesota Re 595), negatively charged groups are neutralized by addition of, e.g., phosphorylethanolamine or glucosamine. The neutralization of the charge may lead to a decrease in LPS membrane stability, but may also give an advantage in the bacterial defense against cationic antibiotic [50].

1.3.2. Polysaccharide

The polysaccharide region of LPS can be divided into two structurally distinctive parts: the core region, which is linked to lipid A, and exposed to the environment O-specific chain (cf. Fig. 1.8). The polysaccharide region is highly heterogeneous, on both, core and O-specific chain level. It differs greatly not only between bacterial species and strains but also shows heterogeneity within single bacterial culture.
Bacterial LPS cores consist of 9-12 sugar units. The saccharide at the reducing end, which is linked to 6′ position of glucosamine of lipid A backbone, is α-3-deoxy-D-manno-oct-2-ulosonic acid (2-keto-3-deoxyoctulosonic acid – Kdo). This 2→6 bond is very sensitive even to mild-acid hydrolysis. There may be one or two further Kdo units (2→4)-linked to the first Kdo [50], substituted further by other saccharide unit. In case of very few non-enteric bacterial species, e.g., *Yersinia pestis*, Kdo is replaced with D-glycero-D-talo-2-ulosonic acid (Ko) [54]. The next sugars in the core region are L-glycero-α-D-manno-heptopyranose (L-α-D-Hep) (1→5)-linked to the first Kdo unit and two further L-α-D-Hep, (1→3)- and (1→7)-linked. This part of LPS core is called an inner core region, and can be substituted by phosphate, pyrophosphate, or phosphoethanolamine. The outer core of enteric LPS is (1→3)-linked to the second L-α-D-Hep from the inner core, and consist of 3-6 sugar units, mainly glucose and galactose, or their derivatives [53]. Non-enteric bacterial species and strains can have cores of different number and kind of sugar units [50]. The heterogeneity in LPS cores is caused by an incomplete synthesis and by a partial biodegradation of the complete product. Another source of the core heterogeneity, found in bacteria, which lack O-specific chain, are changes in genes encoding enzymes responsible for LPS synthesis. This kind of heterogeneity
is called phase variation, and it results in additions to the core of some atypical for bacteria sugar units, like sialic acid, which can improve a bacterial invasiveness [55].

O-specific chains are that part of LPS polysaccharide group that are exposed to the bacterial extracellular space. They consist of up to 50 identical subunits, each composed of 1-8 monosaccharides, as well as non-carbohydrate compounds. The number of subunits varies within single bacterial cell and a presence of molecules with different number of unit repeats give raise to characteristic ladder patter on SDS electrophoresis gels. The saccharide composition (more than 60 monosaccharides), the structure variations (linear or branched), and modifications like phosphorylation, glycosylation, or acetylation, give rise to a great richness of O-specific chains. There is approx. 170, so called, \textit{E. coli} O serotypes, and 46 \textit{Salmonella enterica} serogroups. There can be more than one O-specific chain type in single cell [56]. The main role of the O-specific chain is to protect bacterial cell from toxic agents like antibiotics, antimicrobial peptides by shielding the cell surface. In some bacteria species, the O-specific chains are responsible for adhesion (\textit{Actinobacillus pleuropneumoniae}) [50], they can also mimic eukaryotic cell antigens to escape from immune system detection in case of some pathogens (\textit{Helicbacter pylori}) [51]. Bacterial strains, which contain the O-specific chains are called “smooth” strains. There is also number of bacterial species or strains naturally lacking the O-specific chains (\textit{Haemophilus}, \textit{Neisseria}, \textit{Campylobacter}) but still able to survive in the environment. The lack of the O-specific chain can even help bacteria, e.g., to invade mucosal tissue [50]. Bacterial strains, which lack the O-specific chains are called “rough” strains.

The absence of the O-specific chain is caused by a dysfunction in enzymes responsible for the synthesis of the O-specific chain. Knocking out of enzymes responsible for synthesis of the LPS core region gave rise to set of new laboratory strains, generating so called “deep rough” LPS. The naming convention is that the LPS with a full core is called Ra, and a shortening of the core comes with following letters, i.e., Rb, Rc, Rd1, Rd2, up to Re. LPS Re contains lipid A and only two Kdo units (cf. Fig. 1.8), and it is minimal structure required for \textit{E. coli} growth. Deep rough strains are laboratory strains, and they require special growth conditions, due to increased susceptibility to toxic agents (hydrophobic antibiotics, antimicrobial peptides, immune system) [56].
1.3.3. LPS biosynthesis

All LPS components are synthesized on the inner surface (or in the inner leaflet) of IM. In E. coli, where LPS biosynthesis pathway is best recognized, the first step is the acylation of sugar nucleotide 1-UDP-N-acetyloglucosamine at position 3 by acyltransferase called LpxA, which shows specificity for β-hydroxymyristic acid. The second step is a deacetylation by acyltransferase LpxC. After deacetylation, the second β-hydroxymyristate is attached in position 2 by LpxD. The product's pyrophosphate bond between glucosamine and UDP is cleaved by pyrophosphatase LpxH resulting formation of so called lipid X (2,3-diacylglucosamine-1-phosphate). Lipid X is condensed with another 1-UDP-2,3-diacylglucosamine by synthase LpxB through β-1',6 linkage. The condensation product (lipid IV₅) is phosphorylated at 4' position by LpxK kinase. Two Kdo units are transferred on lipid IV₅ by multifunctional enzyme WaaZ. Two acylations of β-hydroxy-myristates require presence of Kdo units and are done by acyltransferases LpxL abd LpxM. The LPS core polysaccharide is assembled also on the inner side of the IM. It is done by sequential transfer of sugar units from their nucleotide precursors to the lipid A-Kdo₂ acceptor. The transfer is assumed to be facilitated by the IM associated complex of glucotransferases encoded by genes in waa gene locus. Mutations in waa genes cause deep rough phenotypes [56]. Biosynthesis of the O-specific chain also takes place on the inner side of IM. Again, it is sequential transfer of sugar units, performed mainly by glucotransferases encoded in rfb gene locus. However, the acceptor is not rough LPS but undecaprenyl-phosphate, C₅₅-isoprenoid alcohol, used also for synthesis of peptidoglycan and capsular polysaccharide [56]. The ready rough LPS is translocated across the IM, to its outer leaflet by ABC transporter MsbA. The undecaprenyl-phosphate-O-specific chain is translocated independently from rough LPS by Wzx flippase, or, in case of some E. coli strains, by Wzm-Wzt ABC transporter. Rough LPS and O-specific chain are linked on the periplasmic face of the IM by WaaL ligase. Ready smooth LPS (or rough LPS in case of rough mutants) is transported to the outer leaflet of the OM by Lpt pathway. First, the LPS molecule is extracted from the IM by ABC transporter composed of LptB, LptF, LptG, and probably LptC. The transport through the periplasm and assembly in the outer surface of the OM require the periplasmic LptA protein, and two OM proteins: lipoprotein LptE and β-barrel folded LptB [57].
1.3.4. Impact of LPS on innate immunity

LPS can be released from bacterial membrane during cell division, after cell lysis, or cell death. It can be also extracted from bacterial cells by serum LPS-binding proteins. During infection, lipid A portion of LPS can be detected by innate immune system if the host even at picomolar concentrations. The main receptor responsible for lipid A detection is the TLR-4 (toll-like receptor–4). TLR-4 is present on macrophages and endothelial cells. The activation of the macrophages via TLR-4 causes synthesis of pro-inflammatory mediators, like TNF-α or IL1-β, and promotion of the adaptive immune response. In endothelial cells, the activation caused by lipid A/LPS stimulates the production of tissue factors. This system is responsible for a clearing the infection, but sometimes, the over-stimulation can lead to a septic shock, a blood vessels damage, and an organ failure [56, 58].
1.4. SP-D

Pulmonary surfactant-associated protein D (SP-D) is, together with related SP-A, a member of a protein group called collectins. Collectins are calcium-dependent lectins (sugar binding proteins) containing collagenous and C-type lectin domains [59].

1.4.1. SP-D structure

SP-D has a molecular weight of 43 kDa, and consists of four structural domains. The short, N-terminal domain contains two conserved cysteins, which can form inter-subunit disulfide bonds, what determines higher-order oligomerization. The long collagenous domain with conserved Gly-X-Y repeats influences protein oligomerization state and the spacial separation of lectin domains. The collagen domain is glycosylated with a single N-linked oligosaccharide. The α-helical, short neck-domain is responsible for spontaneous trimerization of SP-D monomers into the SP-D subunit by a formation of super-helical structure, and plays some role in the spatial organization of lectin domains in the SP-D trimer. The most important for the protein function is C-terminal C-type lectin carbohydrates recognition domain (CRD). Presence of at least two Ca\(^{2+}\) ions in CRD is required for glucose/mannose type recognition and binding. CRD of SP-D can bind simple or complex sugars (mannose, glucose, inositol), phosphatidylinositol and glucosylceramide, using both their head-group and lipid tails, as well as fatty acids, like palmitate or 3-hydroxy-myristate [5, 60]. SP-D is most often organized in dodecamers, where four trimeric subunits are interconnected by their N-terminus (cf. Fig. 1.9).

Although the dodecameric form of SP-D is most ubiquitous, the trimers and higher-order multimers can be also present. Monomers form tangent trimeric structure crucial for ligand recognition, which allows the interaction of up to three sites on the ligand (trimer of CRD domains have approx. 10-fold greater binding affinity for multivalent ligands than monomeric CRD) [5, 60].
1.4.2. SP-D expression

The major site of SP-D synthesis and secretion is epithelium of respiratory tracks. SP-D is secreted by alveolar type II cells in alveoli, and by non-ciliated Clara cells of bronchial epithelium. Some species synthesises SP-D with epithelial cells or submucosal glands of bronchi and trachea. The synthesis of SP-D seems to be localised mainly in epithelial cells, that are in contact with environment (eustachian tube, lachrimal glands, gastrointestinal and genitourinary tracks, skin), but the SP-D expression was found also in heart, brain, pancreatic islets, and testicular Leyding cells [60, 62].

1.4.3. SP-D function

The main function of collectins in general, and SP-D in particular, is innate host defence against pathogens and organic antigens in lungs through the interactions with the pathogens glycoconjugates. By interactions with macrophages, neutrophiles, and lymphocytes, SP-D participates in immune and inflammatory regulations. Mice deficient in SP-D have decreased resistance against pathogens. Also interactions of some pathogens, such as Klebsiella pneumoniae or influenza A virus, with SP-D correlates inversely with their virulence. SP-D also participates in lipid turnover in lung surfactant. SP-D interacts with small vesicular form of lung surfactant via binding the headgroup of phosphatidylinositol or glucosylerceramide, facilitating surfactant re-uptake by alveolar epithelial cells [60].
1.4.4. Mechanism of SP-D interactions with ligands

SP-D binds to many pathogens, including Gram-negative (via LPS) and Gram-positive (via lipoteichoic acid) bacteria, mycobacteria (lipoarabinomannan), and some viruses (high-mannose oligosaccharides associated with hemagglutinin of influenza A virus) [60, 63]. The interaction with pathogens is mainly via their glycoconjugates. SP-D binds mannose and mannose-rich saccharides in Ca\(^{2+}\)-dependent manner. Ca\(^{2+}\) cations stabilize structure of CRD, sitting on the bottom of sugar binding pocket calcium can neutralize acidic amino-acid residues. Calcium participates in CRD-ligand interactions forming ternary complex of protein, calcium, and carbohydrate [64]. Besides mannose, SP-D can recognize other saccharides, such as glucose, fucose, N-acetyl-glucosamine, N-acetylo-mannosamine, and inositol [5]. Binding of pathogen carbohydrates depends on availability of preferred sugar residues. SP-D binds membrane bound and isolated LPS at its core region, which contains glucose and heptose. The successful binding requires accessibility of SP-D-favored sugar residues, and SP-D prefers binding to LPS with short or no O-specific chain (rough LPS strains) [60]. SP-D can also bind smooth LPS of some Klebsiella serotypes due to the presence of di-mannose in the O-specific antigen [65].

1.4.5. Impact of binding LPS by SP-D

The impact of SP-D binding to bacteria is not entirely clear. The opsonization by SP-D can cause aggregation of bacteria [66] and/or promote uptake by neutrophiles [67]. The SP-D binding to Pseudomonas aeruginosa enhances the internalization of bacterial cell by alveolar macrophages, however, without bacterial aggregation [68]. In case of some phase variants of Klebsiella pneumoniae, SP-D binding promotes killing and internalization of bacterial cells, with various degree of bacteria aggregation, but larger bacterial aggregates cannot be internalized by macrophages [65]. In addition to promotion of the bacterial aggregation and recognition by immune cells, SP-D was suggested to directly kill bacteria by destabilization and permeabilization the bacterial membrane. POPE-liposomes containing deep rough LPS were partially permeabilized upon addition of SP-D. The mechanism of this hypothetical permeabilization is not known, it was suggested that SP-D may perturb bacterial membrane [69]. SP-D can also bind soluble LPS. Soluble LPS, released from bacterial cells, may cause
over-reaction of immune system, which may lead to very severe consequences (cf. 1.3.4). SP-D was suggested to play protective role in the host response to the endotoxin. SP-D can bind free LPS in respiratory tracts, and such complexes are internalized by alveolar macrophages [70]. Moreover, SP-D deficient mice develop more intense inflammatory reaction in response to intratracheal administration of LPS [71].
Chapter 2.
Fundamentals of fluorescence techniques

2.1. Fluorescence

2.1.1. Principles of fluorescence phenomenon

The phenomenon of the light emission by a molecule upon the earlier excitation of its electronic state by a light is called luminescence. Luminescence can be divided into two formal categories, depending on the nature of excited states (and time in which the phenomenon occurs): fluorescence and phosphorescence. In fluorescence, the excited state is a singlet state, which means that the electrons on a ground state orbital and electron on an excited state orbital are paired (have opposite spins). The transition from an excited state to the ground state is then favorable (typical rates of $10^8 \text{ s}^{-1}$) and fast. Typical fluorescence lifetimes are on the order of $10^{-10}$ to $10^{-8}$ s. Phosphorescent emission of light occurs from an excited triplet state and as a transition is forbidden. It results in much lower rates ($10^3 – 10^0 \text{ s}^{-1}$) and much longer lifetimes, on the order of milliseconds to seconds, and even up to minutes.

A good visualization of events occurring in fluorescence phenomenon is given by Jablonski diagram (cf. Fig. 2.1). The first event of fluorescence is absorption of light and excitation of the molecule from its ground state to the one of excited electronic states. This process occurs on femtosecond timescales ($10^{-15}$ s). Absorbed photon have to match the energy difference between ground state ($S_0$) and one of excited electronic states ($S_1$ or $S_2$). The excitation is typical done to the higher vibrational levels. After excitation molecules relaxes to the lowest vibrational level of $S_1$. This process is called internal conversion or vibrational relaxation and occurs on $10^{-12}$ s timescale. After thermal equilibration the molecule returns to $S_0$, emitting photon with energy matching the energy difference between states (emission). The time a molecule spends on $S_1$ ($\sim 10^9$ s) is limiting for the whole process and also for its lifetime. The return usually occurs to the higher vibrational level, and reaches the thermal equilibrium by relaxation ($10^{-12}$ s).
The emission energy is lower than the excitation energy due to thermal relaxation to lowest vibrational levels of $S_1$ and return to higher vibrational level $S_0$ (cf. Fig. 2.1). The loss of energy is the reason for a shift of emission light towards longer wavelengths (cf. Eq. 2.1) in respect to excitation light. This phenomenon is called a Stokes’ Shift. In addition, Stokes’ Shift can be enlarged by solvent effects, energy transfers, etc. The energy of a photon is given by

$$ E = h \nu = \frac{hc}{\lambda} = \frac{hck}{2\pi} \quad [2.1.] $$

$E$ is the energy of the photon, $h$ is the Planck constant, $\nu$ – the light frequency, $\lambda$ – the light wavelength, $c$ – the speed of light in vacuum, $k$ – the wavenumber ($k = \frac{2\pi}{\lambda}$).

Besides fluorescent emission, there are also alternative paths from $S_0$, i.e., phosphorescence and non-radiative relaxation (where energy is dissipated without emission of light). In phosphorescence, molecules undergo spin conversion from $S_1$ to $T_1$ (triplet state) in a process called intersystem crossing. The transition from $T_1$ to $S_0$ is forbidden, and as such has a rate several orders of magnitudes lower than fluorescence. Phosphorescence lifetimes are much longer than fluorescence, and because of an energy difference of the $T_1 \rightarrow S_0$ transition is lower than $S_1 \rightarrow S_0$, the phosphorescence light is shifted towards longer wavelengths (cf. Eq. 2.1) [72, 73].
2.1.2. Emission and excitation spectrum

Fluorescence spectra (excitation and emission) are functions of the light intensity (absorbed or emitted) as a function of the light wavelength (frequency, wavenumber). In other words, it denotes probabilities of transitions to different vibrational and electronic states. Distribution of these probabilities depends on the molecule structure (electronic structure, molecule geometry) and interaction with other molecules (solvent molecules, or neighbor molecules in crystals). When in the gas phase, fluorophores have a distinct excitation and emission peaks, but in liquids, due to interactions with solvent molecules, the spectra are smoothed because of inhomogeneous broadening of spectra. This effect is due to the existence of an almost continues set of vibrational sublevels. Only in few cases (perylene, DPH), the vibrational levels can be distinguished in liquid environment. For each molecular structure, there is a specific resonance energy for dipoles. It means, that there are specific energy levels, to which excitation is favorable. If the transition to some states, e.g., $0^{th}$ vibrational level of $S_0$ to $2^{nd}$ vibrational level of $S_1$, is the most likely to occur, due to the matching the dipole resonance, the reciprocal transition during emission ($0^{th}$ vibrational level of $S_1 \rightarrow 2^{nd}$ vibrational level of $S_0$) is also most probable. It leads to inverted symmetry in excitation and emission spectra (the mirror image rule) (cf. Fig. 2.2).

![Excitation and emission spectra](image)

Fig. 2.2. The excitation and emission spectra image rule and Stokes' shift. Wave number is proportional to light wave energy and reversely proportional to wavelength (cf. Eq. 2.1). If some transition to an excited vibrational state is favorable (according to Franck-Condon rule), the reciprocal transition is also favorable. Only vibrational energy levels with rotational broadening are shown in the figure, the broadening due to solvent interactions is not shown.

Although, the mirror image rule is generally true for most of aromatic hydrocarbons, there are many exceptions, when excited-state reactions and other processes occur, or where excitation to higher electronic states is significant. Emission spectra are independent of the excitation
wavelength, because the fluorescence emission occurs mainly after a relaxation to the lowest vibrational level of $S_1$.

### 2.1.3. Fluorescence lifetime and quantum yield

The fluorescence quantum yield and the fluorescence lifetime are values, which characterize the behavior of specific fluorescent molecular species in the given environment. The fluorescence quantum yield is the ratio of the number of photons emitted by fluorophore to the number of absorbed photons. The quantum yield ($Q$) depends on the relation between rates of fluorescent emission ($\Gamma_F$) and all other, non-radiative processes ($\Gamma_{NR}$).

$$Q = \frac{\Gamma_F}{\Gamma_F + \Gamma_{NR}} \quad [2.2]$$

Some fluorophores, like rhodamines, have quantum yields very close to 1.

The fluorescence lifetime ($\tau$) is an average time, which molecule spends in the excited state before returning to the ground state with a photon emission. The lifetimes of fluorophores depend on the rates of the de-excitation processes (radiative and non-radiative), and can range from picoseconds to hundreds of nanoseconds.

$$\tau = \frac{1}{\Gamma_F + \Gamma_{NR}} \quad [2.3]$$

When the population of fluorophores is excited by a short light pulse (in so-called time domain experiments the length of the light pulses is approx. 100 ps), the lifetime is defined as the time that takes for the excited molecules to decay to a $1/e$ fraction of the original population ($\sim 36.79\%$). The kinetics of the de-excitation is of first-order, and the decay is exponential:

$$\frac{n(t)}{n(0)} = e^{-\frac{t}{\tau}} \quad [2.4]$$

where $n$ is the number of photons emitted in time $t$ after short excitation pulse. $Q$ and $\tau$ can be
affected by temperature, pH, polarity, viscosity, hydrogen bonding, and presence of quenchers. For example, both quantities are decreased by temperature increase, because $\Gamma_{NR}$ is related to processes such as collisions with solvent molecules or intramolecular motion, which are linked to the temperature [72].

2.1.4. Multi-photon excitation

The excitation of a molecule happens, when a photon of energy equivalent to a gap between $S_0$ and $S_1$ levels is absorbed. However, this is not the only possible situation. The excitation can also appear, when two or more photons, with total energy matching the transition energy, are absorbed. This is possible, because photons of smaller energy can excite molecule to an intermediate (virtual) state ($S_i$), which has a lifetime on the order of $10^{-15}$ s. This absorption and formation of virtual state is similar to the light scattering process, especially the Raman scattering, and in general is weakly wavelength specific. In case of scattering, molecule comes back to the ground state, emitting photon in another direction, but if a second photon is absorbed within $10^{-16}$ s, the molecule can be successfully excited to the $S_1$ state (cf. Fig. 2.3).

Fig. 2.3. The Jablonski diagram of 1-photon (a) and 2-photon (b) excitation process. $S_i$ is a short-life virtual state. Due to different selection rules in 1- and 2-photon excitation processes, molecules are excited to different vibrational energy levels, but because fluorescence always occurs from lowest vibrational level of $S_1$, the emission spectra are the same.

The effect of a multi-photon excitation was predicted theoretically by Goeppert-Mayer in 1931 [74], but because of the absence of an appropriate light sources, first experimental observations were performed in 1960's, when the first lasers were developed. The need of a laser source is dictated by a very large photon flux required for the absorption of two (or more) photons within $10^{-16}$ s, and multi-photon excitation. The selection rules for transitions in
one- and two-photon excitation are different, and the same molecule is usually excited to different energy levels. The two-photon excitation spectrum is not a simple transposition of one-photon excitation spectrum, where \( \text{Abs}(\nu_{1\text{ph}}) = \text{Abs}(2\nu_{2\text{ph}}) \), but it is broader and has additional features at shorter wavelengths. Another feature of two-photon excitation is that the dependence of excitation/absorption (and therefore, the emission intensity) on the excitation light intensity is not linear, as in one-photon process, which follows the Lambert-Beer law, but depends on the square of light intensity. The two-photon excitation requires a tightly focused laser beam (typically by objective with high numerical aperture) and intense light, provided by pulsing infrared lasers. The excitation, unlike in one-photon excitation process, is localized to the small focal spot. This feature is used in a microscopy to provide an optical sectioning (cf. Fig. 2.4 and section 2.3) [75].

![Figure 2.4](image.png)

Fig. 2.4. Figure shows the difference between 1- and 2-photon excitation process. In 1-photon process, fluorophores are excited (with changing rate, depending on the excitation light intensity). But in 2-photon process only fluorophores in focus can be excited. This is because of a quadratic dependence of the fluorescence on the excitation light intensity. *Figure taken from ref. [76]*

### 2.1.5. Fluorophores

Molecules, which absorb and emit light in close UV and visible light range are called fluorophores. Organic fluorophores are usually molecules with conjugated system of double bonds, or aromatic bond systems (from simple organic molecules to fluorescent proteins). In organic fluorophores, the transitions responsible for fluorescence are between \( \sigma \) and \( \pi \) orbitals. There are also other type of fluorescent molecules, heavy atoms from the lanthanides group (Eu, Tb) are fluorescent due to transitions between \( f \) orbitals. Organic chelates of Ru and some other rare transition metals, like tri(2,2-bipyridyl)ruthenium(II), are known to be fluorescent.
Ru-based fluorophores exhibit mixed fluorescence and phosphorescence behavior, having long lifetimes of approx. 600 ns [72]. Another class of fluorophores are semiconductive nanoparticles called Quantum Dots. The Quantum Dot core is made of CdSe, shelled with ZnS, and the size of these particles is 1 to 10nm in diameter (core). They have large extinction coefficients, and can absorb any light with wavelength shorter than their emission. The emission spectra are, unlike organic probes, symmetric, and dependent on Quantum Dot core size: the larger core radius, the longer wavelengths of light can be absorbed and emitted [77].

A number of fluorescent molecules are present naturally in a living matter (intrinsic fluorescent probes). Aromatic amino-acids (tryptophan, tyrosin, phenylalanin) are excited and emit in UV range (excitation maxima in 260-280 nm range, emission in 280-350 nm range). Aromatic amino-acids are not good fluorophores (weak absorption and low quantum yield), particularly Phe, which has one benzene ring. Trp and Tyr fluorescence properties strongly depend on polarity of their surrounding. In polar environment, their emission is blue-shifted, and quantum yield and lifetime decreased significantly. These properties are widely used to study protein conformation dynamics. Also other molecules present in living organisms, like reduced nucleotides (NADH, FAD), some porphyrins, specific lipids (like carotenoids), chlorophylls, and many others, are also fluorescent [72, 78]. Besides small organic compounds, the fluorescent protein has been found. Green Fluorescent Protein (GFP) with number of derivatives (cyan – CFP, yellow – YFP, and red – RFP, named after color of light they emit) are fluorescent not due to presence of aromatic amino-acids, but specific fold (β-barrel) with post-translationally cyclized amino-acid side groups (Ser65–Tyr66–Gly67), which is the fluorescent center of the protein. Unlike aromatic amino-acids, GFP and analogs absorb and emit light in visible range, and the fluorescence spectra depend on specific amino-acid groups present in the proximity of fluorescent center [79].
Besides naturally occurring fluorescent compounds, a number of new fluorophores of various properties has been developed. Depending on their properties, fluorophores can be used for reaching various type of information, like molecules mobility (DPH, fluorescently labeled biomolecules), polarity of (local) environment (laurdan, ANS), local pH (fluorescein and derivatives), or specific ions (Ca$^{2+}$, K$^+$, Mg$^{2+}$, Cl$^-$) local concentrations, to name only some of them. Biomolecules can be labeled with fluorescent probes, and the information on their interactions can be obtained. Selection of few fluorescent probes is given in Fig. 2.5. The use of fluorescence microscopy provides additional information of spacial organization of above mentioned features [72].
2.2. Examples of fluorescence techniques

2.2.1. Fluorescence anisotropy

The phenomenon of the fluorescence anisotropy is based on the fact that fluorophores are selectively excited by linearly polarized light only for specific polarization angles. For successful excitation, the electric vector of a light wave has to be align parallel with the absorption transition momentum of the molecule, which is linked to molecular structure of the fluorophore. The actual dependence goes with cosine square of an angle between light polarization vector and the molecule transition momentum for one-photon absorption (and \( \cos^4 \) for 2-photon absorption process) (cf. Fig. 2.6).

![Absorption and transition momenta](image)

**Absorption and transition momenta**

**Photoselection of the absorption process**

- **A)** Maximum absorption
- **B)** No absorption
- **C)** Absorption \( \sim \cos^2 \theta \)

Fig. 2.6. The principles of anisotropy: (upper) Jablonski diagram of the excitation of different transition momenta in the molecule (anthracene). *figure taken from ref. [73]* (lower) The probability of excitation depends on the direction of light polarization (shown by arrow) its position in respect to the molecule transition momentum.
The fluorescence emission is also restricted to the plane of the fluorophore transition momentum. If the molecule stays immobile between excitation and emission processes, the emitted light has the same polarization as absorbed. But, as it happens in liquids, molecules tend to rotate (rotational Brownian motion) while being in the excited state, and therefore, the emission light can have new polarization, according to new orientation of the fluorophore transition momentum. The fluorescence anisotropy is a measure of the polarization of emitted light (the ratio of polarization components to the total intensity) from fluorescent molecules upon the excitation by linearly polarized light, and is given by the equation:

$$r = \frac{I_|| - I_\perp}{I_|| + 2I_\perp}$$  \[2.5.\]

where $r$ is the anisotropy, $I_||$ is an intensity of the emitted light polarized parallel to the the excitation light polarization, and $I_\perp$ is and intensity of the light polarized perpendicular to the the excitation light polarization (cf. Fig. 2.6). The fluorescence polarization ($P$) is a quantity describing the same phenomenon, and its defined as in the equation:

$$P = \frac{I_|| - I_\perp}{I_|| + I_\perp}$$  \[2.6.\]

Fig. 2.7. Scheme of typical setup to measure the anisotropy (or polarization) of the sample. The sample is excited with the linear polarized light (vertical polarization, $I_||$). Fluorescence signal is detected at 90°, ratio of polarization components: parallel to the excitation/parallel ($I_||$) and perpendicular to the excitation/horizontal ($I_\perp$), is measured.

In the light scattering phenomenon, the excited molecules have very short lifetimes ($10^{-15}$ s) and the polarization of emitted light is the same as the polarization of absorbed light ($r \rightarrow 1$).
However, in the fluorescence process, the anisotropy (fundamental anisotropy, cf. below) of $r_0 \approx 0.4$ (for 1-photon, and $r_0 \approx 0.57$ for 2-photon excitation process) already means that the absorption and emission transition momenta are parallel. This is connected to the geometry of the transition momenta in molecules. Symmetries in molecular structures result in even lower $r_0$. As it was stated above, the typical fluorescence lifetime is on the order of 1-10 ns. In non-viscous solution, the rotational correlation time of small molecules is on the order of 0.05-0.1 ns, which means, that they have enough time to rotate few times before emission. In this situation the emission is depolarized and anisotropy approaches 0. In viscous solutions, e.g., membranes or upon binding to large molecules the rotational motility can be impaired to various extents, and the emitted light is more polarized. Assuming that no other processes involved in depolarization, such as an energy transfer, are present, the connection between anisotropy and rotational diffusion is expressed by the Perrin equation (Eq. 2.7), where $r$ is the anisotropy, $r_0$ is the anisotropy in absence of rotational diffusion (fundamental anisotropy), $\tau$ is the fluorescence lifetime, $\Theta$ is the rotational correlation time of the diffusion.

$$r = \frac{r_0}{1 + (\tau/\Theta)}$$

[2.7.]

It is apparent from Eq. 2.7, that the anisotropy is useful to measure changes in rotational diffusion only when the timescale of the molecules' motion matches the fluorescence lifetime. In fact, the rotational motion of macromolecules and lipids in membranes occur on nanosecond timescale. The fluorescence anisotropy has been successfully used for studying different media local viscosity and order parameter, e.g., phase transitions in lipid membranes by DPH fluorescence, due to the different packing and different rotational mobilities in different lamellar phases. It is also a commonly used technique in studying, structure, interactions, and dynamics of macromolecules [72].

### 2.2.2. Fluorophore-solvent interaction (LAURDAN)

The solvent polarity is a quantity describing electrical interactions between solvent and solute molecules. These interactions can be of non-specific dielectric nature, or specific, like hydrogen bonding. The polarity affects processes occurring in solvent, such as chemical reaction rates and its equilibria. It also affects the properties of fluorophores, changing both
absorption and emission spectra. Such behavior is called *solvatochroism*, and can have various extent, depends on the fluorophore molecular structure. The polarity can be associated with the static dielectric constant of the medium, or by the dipole momenta of solvent molecules. There are also empirical scales, based on a solvatochorism of some specific fluorescent probes [73].

Upon the excitation of a fluorophore, electrons can be redistributed (orbital shape can change), which results in change of the molecule dipole momentum (excited state dipole momentum is larger than in the ground state). This process (intramolecular charge transfer) happens in most fluorophores, which exhibit solvatochromic behavior. The change in fluorophore dipole momentum is followed by reorganization of dipolar solvent molecules, so the free energy minimum of the system is achieved (cf. Fig. 2.8). As the consequence, the stronger dipole momenta of solvent molecules, the more energy is lost due to solvent relaxation [73].

![Fig. 2.8. The Jablonski diagram of the dipolar relaxation of the excited state. When excited dipole is larger than the ground state dipole, due to, for instance, intramolecular charge transfer, the solvent dipoles reorient along the excite dipole position. This relaxation process results in lowering the system energy. The longer a molecule lasts in excited state, the greater energy loss (and red-shift of fluorescence F'). *figure taken from ref. [73]*](image)

The example of a fluorophore exhibiting strong solvatochromic behavior is ANS (1-anilino-8-naphthalene sulfonate). ANS in polar solvent, e.g., H₂O, is almost non-fluorescent (very low quantum yield), but highly fluorescent in non-polar environment, such as hydrophobic protein cavities [80]. An another example of a fluorophore, widely used for probing the polarity in lipid membrane, is LAURDAN (6-lauryl-2-(dimethylaminonaphthalene), cf. Fig. 2.9) , the derivative of PRODAN (6-propionyl-2-(dimethylaminonaphthalene)), designed by Weber in 1979 [81]. LAURDAN has an advantage over PRODAN as a membrane probe, because of the higher partition into the hydrophobic phase. LAURDAN position in membranes is also slightly different than PRODAN's, which results in different response of these two probes to a
change in a lipid packing. LAURDAN proved to be an excellent probe for the phase behavior studies of lipid membranes [76, 82, 83]. The spectral shift of LAURDAN is independent on the composition and charge of lipids headgroups, but only on environmental polarity, which is related to penetration of water molecules into the membrane, at the level of glycerol backbone. The extent of a water presence and the extent of a dipolar relaxation of LAURDAN (accompanied by the spectral shift) fits very well the changes in membrane phase, i.e., in solid phase the amount of water molecules penetrating the membrane is minimal, and so the relaxation extent is low (emission spectrum is blue-shifted). But as the membrane melts, and becomes liquid-crystalline, the extent of hydration increase, accompanied by increased dipolar relaxation and emission spectrum shift towards longer wavelength (cf. Fig. 2.10). In order to quantify the membrane polarity using spectral shift of LAURDAN, the LAURDAN General Polarization function (GP) is used (cf. Eq. 2.8, and Fig. 2.10). The GP function is similar to the expression of an emission light polarization (cf. Eq. 2.6), but despite its name it is not related to polarization.

\[
GP = \frac{I_B - I_G}{I_B + I_G}
\]  

[2.8.]

\(I_B\) and \(I_G\) correspond to the intensities of fluorescence of the blue (\(\lambda \sim 440\) nm) and green (\(\lambda \sim 490\) nm) part of the emission spectrum at a given excitation wavelength. The LAURDAN in the membrane of liquid-crystalline phase displays low GP values (-0.1 to 0.2), while in gel phase, GP is much higher (> 0.5) [82].

Fig. 2.9. The emission spectra of LAURDAN in DLPC vesicles at various temperatures (from 0°C to 60°C, 5°C increment). The spectral shift towards longer wavelengths is due to membrane melting (transition from gel to liquid-crystalline phase), which results in an increase of the membrane hydration, and the higher dipolar relaxation of LAURDAN. figure redrawn after ref. [82]
2.3. Fluorescence microscopy

Spectroscopic techniques provide important information about different samples, but they lack spacial resolution. In order to obtain spatially resolved information, the use of microscope is required. In simple words, the microscope is a magnifying device. The origin of light microscopes dates back to the beginning of 17th Century, when the work of Anton von Leeuwenhoek, Robert Hooke, and others gave a great impact on the understanding of nature in micro-scale. The key principles of the microscope are: (i) the carrier of information (light in light microscopy, electrons in electron microscopy, etc.), (ii) contrast (various stainings in bright field microscopy, fluorescent probes in fluorescence microscopy), and (iii) magnification (limited, e.g., by the physical properties of light, or technology).

In the fluorescence microscopy, the contrast, which allows seeing features of the studied object, arises from the presence of specific staining (fluorescent probes). The advantage of the fluorescence microscope is the high contrast (between light emitting label and dark background), and the specificity of labeling. The fluorescent probes can bind specifically to lipid membranes (both non-specifically to the membrane hydrophobic core and in specific way to the given lipid species), DNA (or defined DNA sequences), even to specific proteins. This extremely useful feature is due to special tagging of fluorescent probes, which allows them to recognize molecules of interest. The other advantage of using fluorescence and fluorescent probes is their sensitivity to environmental conditions (cf. sections 2.1.5 and 2.2.2). Utilizing this feature can provide information about local conditions (pH, ion content and concentration, polarity) in defined areas of the studied objects (spatially resolved information) [75].

Fluorescence microscopy techniques have some common principles of their action. The excitation light is focused on the specimen by objective lenses. The light emitted by fluorescent probes is collected, typically by the same objective. A set of optical filters or/and monochromators separates the emitted light from the excitation light. It can also separates fluorescence of distinct probes. The signal is detected by a photosensitive device (CCD camera, photomultiplier tube, or avalanche photo-diode). There are two main techniques of imaging in fluorescence microscopy: (i) wide field microscopy, where the entire field of view is illuminated at the same time, and the image is recovered by a CCD camera, or (ii) laser
scanning microscopy (LSM), in which a laser light beam is tightly focus to a single light 
diffraction limited spot, and moves through the specimen, illuminating single points of the 
image, which then are used to built the image frame (raster scan). Fluorescence microscopy 
has inherited resolution in lateral plane (limited by light diffraction) but the optical resolution 
along optical axis (the optical sectioning) requires specially constructed microscopes. The 
optical sectioning can be obtained in two ways: (i) the use of pinholes in confocal LSM to 
block the light out of focal plane [84], and (ii) the nonlinear excitation dependence on laser 
beam intensity (quadratic in the two-photon process) in multi-photon LSM [85]. In both 
systems, the scanning in the axial direction is done by changing the position of the focal plane in respect to the sample. The image acquisition in LSM systems is slow due to a pixel-to-pixel 
scanning process (the raster scanning). An improvement in scanning speed with some 
decrease in a resolution is offered by the spinning disc confocal microscope, where the rapid 
rotation of a disc with series of pinholes rather than a single pinhole with fixed position is 
used to illuminate several pixels simultaneously. The signal in this system is detected by a 
CCD camera. The spinning disc confocal microscope, in both speed and optical resolution with an ability to do the optical sectioning is a compromise between wide field and laser 
scanning systems [86].

The multi-photon LSM has several advantages over the confocal LSM: (i) the excitation is 
exclusively limited to the small focal volume (~0.1 fL = 10^{-16} \text{ dm}^3), there is no out-of-focus 
excitation, the background fluorescence, and the out-of-focus bleaching is limited. (ii) no 
confocal spatial filter (pinhole) is required to obtain optical sectioning. (iii) the UV probes 
(classical fluorescence spectroscopy probes, such as DPH or LAURDAN) and intrinsic probes 
(NADH) can be excited with infrared laser [87, 88]. (iv) the light scattering is wavelength 
dependent, infrared light is scattered less than visible light, what allows deeper sample 
penetration [89]. The multi-photon LSM has also disadvantages: (i) expensive light sources 
(infrared lasers capable to generate ultra-short pulses (~10^{-12} \text{ s}) with high repetition rate (~100 
MHz)), (ii) the resolution of multi-photon LSM is worse than confocal LSM due to the longer 
wavelength used for excitation (cf. below) [90].

Although the fluorescence microscopy offers great possibilities to visualize specimens on 
micrometer length scales, there are some limits in obtainable optical resolution due to the wave nature of light (diffraction). The point light source can be detected only as a distribution
of light intensity spread over larger area. This distribution profile is called the point spread function (PSF) and its characteristic for a given microscopy system. The PSF size and optical resolution taken as minimal distance ($d$) between two resolvable point sources depends on the excitation wavelength in vacuum ($\lambda$) and numerical aperture of the objective ($NA$), and according to the Rayleigh criterion is:

$$d = \frac{1.22 \lambda}{NA_{\text{condenser}} + NA_{\text{objective}}}$$  \[2.9.\]

$NA = n \cos \theta$, where $n$ is refractive index of media (air, water, or oil), $\theta$ is a half of the included angle of the lens, which depends on the diameter of the lens and its focal length, and is usually not higher than 70°. The ideal lateral resolution for an objective $NA$ 1.3 is 0.16 μm for $\lambda = 488$ nm, and ~0.25 for $\lambda = 780$ nm. The use of pinhole can improve the resolution of multi-photon LSM to the values close to confocal LSM (two-photon confocal LSM). The resolution in axial direction is usually 3-4 times worse than in the lateral plane [90].

### 2.3.1. LAURDAN GP Imaging

Most of the spectroscopic measurements available for fluorometers can be performed using microscopy. Although technically more demanding, such studies provide additional information on spatial distribution of the parameter of interest. The unique properties of LAURDAN to sense the environment's polarity (described in section 2.2.3) was successfully used to study model membranes (planar membranes and giant unilamellar vesicles - GUVs) and cells [76, 91-93]. The spectral shift in the fluorescent emission of LAURDAN is detected by using a set of optical filters in front of the detectors, isolating blue (~440 nm) and green (~490 nm) components. The image of the spatial distribution of the LAURDAN GP function can be calculated using Eq. 2.8 for intensities of each pixel separately. The resulting LAURDAN GP map has optical resolution of LSM (cf. above). LAURDAN is an UV excited probe (the strongest absorption occurs between 350 and 390 nm), but the use of UV light excitation in microscopy causes severe photobleaching, interfering with reliable image acquisition. The solution for the inconvenient use of UV light is two-photon excitation and multi-photon LSM. Typically, the laser beam of 780 nm is used to efficiently excite LAURDAN. LAURDAN usually partitions similarly in both gel and liquid-crystalline phases.
in lipid membranes [87, 94] (the exception is given in chapter 7). Another feature of LAURDAN is a parallel orientation of the electronic transition momentum to the fatty-acid chains of phospholipids. The different photoselection effect (cf. section 2.2.1, Fig. 2.6 and 2.10) in gel phase, where fatty-acid chains are tightly packed and LAURDAN is strictly aligned to the membrane normal (photoselection effect is pronounced), and liquid-crystalline phase, where the LAURDAN's transition momenta are less organized (more tilted - no photoselection) can be also utilized to extract information on the lipids organization when linear polarized light is used for excitation [87, 94].

Fig. 2.10. Figure shows the effect of a photoselection in the LAURDAN excitation on the image formation (A). in GUV. At the equatorial plane the LAURDAN transition momenta are organized perpendicular to the laser beam (high excitation, depending however on the excitation light polarization). In the polar region, the LAURDAN transition momenta are organized parallel to the laser beam, and the excitation, particularly in a gel phase, is somewhat lower. figure redrawn after ref. [76] Figure (B) shows how the LAURDAN GP image is created. The formula (Eq. 2.8) is applied to each pixel of the two original frames.

The drawback of the strong photoselection effect is that obtaining reliable LAURDAN GP function data in planar membranes and polar regions of GUVs is difficult, because LAURDAN's transition momenta are organized parallel to the light beam (disregarding the excitation light polarization), especially in gel phase excitation of LAURDAN (and fluorescence signal) is very weak [95].
2.3.2. Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) is a spectroscopic (not microscopic) technique available on LSMs. In basics, FCS utilizes the correlation of fluorescence signal fluctuations in a small volume (such as PSF of confocal or multi-photon LSM) to obtain information on i) diffusion (translational, or rotational, when polarizers are used), ii) concentration of fluorescent species, and iii) dynamics of fluorescent molecules (blinking, binding, conformational dynamics and protein folding, etc.). Each of the aforementioned phenomenon has its own characteristic dynamics and can generate specific fluorescence signal fluctuations, which can be recovered by FCS [96]. Although FCS is not an imaging technique, the spatial location in inhomogeneous systems (membranes, cells) effects the the fluctuations correlation, because only the local environment in the proximity of the PSF is accessible. The PSF has a fixed location in the experiment, but any location within the investigated specimen can be chosen.

The signal fluctuations have two characteristic features: i) the amplitude and ii) the duration or the time scale on which fluctuations occur. The amplitude of fluctuations depends on the number of particles in the observation volume (microscope PSF) and follows Poisson statistics, providing particles do not interact. The duration or the characteristic time of fluctuations depends on the rates of the process responsible for fluctuations (diffusion, binding) [96]. The intensity of fluorescence signal depends on the set of factors, expressed in Eq. 2.10:

\[ F(t) = \kappa Q \int W(r)C(r,t)dr \]  \hspace{1cm} [2.10.]

where \( \kappa \) is the detector sensitivity, \( Q \) is the quantum yield of the fluorescent probe (may contain fluctuations due to internal processes), \( W(r) \) describes the illumination profile (shape of PSF), and \( C(r,t) \) is the (local) concentration of fluorophore, which is changing over time because of the diffusion processes. The shape of the illumination profile is important because fluorescence intensity depends on excitation light intensity (1-photon process) or square of excitation intensity (2-photon process), which varies within the PSF. The intensity of the fluorescence signal as a function of position (\( I(r,z) \), r in the lateral plane and z in the axial direction) can be described by 3-dimensional Gaussian approximation of the PSF:
for 1-one photon excitation, or

\[ I(r, z) = I_0 \exp\left(-\frac{4r^2}{\omega_0^2} - \frac{4z^2}{z_0^2}\right) \]  

[2.11b.] for 2-photon process, where \( I_0 \) is the intensity in the center of PSF and is determined by the laser beam intensity (or squared laser beam intensity for 2-photon process), \( \kappa \), and \( Q \). \( \omega \) and \( z \) are the widths of PSF in the lateral plane and the axial direction, respectively. For two-photon excitation, the Gaussian-Lorentzian approximation of the PSF can be also used (for axial and lateral directions, respectively.

The signal fluctuation amplitude and duration are extracted by use of an autocorrelation function \( G(\tau) \) (Eq. 2.12 and Fig. 2.11):

\[ G(\tau) = \frac{\langle \delta F(t) - \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} \]  

[2.12.] where \( F(t) \) is the fluorescence signal intensity at time \( t \), \( \langle F(t) \rangle \) denotes the average value of \( F(t) \), and \( \delta F(t) = F(t) - \langle F(t) \rangle \).

![Fig. 2.11. An example of the fluorescence signal fluctuations used for calculation of autocorrelation function G(τ), according to Eq. 2.12. figure redrawn after E. Gratton (LFD Workshop, www.lfd.uci.edu)](image)

In the simplest situation, when the fluctuations are caused only by diffusion, \( G(\tau) \) has only two characteristic values, \( G(\tau) \) as \( \tau \) approaches 0, \( G(0) \) (fluctuations amplitude), and characteristic relaxation time of the fluctuations (correlation time), \( \tau_D \). (cf. Fig. 2.12).
The fluctuation of the number of molecules ($N$) in the PSF follows the Poisson statistics, and therefore a variance of the distribution is proportional to the average number of molecules. When $\tau$ approaches 0:

$$G(0) = \frac{\langle \delta F(t)^2 \rangle}{\langle F(t)^2 \rangle} = \frac{\langle (F(t) - \langle F(t) \rangle)^2 \rangle}{\langle F(t)^2 \rangle} = Variance \frac{\langle N \rangle}{\langle N \rangle^2} = \frac{1}{\langle N \rangle}$$  \[2.13.\]

The amplitude of the fluctuation is then invertible proportional to the average number of molecules in the PSF volume (the concentration of fluorescent molecules).

The correlation time is the average time fluorescent molecules spend in the PSF, and it depends on the diffusion speed (diffusion coefficient, $D_{coef}$) and size of the PSF ($\omega, z$):

$$\tau_D = \frac{\omega^2}{4 D_{coef}}$$  \[2.14.\]

In order to obtain the data about $D_{coef}$ and concentration of the fluorescent molecules, recorded $G(\tau)$ is fitted with the appropriate model, e.g., a model for pure diffusion (one or many species with different $D_{coef}$), 2-dimensional diffusion, diffusion with triplet state formation, blinking, binding, etc.

For the single species diffusing in 3-dimensional Gaussian excitation volume, the model is:
\[ G(\tau) = \frac{1}{N} \left( 1 + \frac{4 D_{\text{coef}} \tau}{\omega_0^2} \right)^{-1} \left( 1 + \frac{4 D_{\text{coef}} \tau}{z_0^2} \right)^{-\frac{1}{2}} \]  

[2.15a.]

for 1-one photon excitation, or

\[ G(\tau) = \frac{1}{N} \left( 1 + \frac{8 D_{\text{coef}} \tau}{\omega_0^2} \right)^{-1} \left( 1 + \frac{8 D_{\text{coef}} \tau}{z_0^2} \right)^{-\frac{1}{2}} \]  

[2.15b.]

for 2-photon process. If the diffusion process occurs in 2-dimensional system, e.g., lipid membrane, the model with a 2-dimensional Gaussian approximation of PSF can be used:

\[ G(\tau) = \frac{1}{N} \left( 1 + \frac{4 D_{\text{coef}} \tau}{\omega_0^2} \right)^{-1} \quad \text{or} \quad G(\tau) = \frac{1}{N} \left( 1 + \frac{8 D_{\text{coef}} \tau}{\omega_0^2} \right)^{-1} \]  

[2.16.]

for 1-photon and 2-photon excitation process, respectively. Other processes, such as triplet state formation can be also included into \( G(\tau) \) models:

\[ G(\tau) = (1 + \frac{T}{1-T} \exp(-\frac{\tau}{\tau_T})) \]  

[2.17.]

where \( T \) is the triplet state amplitude, and \( \tau_T \) is the triplet state lifetime. If more species of different \( D_{\text{coef}} \) and concentration is present, or the other processes are present, the \( G(\tau) \) becomes a sum of these components [96]:

\[ G(\tau) = y G_1(\tau) + (1-y) G_2(\tau) \]  

[2.18.]

\( D_{\text{coef}} \) provides information on the mobility of fluorescent molecules, which can be further used to obtain information on the size of the molecule (or aggregate) or local conditions (viscosity, membrane phase, etc.). For the molecules undergoing 3-dimensional Brownian diffusion, the Einstein-Smoluchowski relation links the molecule and system properties to the diffusion. Providing, the molecules of interested are in diluted conditions, they are much bigger than solvent molecules, Reynolds number is small, the \( D_{\text{coef}} \) is given by so-called Einstein-Stokes equation:
\[
D_{\text{coef}} = \frac{k_B T}{6 \pi \eta R_h}
\]  
[2.19.]

where \(k_B\) is the Boltzmann constant, \(T\) is the temperature, \(\eta\) is the medium viscosity, and \(R_h\) is the hydrodynamic radius (radius of spherical molecule). The equivalent of the Einstein-Stokes equation for 3-dimensional diffusion in 2-dimensional systems, is contemplated by the Saffman-Delbrück model [97]:

\[
D = \frac{k_B T}{4 \pi \mu_m h} \left( \ln \left( \frac{\mu_m h}{\mu_w R} \right) - 0.577 \right)
\]  
[2.20.]

where \(h\) is the thickness of the membrane, \(\mu_m\) is the viscosity of the membrane, \(\mu_w\) is the viscosity of surrounding solution, and \(R\) is the radius of the diffusing cylindrical object. The model assumes that the object's radius \(R > h\), and diluted conditions. The validity of Saffman-Delbrück model was confirmed for particles of relatively small radii (transmembrane proteins: \(R\) of 0.5-4 nm), as well as for much larger objects (microscopic-size domains: \(R\) of 0.5-10 \(\mu\)m) [98, 99]. The directional flow, a high viscosity or crowding (high density of big molecules) change the ideal diffusion behavior. As an example, high concentration of peptides in the lipid membrane causes decrease in their \(D_{\text{coef}}\) (anomalous diffusion) [98].

The FCS technique, since its development by Madge et al. [100], has been successfully used to study dynamic processes in various systems, both \textit{in vitro} and \textit{in vivo} [101]. FCS has been widely used for studies of polymer dynamics [102], aggregation behavior of synthetic molecules and biopolymers [103, 104], protein-ligand interaction dynamics [105], cell components [101, 106]. FCS has been applied to study the membrane phases and the membrane component dynamics (lipids and proteins) in GUVs and planar membrane systems [98, 107-109]. Many techniques have been derived from the basic form of the FCS, some of them have been developed to access slower processes (diffusion in the cell or cellular membrane), like scanning FCS [108] or image correlation techniques, e.g., raster image correlation spectroscopy - RICS) [110]. The FCS techniques have also been refined towards greater precision or resolution, like in the case of two-focus FCS [111, 112] or STED(stimulated emission depletion)-FCS [113, 114].

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II. METHODOLOGY
Chapter 3. Materials

E. coli Polar Lipid Extract (E. coli lipids) as a chloroform solution and other phospholipids as a powder: 1,2-di-palmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1',3'-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol (cardiolipin, CA) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL).

LPS from Escherichia coli O55:B5 (LPS smooth), LPS E. coli O55:B5 fluorescein isothiocyanate conjugate (LPS-FITC), E. coli EH-100 (LPS Ra mutant), E. coli J5 (LPS Rc mutant), E. coli F-583 (LPS Rd2 mutant), Lipid A monophosphoryl from E. coli F583 (Rd mutant), Salmonella typhosa LPS, Lipoteichoic acid (LPA) from Bacillus subtilis, and 2-keto-3-deoxyoctonoic ammonium salt (Kdo) were obtained from Sigma (Sigma-Aldrich, St. Louis, MO).

Chloroform (HPLC-grade) and methanol (HPLC-grade) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Trizma base, HEPES, sodium phosphate, sodium chloride, calcium chloride, ethylenediaminetetraacetic acid (EDTA), and other basic chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO).

6-dodecanoyl-2-dimethylaminonaphthalene (LAURDAN), Lissamine-rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Rhodamine-DPPE), Alexa Fluor488 hydrazide sodium salt, Rhodamine B, and Dextran 3000 MW conjugated to tetramethylrhodamine (Dextran-TMR) were purchased from Molecular Probes (Eugene, OR).

All water solutions were made using deionized water (18.3 MΩ·cm) from a MiliQ system (Millipore Inc., Bedford, MA).

Bacterial membrane vesicles were kindly provided by Dr Ben Berks (Department of Biochemistry, University of Oxford, UK).

Recombinant mouse surfactant protein D (SP-D) and monoclonal antibodies anti-mouse SP-D were kindly provided by Dr Søren Hansen (Department of Cancer and Inflammation Research, University of Southern Denmark, Odense, DK).
Chapter 4. Methods

4.1. Phosphate determination

Inorganic phosphate concentration determination was performed to obtain concentration of phospholipids in stock solutions, as well as concentration of lipids in liposomes suspensions. The protocol is based on the Bartlett method of a colorimetric determination of free phosphate.

Known volumes of the sample and phosphate standards (0 – 100 nmol of sodium phosphate per sample) were adjusted to 50 μL with deionized water. Release of the phosphate from phospholipids was performed by addition of 50 μL of 70% perchloric acid and heating in sand bath at >250°C for >30min. After cooling, 200 μL of 10 mg/mL ammonium molybdate followed by 200 μL of 40 mg/mL ascorbic acid was added. The samples were incubated at 37°C for 1 hour, and then placed in a 96-well plate (200 μL per well). The absorbance was measured at 820 nm in a plate reader (FLUOstar, BMG LABTECH GmbH, Offenburg, Germany). The protocol sensitivity limit is 1-2 nmol of phosphate per sample. If the absorbance at 820 nm was too high (usually when phosphate exceeds 100 nmol), the absorbance measurement was repeated at 700 nm.
4.2. Kdo determination

Kdo (2-keto-3-deoxyoctonoic acid) is a monosaccharide unit, typical for the inner core of the LPS molecules. The protocol is based on the Karkhanis method of a colorimetric determination of Kdo [116, 117].

Known volumes of the Kdo containing sample and Kdo standards (0, 2.5-25 nmol of Kdo per sample) were adjusted to 45 μL with deionized water, and 5 μL of 90 mM H₂SO₄ was added. To release the monosaccharide units from LPS molecules, the acidic hydrolysis was performed at 100°C for 20 min. After cooling, 25 μL of 9.1 mg/mL H₃IO₄ in 68 mM H₂SO₄ was added and samples were incubated at room temperature for 20 min in the dark. Subsequently, 50 μL of 26 mg/mL NaAsO₂ in 0,5 M HCl was added and samples were vortexed till the disappearance of any yellow color. 250 μL of 3 mg/mL thiobarbituric acid was added and samples were incubated at 100°C for 10 min. 125 μL of DMSO was added to still warm samples, and the reaction mixtures were placed in 96-well plate (200 μL per well). The absorbance was read at 550 nm in a plate reader (FLUOstar, BMG LABTECH GmbH, Offenburg, Germany).

The LPS molecules contain different number of Kdo units. For determination of LPS isolated from E. coli, the average number of 2 Kdo units per LPS molecule is assumed [117].

4.3. Labeling of LPS with Alexa Fluor488 hydrazide

LPS (smooth) from E. coli O55:B5 was labeled with Alexa Fluor 488 hydrazide according to the protocol based on combined methods described by Luk and Triantafilou [118, 119].

LPS was oxidized with 10 mM NaIO₄ in 100 mM carbonate buffer (NaHCO₃) pH 5 at 4°C for 20 min. The reaction was stopped by addition of glycerol to a final concentration of 15 mM. Oxidized LPS was purified by extensive dialysis in a 3.5kDa cut-off Slide-A-Lyzer Cassette (Pierce, Rockford, IL) against 10 mM sodium phosphate buffer 150 mM NaCl pH 7.4 at 4°C. Conjugation with Alexa Fluor 488 hydrazide was performed by overnight incubation in 10 mM sodium phosphate buffer 150 mM NaCl pH 7.4 at 4°C. Labeled LPS was separated from free probe by size exclusion chromatography on Sephadex G-100 (Sigma-Aldrich, St. Louis, MO). The degree of labeling and purity was validated by FCS measurements.
4.4a. SP-D/LPS ELISA

The binding preference of SP-D to various LPS species was measured using the enzyme-linked immunosorbent assay (ELISA) method of Hansen et al. [120]. The assay is a non-competitive indirect sandwich ELISA using LPS/LPA for coating, biotinylated MAb-403 (anti-SP-D antibodies) conjugated with hydrazine biotin (provided kindly by Dr. Soren Hansen), and streptavidin conjugated horseradish peroxidase (HRP) for detection, and OPD reagent (Kementec, Taastrup, Denmark) conversion as the read-out parameter for HRP enzymatic activity, which is proportional to SP-D binding [120].

Certified 96-well Maxisorb plates (Nunc, Roskilde, Denmark) were coated with LPS and LPA. 100 μL of LPS (LPA) solutions of app. 20 μg/mL in 1M NaCl per plate well was incubated overnight at 4°C. After removal of the LPS solutions, wells were incubated in a washing buffer (10 mM TrisHCl buffer, pH 7.4, 145 mM NaCl, 5 mM CaCl$_2$ (or 10 mM EDTA for control samples without Ca$^{2+}$), 0.05% (V/V) Emulfogene) at room temperature for 30 min. After this step wells were emptied and washed (four times) with washing buffer. Samples were incubated with SP-D (3 μg/mL, and series of subsequent double dilutions, i.e., 1.5 μg/mL, 0.75 μg/mL, 0.375 μg/mL, 0.188 μg/mL, 93.8 ng/mL, 46.9 ng/mL, and control without SP-D) in a washing buffer overnight at 4°C. Control without Ca$^{2+}$ was included to exclude non-specific (or not saccharide-CDR dependent) binding of SP-D to the plates. After this incubation, samples were emptied and washed with washing buffer (the control without Ca$^{2+}$ was also washed with Ca$^{2+}$ containing buffer). Wells were incubated with 100 μL of 0.25 μg/mL biotinylated Mab-403 antibodies (anti-SP-D) in washing buffer at room temperature for 1 hour with shaking. Wells were emptied, washed four times in washing buffer, and incubated at room temperature with 100 μl of 60 ng/mL streptavidin conjugated HRP (Zymed, Invitrogen) in washing buffer with 1 mg/mL bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) at room temperature for 30 min with shaking. Wells were emptied, washed four times with washing buffer, and incubated at room temperature for 15 min with 100 μl of OPD reagent in substrate buffer with 0.03% H$_2$O$_2$, according to the manufacturer's recommendations (Kementec, Taastrup, Denmark). Development was stopped by adding 100 μL of 1 M H$_2$SO$_4$, and absorbance were read at 492 nm with the absorbance at 650 nm as a subtractive reference in a plate reader.
4.4b. SP-D/LPS vesicles

The assay for the SP-D/LPS-containing vesicles was performed using the bulk FCS measurements (cf. section 4.13). The LPS-containing vesicles labeled with 0.05 mol% Rhodamine-DPPE was prepared according to the procedure described in section 4.7. The vesicles were extruded through the polycarbonate filter with the pore size of 50 or 100 nm (cf. section 4.6). The concentration of the extruded vesicles was adjusted to 0.3 mM (phospholipids) and incubated with SP-D (2 nM and series of subsequent double dilutions, i.e., 1 nM, 0.5 nM, 0.25 mM, 0.125 mM, 0.0625 nM, and control without SP-D; the indicated values indicate the molar concentration of the SP-D dodecamers) in presence of either 1 mM Ca\textsuperscript{2+} or 1mM EDTA for >2 hours. The FCS measurements were performed to evaluate the diffusion and size of the particles.

4.5. Size exclusion chromatography

Size exclusion chromatography allows a separation of polymers (proteins, polysaccharides, DNA, liposomes) according to their hydrodynamic radius. Size exclusion chromatography was used to purify LPS conjugated with Alexa488 hydrazide after a conjugation reaction, and to separate liposomes containing incorporated LPS from non-incorporated aggregates of free LPS. For these two purposes, different resins were used, according to different needs for the chromatography resolution range: Sephadex G-100 (Sigma-Aldrich, St. Louis, MO) and Sephacryl S-400 (Amersham Biosciences AB, Uppsala, Sweden) for labeled LPS-Alexa488 and LPS vesicles, respectively. Glass columns of size 5*130 mm were used. The calibration of both chromatography columns was done with Blue Dextran (Sigma-Aldrich, St. Louis, MO) and CoCl\textsubscript{2} solutions, as well as with free Alexa Fluor488 (Sephadex G-100) and liposomes and free labeled LPS (Sephacryl S-400). Column packing and maintenance was performed according to the manufacturers' recommendations. During the separation, samples of 100 μL were used to load the column, and fractions of app. 100 μL were collected. For sample detection in the column fractions, either absorbance (Blue Dextra, CoCl\textsubscript{2}) or fluorescence (Alexa Fluor488, LPS-Alexa488, fluorescently labeled liposomes) was measured in a plate reader (FLUOstar, BMG LABTECH GmbH, Offenburg, Germany).
4.6. Liposomes

Liposomes (large multilamellar vesicles MLVs, large unilamellar vesicles LUVs, small unilamellar vesicles SUVs) were prepared using the thin lipid film hydration method. The lipid stocks in a CHCl$_3$/CH$_3$OH 2:1 (V/V) solvent were mixed with fluorescent probes (rhodamine-DPPE, LAURDAN, or DPH) in CH$_3$OH to desired ratios and concentrations. The desired amount of mixed lipid solution was deposited on the bottom of a glass vial, and solvent was evaporated under a stream of nitrogen. The resulting dry lipid films were kept in the desiccator overnight, to remove residues of the organic solvent. Hydration of the dry lipid film was accomplished by addition of an aqueous solution (buffer or aqueous solution of LPS, typically to about 10mM phospholipids concentration) to the container followed by vortexing/sonication in the bath sonicator (Branson 1510, Branson Ultrasonic Corporation, Danbury, CT) above lipids the $T_m$ for 20 min. Resulting vesicles (mainly MLVs) were used to either continue the LPS incorporation procedure or extruded through polycarbonate filters (Avanti Mini-Extruder, Avanti Polar Lipids Inc., Alabaster, AL and Whatman Nuclepore Track-Etch membrane, Whatman, Florham Park, NJ) in order to obtain LUVs of defined size. Polycarbonate filter pores were 50 nm or 100 nm in diameter, the liposome suspension was passed through a filter 30 times, the temperature was kept above lipids $T_n$.

4.7. Incorporation of LPS into oligolamellar vesicles

To incorporate the LPS into the liposomal membrane, the dehydration-rehydration method (proposed by Dijkstra et al. [4]) with some modifications was used. The dehydration-rehydration method is more efficient in the incorporation of the LPS into liposomal membranes (particularly those containing negative charges), particularly, when compared with prolonged sonication methods (ref. [4, 121, 120], and our findings). The phospholipid vesicles were prepared from stocks of $E. coli$ lipids mixed with fluorescent probes (either rhodamine-DHPE or LAURDAN) in CHCl$_3$/CH$_3$OH 2:1 (vol). The organic stocks of fluorescently labeled $E. coli$ lipids were deposited in borosilicate-glass vials in various amounts (in order to obtain desired LPS-to-$E. coli$ lipids ratio), dried under a N$_2$ stream and exposed to a low pressure in a desiccator for at least 2 hours to remove residuals of the organic solvent. The lipid films were hydrated in 0.4-0.5 mM LPS (LPS-smooth, LPS Ra,
LPS Rc, or LPS Rd) water solutions at 55°C for 20 minutes, using continuous vortexing (Eppendorf Thermomixer Comfort R). These samples were further sonicated in a bath sonicator (Branson 1510, Branson Ultrasonic Corporation, Danbury, CT) at 55°C for 20 minutes, frozen in liquid nitrogen, and lyophilized for 5 hours. Dried samples were re-suspended in 150 mM NaCl to a final phospholipids concentrations of 2 mM, vortexed and further sonicated in bath sonicator (Branson 1510) at 55°C for 20 minutes. The LPS-containing liposomes were separated from non-incorporated LPS (both aggregates and monomers) by a size exclusion chromatography using a chromatography column loaded with Sephacryl S-400. The separation of LPS-containing liposomes from LPS monomers and aggregates on the column was evaluated using controls with fluorescently labeled liposomes and LPS, i.e. the liposomal fraction was detected in the column void volume and LPS eluted later (cf. Fig. 6.2). After this procedure, the liposomal fraction was taken for further analysis, and finally used to prepare GUVs. To evaluated the incorporation, the LPS-to-phospholipid molar ratio in prepared vesicles was measured. The total phospholipid concentration and the LPS concentration in these samples were determined by inorganic phosphate assay (cf. section 4.1) and 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) assay (cf. section 4.2), respectively. The LPS molecules from E. coli contains 2-4 phosphate groups (cf. Fig. 1.3.2), the LPS phosphate was subtracted from inorganic phosphate assay, to obtain the true E. coli-lipids concentration. The preparation of liposomes containing lipid A were done by mixing E. coli polar lipid extract with Lipid A in CHCl3/CH3OH/H2O 74:23:3 (vol), and fluorescent probes (either rhodamine-DHPE or LAURDAN) in various amounts (in order to obtain desired lipid A to E. coli lipids ratio). Organic solvent was removed as described above, and samples were hydrated with 10 mM phosphate buffer 150 mM NaCl pH 7.4 at 55°C for 20 minutes using continuous vortexing (Eppendorf Thermomixer Comfort R). These samples were further sonicated in a bath sonicator (Branson 1510) at 55°C for 20 minutes. The incorporation of lipid A into resulted liposomes was assumed to be 100% efficient.

4.8. Giant unilamellar vesicles

The protocol of GUVs formation was based on the electroformation method developed initially by Dimitrov and Angelova [123] and further refined by Méléard et al. [93, 124]. The protocol is based on using 500 Hz AC electric field and physiological ionic strength.
Aqueous solutions containing liposomes (0.1-0.2 mM phospholipid concentration) were deposited in small aliquots (~1 μL) on the surface of Pt wires in a home-made chamber, that allows visualization in the microscope (cf. Fig. 4.1).

Fig. 4.1. The home-made GUVs electroformation chamber, that allows a visualization of the GUVs directly on the Pt wires in the microscope. The chamber is made of Teflon. Lipid samples are deposited onto the Pt wire (1). Temperature can be adjust by a water circulated inside metal ring (3) connected to a water bath. Visualization of the GUVs on the Pt wires is possible thanks to a cover slide (2) glued to the chamber (4). Figure taken form ref. [125]

The chamber was kept in the desiccator for app. 5 min to remove water. The procedure was repeated 3-4 times. The aqueous suspensions of liposomes rather than chloroform solutions of lipids were used for three reasons: i) the use of biological samples (bacterial membrane vesicles) and compounds that are poorly soluble in organic solvents (LPS); ii) a quantification of the real incorporation into the liposomal membranes was possible only by using smaller liposomes (not GUVs); iii) the electroformation from an aqueous suspension of liposomes gives a higher yield of GUVs. After this last step, 500 µl of buffer containing 150 mM NaCl (10 mM sodium phosphate, 10mM TrisHCl, or 25 mM HEPES, pH 7.4), was added to the chamber containing the Pt electrodes, and an AC field was applied using a function generator (Vann Draper Digimess Fg 100, Stenson Derby, UK). The GUVs electroformation procedure had three main steps: 1), frequency 500 Hz, amplitude 35 V/m for 5 min; 2), frequency 500 Hz, amplitude 313 V/m for 20 min; 3), frequency 500 Hz, amplitude 870 V/m for 90 min. In all the electroformation steps, the temperature was kept at 55°C, except for the samples...
containing preparations of bacterial membrane vesicles, where temperature was kept at 37°C to minimize the denaturation of membrane associated proteins. GUVs were visualized in the electroformation chamber using the confocal LSM and 2-photon LSM (for details, cf. sections 4.11 and 4.12).

4.9. LAURDAN GP (spectrofluorometer)

The LAURDAN GP measurements for oligolamellar vesicles, LPS aggregates, and the reference solution for the 2-photon GP imaging were done using an ISS ChronosFD spectrofluorometer (ISS, Champaign, IL), connected to a water bath (Julabo F25-MV Refrigerating Circulating Bath, JULABO Labortechnik GmbH, Germany). The 374 nm light emitting diode (ISS) with a bandpass filter (370 ± 18 nm) was used as an excitation light source. The fluorescence light was read in L configuration (cf. Fig. 4.2), at 440 nm and 490 nm \(I_B\) and \(I_G\), according to Eq. 4.1, respectively), using a monochromator with 2 mm slits (corresponding to app. 8 nm bands) and a photomultiplier as a detector.

Fig. 4.2. Sketch showing schematic ChronosFD spectrofluorometer used for the LAURDAN GP and DPH anisotropy measurements. The blue line indicates the excitation light produced by the light emitting diode (LED 374 nm) with a bandpass filer (370 ± 18 nm) placed between the Beam Splitter and the Excitation Polarizer. In the LAURDAN GP measurements, the emission monochromator and detector (PMT) on the right side were used (L configuration). In the DPH anisotropy measurements the left side of the setup was used (T configuration). The longpass filter (KV 408 nm) was placed between the left emission polarizer and the left PMT. The sample holding unit (marked in grey) was connected to the water bath with the temperature control unit (Julabo F25-MV). The system detection was in photon-counting mode. *figure taken from manufacturer's webpage (www.iss.com)*
The measurements were performed in the photon counting mode. Crossed polarizers were used to minimize the effect from scattered light. The data were corrected using spectra correction factors provided by manufacturer (ISS):

\[ GP = \frac{I_B - (G \times I_G)}{I_B + (G \times I_G)} \]  

\[ [4.1] \]

\( G \) is the instrumental correcting factor, similar to the \( G \) factor in classical anisotropy/polarization equation. The use of \( G \) factor is important because of the differences in transmission of light in different polarization planes by monochromator (cf. ref. [78]).

The samples (LPS or phospholipids) for the LAURDAN GP measurements were mixed with an appropriate amount of LAURDAN (in CH\(_3\)OH) in 400:1 lipids to LAURDAN molar ratio. The samples were dried and rehydrated in phosphate buffer to obtain a 0.5 \( \mu \)M LAURDAN concentration. The concentration of all lipids, particularly LPS, was kept well above the critical aggregation concentration (cf. section 5.3). Sonication in a bath sonicator (Branson 1510) at 55°C was applied to all samples. This procedure resulted in formation of micellar aggregates (LPS) or liposomes (phospholipids). Samples were transferred to quartz cuvettes and placed in spectrofluorometer sample holder with a temperature control. To ensure a system equilibration, the samples were incubated at the measurement temperature for > 10 min before each measurement, for all temperature points. No change in LAURDAN GP was observed in between measurements at given temperature, indicating that the system was in equilibrium.

4.10. Anisotropy (spectrofluorometer)

The DPH anisotropy measurements for oligolamellar vesicles and LPS aggregates were performed using an ISS ChronosFD spectrofluorometer (ISS, Champaign, IL), connected to a water bath (Julabo F25-MV Refrigerating Circulating Bath, JULABO Labortechnik GmbH, Germany). The 374 nm light emitting diode (ISS) with a bandpass filter (370 ± 18 nm) was used as an excitation light source. The fluorescence light was read in T configuration (cf. Fig. 4.2), with a longpass filter (LP 408 nm) and a photomultiplier as a detector. The measurements were performed in the photon counting mode. \( G \) factor (cf. section 4.9) was
not applied because the monochromators were not used.

The samples (LPS or phospholipids) for the DPH anisotropy were prepared using the same routine as for LAURDAN GP measurements (cf. section 4.9).

4.11. Confocal microscopy

Visualization of the GUVs and the detection of fluorophores presence (LPS-Alexa488 or GFP-Tat) in GUVs membranes was performed using a confocal LSM: Zeiss Axiovert 200M equipped with Zeiss LSM 510 META confocal unit (Carl Zeiss MicroImaging GmbH, Jena, Germany). The objective was a Zeiss C-Apochromat 40x water immersion, NA 1.2 (Zeiss). Excitation of the green fluorophores (Alexa488, GFP, fluorescein) was done with the 488 nm line of an Argon laser, guided to the sample by a dichromatic mirror 1 (HFT UV/488/543/633). The scattered laser light was removed by dichromatic mirror 2 (NFT 490), and the detection of the fluorescence signal was done using a 515 ± 15 nm or 525 ± 25 nm bandpass filter and a photomultiplier (Zeiss). The excitation of Rhodamine-DHPE was done with a HeNe laser (543 nm), the scattered laser light was removed by a dichromatic mirror 2 (NFT 545), and the detection was done using a 590 ± 25 nm and a photomultiplier (Fig. 4.3.).

![Diagram of beam path setup](image)

**Fig. 4.3.** Schematic of the beam path setup used in the confocal LSM. The light source is an Argon (488 nm line) or a HeNe (543 nm) laser. Both, the laser beam and the scattered laser light is indicated in a blue color, the fluorescence light is indicated in a green color. The dichromatic mirror 1 is a HFT UV/488/543/633, the dichromatic mirror 2 is a NFT 490 (Alexa Fluor488, fluorescein) or a NFT 545 (Rhodamine-DHPE), and the bandpass filter is a BP 515 ± 15, 525 ± 25 nm (Alexa Fluor488, fluorescein), or a BP 590 ± 25 nm (rhodamine-DHPE).
When both, the green probe and the rhodamine-DHPE were present in the sample, the multi-track scanning mode was used to minimize interference between channels. GUVs were visualized at their equatorial planes, or stacks of confocal pictures were used for a 3-dimensional reconstruction of the GUVs. For the 3D reconstruction, the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, http://rsb.info.nih.gov/ij/) was used.

4.12. Multi-photon microscopy and LAUDRAN GP imaging

The LAURDAN GP measurements were performed in a custom built two photon excitation fluorescence microscope based on Olympus IX70 (for description cf. Fig. 4.4 and ref. [95]) and operated with the software package SimFCS (Laboratory for Fluorescence Dynamics, University of California at Irvine, CA). The objective used in the experiments was a 60X water immersion objective with an NA of 1.2 (Olympus, UPlanSApo 60x/1.20W). The excitation light source was a femtosecond Ti:Sa laser (Broadband Mai Tai XF-W2S with 10 W Millennia pump laser, tunable excitation range 710-980 nm, Spectra Physics, Mountain View, CA) and the excitation wavelength was 780 nm. The excitation light was circularly polarized to avoid photoselection effects in the image plane. For the calculation of the LAURDAN GP function, the fluorescence signal from the sample was split into two different channels using a dichromatic mirror (475 nm). Each channel contained a bandpass filter (438 ± 12 nm or 494 ± 10 nm, which corresponds to $I_B$ and $I_C$ in Eq. 4.1, respectively) and a photomultiplier (Hamamatsu H7422P-40). The LAURDAN GP images were calculated using SimFCS (Laboratory for Fluorescence Dynamics).
Corrections using the $G$ factor were performed according to Brewer at al. [95], using a 160 µM LAURDAN solution in DMSO as a reference ($GP_{ref} = 0.006$ at room temperature). The LAURDAN GP for the reference solution (LAURDAN in DMSO) was measured using the same instrumental conditions (detector gain for both photomultipliers) as in the GUVs measurements. The LAURDAN GP value measured on the microscope was then corrected with the $G$ factor to match the value measured on the spectrofluorometer ($GP_{ref} = 0.006$, cf. section 4.8), according to Eq. 4.2:

$$G = \frac{I_B (1 - GP_{ref})}{I_G (1 + GP_{ref})}$$  \hspace{1cm} [4.2]

The GP values obtained in the GUVs experiments were computed from the distinct membrane regions at the GUVs equatorial plane using a ROI routine. Approximately 20-25 GUVs for every concentration were analyzed and the average GP and the standard deviation reported.
4.13. Fluorescence correlation spectroscopy

The FCS measurements were performed using a custom built two photon excitation fluorescence microscope described above (section 4.11). The excitation wavelength was 840 nm for Rhodamine B and 930 nm for Alexa Fluor488, the signal was collected through a bandpass filter of 605 ± 35 nm for Rhodamine B and 525 ± 25 nm for Alexa Fluor488 using a photomultiplier (Hamamatsu H7422P-40).

The data collection and calculations of auto-correlation function (Eq. 2.3.3) were performed using SimFCS (Laboratory for Fluorescence Dynamics). The calculated ACF were fitted globally to the model of a single (or two) species of molecules diffusing in a 2-dimensional (membrane diffusion) or 3-dimensional (bulk diffusion) Gaussian two-photon excitation volume (Eq. 2.3.6b and 2.3.7b) using Globals for Spectroscopy (Laboratory for Fluorescence Dynamics). The size of the point spread function ($\omega_0$ and $z_0$) was calibrated before the FCS measurements using fluorescent polystyrene beads and calibration standards with known diffusion coefficient: Rhodamine B, $D_{\text{coef}} = 271 \ \mu\text{m}^2\text{s}^{-1}$; Dextran-TMR, $D_{\text{coef}} = 128 \ \mu\text{m}^2\text{s}^{-1}$ (determined using calibration with fluorescent polystyrene beads); Alexa Fluor488 hydrazide $D_{\text{coef}} = 430 \ \mu\text{m}^2\text{s}^{-1}$ [101].

In the bulk diffusion studies the laser beam was focused inside the sample solution. For each measurement, typically 5 – 10 per sample, the individual $D_{\text{coef}}$ was modeled and used to calculate average $D_{\text{coef}}$.

In the membrane diffusion studies the laser beam was focused on the membrane in the polar regions of GUVs. The right focus position is crucial for the precise FCS measurements on the membrane [126]. The Z-Stack FCS experiment using diffusion of rhodamine-DHPE in GUVs membranes revealed that simply finding the highest intensity of the fluorescence signal gave enough precision to measure the $D_{\text{coef}}$s, therefore, the Z-Stack FCS routine was not followed (cf. section 5.1.2). Approximately 20 vesicles per sample were analyzed. The $D_{\text{coef}}$ was modeled for each vesicle, and used to calculate the sample average.
III. RESULTS
Characterization of the system components

The first stage of the project was the characterization of the system components (LPS) in their pure forms, as well as validation of main techniques used in the later stages of the project. The thermotropic phase behavior of E. coli polar lipid extracts (called later as E. coli phospholipids) and selected LPS species, together with aggregation behavior of LPS molecules (CMC) was evaluated. The existing data on LPS CMC [127-130] and \( T_m \) [2, 3, 52] are limited to a few specific LPS species. Considering the variety in LPS structures and the effect of LPS structure differences on the above mentioned parameters, the characterization of selected LPS chemotypes using consistent methodology can help in understanding the features of the model system, presented in following sections.

5.1.1. Characterization of microscope PSF for FCS measurements

Information about the exact size of PSF (\( \omega_0 \) and \( z_0 \)) in the microscope system is crucial for proper interpretation of FCS experiments results, because the only parameter measured independently is the correlation time of the process (\( \tau_0 \)), but the diffusion is both -time and PSF size-dependent.

The calibration of the microscope PSF is usually done by imaging a point sources of fluorescence light, e.g., fluorescent beads or quantum dots. The image of such small particles
recreates the actual PSF of the system, providing that fluorescent particles are separated. This method of PSF calibration is time consuming, and the calibration should be perform before each series of experiments, because even slight changes in a microscopic system alignment, correction setting of the objective, or cover slide thickness may change the PSF. Therefore, the most common routine is to measure diffusion coefficient of a probe with known parameters. However, there is large discrepancy in the literature about $D_{\text{coef}}$ of the commonly used fluorophores, e.g., for free Alexa488 several $D_{\text{coef}}$ were reported:

Table 5.1. $D_{\text{coef}}$ of free Alexa Fluor 488 reported in the literature

<table>
<thead>
<tr>
<th>$D_{\text{coef}}$ (μm$^2$/s$^{-1}$)</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>196</td>
<td>Pristinski et al. (2005) [102]</td>
</tr>
<tr>
<td>245</td>
<td>Masuda et al. (2005) [131]</td>
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<td>380</td>
<td>Petrasek et al. (2008) [132]</td>
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<td>414</td>
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<td>430</td>
<td>Jameson et al. (2010) [101]</td>
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<td>435</td>
<td>Petrasek et al. (2008) [134]</td>
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In the FCS experiments two excitation wavelengths were used: 837 nm and 930 nm, exciting Rhodamines and Alexa488, respectively. The PSF is wavelength dependent, therefore two individual diffusion standards were used: Rhodamine B in phosphate buffer (later changed to Dextran-TMR), and free Alexa Fluor 488 hydrazide in phosphate buffer. The $D_{\text{coef}}$ of those probes were measured subsequently after PSF calibration using fluorescent beads. In this calibration, the series of images with known pixel size (>100) was taken, and Gaussian distribution was fitted to the averaged fluorescent bead signal using SimFCS package (LFD) (Fig. 5.1). The procedure was repeated 5 times for each wavelength. The PSF size was estimated to $0.368 \pm 0.007 \mu m$ (837 nm) and $0.423 \pm 0.014$ (930 nm).

![Fig. 5.1. The average intensities of pixels as a function of the distance from the center of fluorescent beads (excited with 837 nm), modeled with Gaussian distribution. This plot is an average of >100 frames, with several well-separated beads. Because the fluorescent signal is emitted by an immobile point-source, this can be treated as treated as a microscope PSF.](image-url)
The $D_{\text{coef}}$ of rhodamine B was measured to be $271 \pm 4 \, \mu\text{m}^2\text{s}^{-1}$, which is smaller than values given in literature ($420 \, \mu\text{m}^2\text{s}^{-1}$ [135]). The FCS measurements on the small, fast diffusing molecules are more difficult to conduct, because only a part of the time correlation is available for fitting. This is due to the limitation in the scanning frequency. Therefore, slower diffusing molecules are more convenient as a diffusion standards for PSF determination. As a new standard, Dextran-TMR (MW 3000) was chosen, and its $D_{\text{coef}}$ was determined to be $129 \pm 5 \, \mu\text{m}^2\text{s}^{-1}$. The $D_{\text{coef}}$ of Alexa Fluor 488 was chosen after ref. [101] ($430 \, \mu\text{m}^2\text{s}^{-1}$). The PSF of the microscopy system was determined using $D_{\text{coef}}$ of above mentioned standard molecules, prior to each FCS experiment.

![Figure 5.2](image.png)

Fig. 5.2. The experimental (gray) and modeled auto-correlation functions (black) of rhodamine B, Alexa Fluor 488 hydrazide, and Dextran-TMR. Each of the measurement was performed with 100 kHz acquisition frequency. Faster acquisition frequencies generate worse data quality due to the lack of photons and detector response. Slower diffusing standards allow better auto-correlation modeling due to a larger amount of meaningful data for a fitting procedure.

The proper determination of PSF is an important issue. The lack of standardization in this procedure, together with an existence of no absolute value of $D_{\text{coef}}$ for standard fluorophores makes the absolute measurement of $D_{\text{coef}}$ difficult, and result values have a relative character.
5.1.2. FCS in membrane system, Z-scan FCS

The FCS technique allows for a determination of the $D_{\text{coef}}$ for a free 3-dimensional diffusion of fluorescent molecules, as well as the lateral (2-dimensional) diffusion in membrane systems, e.g., planar membranes, top surfaces of GUV, or flat areas of cell membranes. The importance of the proper PSF calibration for the accuracy of the $D_{\text{coef}}$ determination was discussed in previous section (5.1.1). In the FCS experiments in the planar systems, there is another factor determining the measurement accuracy: the vertical ($z$)-position of the laser focus relative to the membrane surface. This is because the size of the PSF ($\omega_0$) changes as a function of the focal spot position in respect to the membrane in axial direction ($z$): the $\omega$ is smallest in the center and broaden as focal spot center is moving away. The Z-scan (per analogy to the routine of obtaining 3-dimensional images in confocal or 2-photon LSM) involves the determination of $\tau_D$ and particle numbers in 0.2 $\mu$m steps along the $z$-axis [126]. From the dependence of those parameters on the position of the focus, $D_{\text{coef}}$ can be determined, or the precise focus easily found.

Fig. 5.3. Z-projection of the GUV composed of *E. coli* lipids labeled with 0.001 mol% rhodamine-DPPE in phosphate buffer. In the FCS measurement, laser was focused on the polar region of vesicle.

The lateral diffusion of Rhodamine-DHPE was determined in free standing membranes (GUVs) composed of *E. coli* lipids. The concentration of rhodamine-DHPE in the membrane was 0.001 mol%. The Z-scan FCS was compared with the FCS data made by focusing at the maximum intensity. The lateral $D_{\text{coef}}$ of rhodamine-DHPE in *E. coli* lipids, in free standing bilayer system, was determined to be $4.72 \pm 0.20 \mu m^2 s^{-1}$, which is in line with the values of lateral diffusion of phospholipids in model membrane systems reported in literature [109, 126].
The Z-Stack FCS routine is reported to have advantage over the focusing at higher signal intensity in supported bilayer [126]. It is likely, that this effect is due to the contribution to the signal from the solid support, i.e., reflection, or intrinsic fluorescence. However, this is not the case in free standing bilayer models, where the focusing at a spot of highest fluorescence intensity is simpler and less time consuming, and therefore this routine is follow in further FCS experiments on model membranes. The margin of possible error is also smaller in 2-photon LSM, due to elongation of PSF in axial direction (typically $z \sim 3-4$ times larger than $\omega \sim 0.37 \, \mu m$), which in our microscopy system is approx. 1.5 $\mu m$.

### 5.2. Labeling of LPS (O55:B5) with Alexa Fluor 488 hydrazide

Labeling of LPS (O55:B5) was performed, because commercially available conjugates of LPS with fluorescein isothiocyanate or Oregon Green were unsuitable for the experiments due to a low degree of the labeling (only fraction of the LPS molecules in commercial preparations was labeled), the contamination of the commercial preparations with free probe, and low photostability of the probes (particularly fluorescein). Labeling of LPS is a straightforward procedure [118, 119], and conjugating the LPS with the probe, either fluorophore or biotin
does not change significantly the recognition of LPS by the host immune system [136, 137].
Isothiocyanate or hydrazide is commonly used as a reactive group to conjugate the probe
molecules with LPS. In the second case, a hydrazide group reacts with aldehyde groups in the
core region of LPS, which are introduced there by a mild oxidation of free hydroxide groups
on the galactose units.

Two LPS species were chosen for the labeling procedure: *E. coli* O55:B5 (smooth) and *E. coli*
J5 (LPS Rd2), according to the procedure described in section 4.3. Labeling of LPS Rd2 was
unsuccessful because of very low degree of labeling. The first choice of the column for final
purification was a PD-10 with Sephacryl G25 (Sigma Aldrich) used to purify LPS in [119].
The column elution profiles, where the fluorescence of the conjugated probe was monitored
pointed out that the column with Sephacryl G25 resolution range prevents the successful
separation of LPS-Alexa488 from free Alexa488. A column loaded with Sephacryl G100 was
used and the LPS fraction was evaluated by FCS measurements (Fig. 5.5). The LPS-Alexa488
was diluted to match approx. 10 nM concentration. That low concentrations are necessary for
the FCS technique. The PSF was calibrated with free Alexa Fluor 488 hydrazide, with a $D_{\text{coef}}$
= 430 $\mu \text{m}^2 \text{s}^{-1}$ [101]. The FCS results on LPS-Alexa488 could be analyzed with 1-species
diffusion model (using a 2-species model with fixed parameters for free Alexa Fluor 488
hydrazide gave no advantage for the accuracy of the fit), and $D_{\text{coef}}$ for LPS-Alexa488 was
found to be 26.0 ± 1.5 $\mu \text{m}^2 \text{s}^{-1}$, which corresponds to a sphere with the radius of approx. 8.2
nm (according to Eq. 2.19, cf. section 2.3.2):

![Fig. 5.5. The experimental ACFs (gray) and modeled ACFs (black) of LPS-Alexa488 (right) and free Alexa Fluor 488 hydrazide (left). The experimental ACF were modeled as a single species diffusing in a 3-dimensional excitation volume. All ACF were normalized.](image-url)
The PCH analysis and comparison between LPS-Alexa488 and free Alexa488 hydrazide showed that the brightness parameter of LPS-Alexa488 is approx. 3 times greater than the brightness parameter of free Alexa488 under the same instrumental conditions, which means that the degree of labeling is close to 3 Alexa488 per 1 LPS molecule.

The knowledge of the molecules degree of labeling and their purity is an important factor for the proper conduction of experiments, but surprisingly, little attention to this parameter is paid. Usually, it is assumed, that purification techniques (dialysis or size-exclusion chromatography) provide sufficient degree of purity. The degree of labeling is usually evaluated by measuring the ratio of labeled molecule to the probe concentration, which is true only when free probe is completely removed. The FCS technique requires sophisticated and expensive instrumentation, but provides detailed information about the aforementioned features. It is particularly interesting, that commercially available fluorescent conjugates (LPS-FITC, LPS-Oregon Green) reveal relatively low quality when thoroughly investigated. LPS-Alexa488 was further used for determination of critical micelle concentration of LPS, evaluation of the presence of LPS in GUV membranes, purification procedures of liposomes containing LPS, and finally to study lateral diffusion of LPS in free standing membranes.

5.3. The critical micelle concentration of LPS

Critical micelle concentration (CMC) is an important parameter describing the behavior of amphiphilic molecules in aqueous solutions. The CMC is a bulk concentration of amphiphilic molecules below which amphiphiles exist in monomeric form. Above CMC amphiphiles tend to aggregate in micelles or other type of aggregates.

A series of LPS smooth (O55:B5) dilutions, all containing 10 nM LPS-Alexa488, were prepared in phosphate buffer. After sonication and equilibration (>2 hours) the samples were placed in glass chambers, and the $D_{\text{coef}}$ was determined by FCS. The experiment was conducted at room temperature (below $T_m$ of LPS). For tLPS concentrations lower than 5 μM, no deviation from monomeric LPS diffusion was observed ($D_{\text{coef}} \sim 26.0 \, \mu m^2 s^{-1}$).
Fig. 5.6. The experimental ACFs (gray) and modeled ACF (black) of 10 nM LPS-Alexa488 in presence of different concentrations of unlabeled LPS (O55:B5): 0.1 to 5 μM. No aggregation was observed.

When the concentration of LPS exceeded 5 μM (10 and 100 μM) formation of large and inhomogeneous aggregates was observed. It was difficult to asset precise diffusion data, due to sample inhomogeneity (D\text{coef} of the slowest component could be estimated to approx. 0.2 \(\mu\text{m}^2\text{s}^{-1}\)), but clearly, the critical aggregation concentration was reached.

Fig. 5.7. The experimental ACFs (gray) and modeled ACF (black) of 10 nM LPS-Alexa488 in presence of different concentrations of unlabeled LPS (O55:B5): 10 μM (A) and 100 μM (B). Some degree of aggregation (A) and massive inhomogeneous aggregation (B) observed in the samples. ACF was modeled for D\text{coef} 0.2 \(\mu\text{m}2\text{s}^{-1}\).

Transition between the monomeric form of LPS to the aggregated one was estimated to be in range of 10 μM. This order of magnitude is in agreement with other reports using FCS, Dynamic Light Scattering, and calorimetry. The DLS and nuclear magnetic resonance (NMR) diffusometry revealed the transition from monomeric to aggregated form of LPS (O55:B5) to occur in range of 10-300 mg/L (1-0 μM, assuming the MW of LPS 10 kDa) at room
temperature [129]. CMC of rough LPS (Re) was reported to be 8 μM at 37°C, but 42 μM at 25°C [130]. Other report, using fluorescence assay, determined CMC of several LPS species (both smooth and rough) to be in range of 1-10 μM (LPS O55:B5 CMC ~ 4 μM) [127]. Different sizes of the LPS aggregates were reported. Size dependence on the LPS concentration was reported by Santos et al. (radii of 60 and 100 μm at 37°C) [128]. In work of Bergstrand et al., presence of bimodal distribution of aggregate sizes was found (radii of 25 and 320 μm at 25°C) [129]. The existence of large LPS aggregates (D_{coeff} of 0.2 μm²s⁻¹ points to a radius in the range of 1 μm) is probably due to the clustering of small aggregates.

Although FCS was used to determine amphiphiles CMC (block copolymers) [138], the use of this technique to study more complex molecules, e.g., LPS, has some important limitations. FCS is sensitive to the low concentrations of fluorescent molecules, but also lacks statistics. As long as there is a narrow distribution of a few (1-3) particles of different D_{coeff}, the diffusion can be easily determined. However, the particle size distribution, easily accessible in DLS experiments, cannot be obtained by the FCS technique. The CMC of LPS O55:B5 was estimated to be in range of 10 μM, in agreement with other reports [127, 129, 130]. For further experiments: for a determination of fatty-acids packing in LPS aggregates, or for an incorporation of LPS into liposomal membranes, concentrations above CMC of LPS were chosen (>100 μM) to ensure an existence of LPS aggregates in the sample. The concentration of LPS above CMC may be particularly important for the incorporation procedure, high concentrations may promote the partition of LPS molecules into the hydrophobic -hydrophilic interface (membrane).

5.4. Molecular packing and order in LPS aggregates

LPS molecules show complicated phase behavior, as other amphiphilic molecules, its phase depends on a set of physicochemical parameters, such as the degree of hydration, temperature, and divalent cations (e.g., Mg²⁺) concentration [139-141]. A selection of LPS molecules from *E. coli* (LPS smooth (O55:B5), and rough strains: Ra, Rc, Rd2, including lipid A from *E. coli*) was characterized by fluorescence techniques (LAURDAN GP and anisotropy). The dependence of the molecular packing in the LPS aggregates on the temperature and
polysaccharide chain length (notice that the hydrophobic part of LPS, lipid A, is the same in all selected LPS chemotypes) was evaluated and compared with analogous parameters in \textit{E. coli} lipids, DPPC and POPC vesicles.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.8.png}
\caption{Dependence of LAURDAN GP and DPH anisotropy in vesicles composed of \textit{E. coli} lipids, DPPC and POPC on the temperature. Measured values of LAURDAN GP of DPPC in gel phase, DPPC \(T_m\), etc., are in agreement with literature data (ref. [87, 94]).}
\end{figure}

The DPH anisotropy and LAURDAN GP of DPPC and POPC vesicles was used to compared packing and order in membranes made of well known single components (DPPC and POPC) and the mixture of many components (PE, PG, CA of various fatty-acid composition). LAURDAN GP is sensitive for the hydration of the probe surroundings, which changes along with the lipids packing. The values of LAURDAN GP of DPPC below the \(T_m\) (~0.5), the GP of the liquid membranes, i.e., DPPC above \(T_m\), POPC above \(T_m\) (< 0) match the values reported in the literature (cf. ref. [87, 94]). The phase transition of DPPC is sharp \((T_m \sim 43^\circ C)\), what reflects the cooperativity of the DPPC melting in single component liposomal system. In contrast, the phase transition of \textit{E. coli} lipids which occurs close to 25\(^\circ\)C (lipids are extracted from \textit{E. coli} grown at 37\(^\circ\)C) is very poorly marked. This is due to the lack of cooperativity in phospholipids melting, since \textit{E. coli} lipids consist of mixture of lipids with many different \(T_m\) (PE, PG, and CA with various fatty-acid composition). At 20\(^\circ\)C \textit{E. coli} lipids are not as fluid as membranes in a clear liquid-disordered phase (GP < 0), but they are less densely packed than lipids in gel or liquid-ordered phase.
Selected LSP chemotypes show similar lack of melting cooperativity as *E. coli* lipids. This fact cannot be explained by the sample heterogeneity, with the exception of smooth LPS, which contains different number of repeats of the O-specific antigen. The broad transition in LPS aggregates may be due to the small number of molecules per aggregate. Although the precise $T_m$ of LSP chemotypes studied here could not be determined, the general tendency in the dependence of $T_m$ on polysaccharide chain: $T_m$ of lipid A $> T_m$ of smooth LPS $> T_m$ of rough LPS, is visible in the LAURDAN GP data. LPSs and lipid A $T_m$ is substantially higher than the $T_m$ of the phospholipids of *E. coli*, the difference in LAURDAN GP is approx. 0.19-0.27 at 20°C and 0.18-0.39 at 37°C, depending on LPS chemotype. The large difference of the lipid packing preference may cause the phase separation in the membrane system. The anisotropy of DPH, a fluorophore located in the fatty-acid chains region, reflects the rotational freedom (or order) in this area. The anisotropy in aggregates of LPS chemotypes is in line with the LAURDAN GP data, except for lipid A. The reason for this fact is unknown. One can speculate that possibly the small size of the aggregates formed by lipid A may allow fast rotational motion of the whole aggregates decreasing the anisotropy of the probe.

The results are in line with the gel to liquid-crystalline transition temperatures: the same tendency in the dependence of $T_m$ on the polysaccharide group length reported for the
selection of rough LPS chemotypes from *Salmonella minnesota* was observed [3]. The $T_m$ of smooth LPS was reported to be at 37°C, and decreases with the shortening of the polysaccharide group. However, in the case of lipid A, (which lacks the polysaccharide group), the $T_m$ raises to 45°C. This transition occurs when the LPS is fully hydrated, and no divalent cations are present. Decrease in the LPS hydration and increase in the divalent cations concentration lead to an elevation of $T_m$. Decrease in the hydration of the LPS molecules makes a stronger tendency to form lamellar structures, but above $T_m$ the tendency to form different non-lamellar phases (H$_{II}$ or cubic) increases, particularly for the deep rough LPS chemotypes [2, 3].

5.6. Summary

In this section the preliminary characterization of the model system, described in the following sections, was performed. Purity and diffusion in solution of labeled LPS was characterized. Sets of LPS properties: critical aggregation concentration and lateral packing of LPS molecules, particularly packing and order parameters of the LPS molecules in relation to *E. coli* lipids, DPPC and POPC, were characterized. In all further experiments, the concentration of LPS molecules is used above their CMC. Under my experimental conditions (no divalent cations, full hydration, temperature < $T_m$) all LPS chemotypes are likely to favor lamellar phase. In the procedures requiring elevated temperature (lipid mixing and GUVs formation) the formation of H$_{II}$ or cubic phase may occur, particularly for lipid A.

Although no original result were found, the collected data on the system components and the control of the techniques and methodology used here proved to provide information in line with literature data, obtained with different techniques. The collected information and expertise was used for the characterization of the LPS in an original model system described in following sections.
Chapter 6.

Incorporation of LPS into *E. coli*-phospholipid membranes and preparation of GUVs containing bacterial membrane components

In this section, an efficient method of preparation of GUVs composed of *E. coli* membrane components (PE, PG, CA, and LPS) under physiological salt and pH conditions is presented. The focus of the method is to ascertain the presence of LPS in the membrane together with a determination of LPS concentration in the final membranes. The impact of LPS chemotypes and initial LPS to phospholipids ratio on the LPS incorporation efficiency is discussed. The evaluation of LPS incorporation ratio was carried carefully, and the amount of LPS, particularly the chemotypes with large polysaccharide group, was significantly higher than reported elsewhere. Liposomes containing LPS incorporated into their membranes were used mainly for studies on the LPS toxicity [4, 121, 142] and as simple models of bacterial membranes [69]. Although in most of those studies the incorporation rates and the final product purity were investigated (cf. ref. [4, 121, 143]), the range of LPS chemotypes and concentrations were very limited (typically to 2 mol%). The formation of LPS-containing GUVs was reported by Henning et al. but the evaluation of this model was very limited, hindering the comparison with the model system presented in this work [144]. In addition, the method of preparing GUV composed of inner bacterial membrane components, phospholipids and membrane associated proteins, is presented in this section. The substrates for GUVs formation are natural membranes isolated from *E. coli* cells. This method may be an alternative for protein renaturation in the liposomes, particularly because it allows to preserve relatively unchanged proteins surrounding, and to study more complex systems.
6.1. Preparation of GUVs composed of E. coli lipids

GUVs were formed exclusively by a variant of the electroformation method of Méléard et al. [124]. This method employs an high frequency (500 Hz) alternating electric field, and allows the formation of GUVs at a physiological ionic strength (> 150 mM NaCl). The physiological ionic strength conditions are particularly beneficial for the preparation of GUVs containing high ratios of charged lipids. GUVs containing charged lipids form easier at the high salt concentrations, probably because their surface charge is screened by counter-ions. The phospholipids (and lipid A) mixtures can be used for the electroformation of GUVs by a direct deposition on the Pt wires (or ITO plates) from organic solvent solutions. However, the formation of GUVs from liposomes suspension was chosen because this procedure results in higher yield of formed GUVs. Moreover, organic solvents cannot be used because LPS with large polysaccharide groups (smooth, Ra – Rc) are very poorly soluble, e.g., LPS in CH₃OH gives an inhomogeneous suspension with floaters (cf. the manufacturer's product vendor of LPS). LPS also forms aggregates in aqueous solutions, where its concentration is above the CMC, which is in 1-10 μM range, but these aggregates are smaller and have a more uniformed size [127, 129, 130]. The presence of the membrane associated proteins also imposes the use of aqueous solutions on all steps of the GUVs formation procedure.

![Fig. 6.1. LSM images taken at equatorial plane of GUVs (false color representation). A) POPC, B) POPE:POPG 7:3 molar, C) POPE:POPG:tetramirystoyl-CA 70:25:5 molar, D) E. coli polar lipid extract. GUVs on this figure have approx. 15 – 20 μm in diameter (on images with several vesicles given diameter refers to bigger vesicles). All samples were prepared by a co-sonication of phospholipid vesicles with LPS-Alexa488 in approx. 100:1 molar ratio, but the LPS concentration in GUVs has not been determined. The fluorescence signal recorded for these images comes from Alexa 488 conjugated with smooth LPS chemotype (O55:B5). No inhomogeneities in LPS distribution was observed.](image-url)
First attempt to incorporate LPS molecules in the GUVs membrane combined simple mixing of liposomes with LPS in aqueous suspension followed by electroformation procedure. Liposomes composed of different phospholipids, both synthetic (POPC, POPE, POPG, CA) and natural extract of bacterial membranes were mixed with LPS-Alexa488 and co-sonicated. The molar ratio between phospholipids and LPS was approx. 100:1. The presence of LPS in the membrane was confirmed: LPS-Alexa488 was the only fluorophore present in the visualized samples, and fluorescent signal comes only from LPS incorporated into GUVs membranes. Association of LPS with membrane by the other than hydrophobic interaction, e.g., electrostatic, is unlikely because both LPS and membranes carry a negative charge. Moreover, high ionic strength of the buffer hinders the electrostatic interactions. The final concentration of LPS in GUVs membranes has not been determined due to a lack of proper experimental method, but the assumption that all LPS is incorporated cannot be held (cf. next section). The presence of LPS molecules in the membrane without determination on its concentration may be enough for, e.g., LPS – proteins binding studies, but give no insight into effect of LPS on its behavior or the lateral packing in membranes. The electroformation method provided good yield of GUVs for selected phospholipid compositions in physiological ionic strength (>150 mM NaCl equivalent). The selection of buffers used for a GUVs formation was: 10mM HEPES, 10mM TrisHCl, and 10mM sodium phosphate; all with 150 mM NaCl and pH 7.4. No apparent difference in the outcome of the electroformation procedure was seen. The protocol was also successfully used for the buffers containing the divalent cations, 2 mM Ca\(^{2+}\) did not change the GUVS formation results. Presence of 5 mM Ca\(^{2+}\) was found to cause precipitation of POPE/POPG and POPE/POPG/CA lipid mixtures, but not the *E. coli*-lipid extract.

6.2. Incorporation of LPS into the *E. coli*-lipid membranes

In order to evaluate the concentrations of LPS incorporated into the membrane several additional steps were included. The steps in the new procedure were as follow: i) mixing the lipids (liposomes) with aqueous suspension of LPS (by co-sonication or other methods, cf. below), ii) separation of liposomes (with LPS molecules incorporated into vesicles membranes) from non-incorporated free LPS molecules, iii) determination of phospholipids
and LPS concentration in purified liposomes. I found the choose of a proper LPS incorporation method most controversial. I decided to follow dehydration-rehydration method by Dijkstra et al. [4] as the one offering highest incorporation efficiency. A selection of different LPS chemotypes containing polysaccharide groups of various length, but the same lipid A part was chosen to study the impact of polysaccharide group length on the incorporation efficiency.

To separate vesicles from non-incorporated LPS size exclusion chromatography was chosen, as much faster than dialysis. Using fluorescently labeled compounds (lipids and LPS), resins of different size resolutions were tested. I found Sephacryl S-400 (Amersham Biosciences) the most useful for the separation of vesicles and LPS molecules (Fig. 6.2).

![Fig. 6.2. Comparison between size exclusion elution profiles of different samples containing liposomes labeled with 0.1 mol% rhodamine-DPPE (black line) and LPS (indicated by gray line). FRI is fluorescence relative units. In A, the ability of the column to separate vesicles and free LPS (LPS-FITC) is shown. In this sample no LPS incorporation procedure was applied, vesicles and LPS-FITC (in 1:1000 molar ratio) were mixed. Single elution peak for LPS-FITC (gray line) indicates a presence of mostly monomeric LPS. B and C show the comparison between sonication (B) and dehydration-rehydration (C) method. Vesicles and smooth LPS in 1:100 molar ratio, doped with LPS-Alexa488. Almost no incorporation is observed after sonication procedure. Additional elution peak (with maximum close to 37th - 38th fraction) is probably due to existence of LPS aggregates, as the concentration of LPS is above CMC.](image_url)

After selecting the most appropriate purification procedure, the two methods of mixing LPS with lipids were compared: i) prolonged co-sonication method [143] and ii) dehydration – rehydration [4]. For this procedure, phospholipids were mixed with unlabeled smooth LPS (O55:B5) and LPA-Alexa488. The co-sonication procedure relies on the prolonged sonication...
(~30 min) of sample containing suspension vesicles and LPS with high energy input (tip sonicators are usually used). In my experiment, I co-sonicated *E. coli*-lipids vesicles labeled with 0.1 mol% rhodamine-DPPE and smooth LPS (O55:B5) mixed with LPS-Alexa488. The molar ratio of phospholipids to LPS was approx. 100:1 (LPS concentration in these solutions was approx. 100 μM). The same ratios where used in the dehydration – rehydration method, and both samples were purified and compared. In agreement with previous reports [4, 121], I found the dehydration – rehydration method more efficient in incorporating LSP into the vesicles. Moreover, the prolonged sonication results in formation of smaller liposomes (notice the elongated elution profile of sonicated vesicles in Fig 6.2 and 6.3), which cannot be separate from LPS aggregates. Vesicles containing lipid A were prepared directly from CHCl₃/CH₃OH solutions because all components were soluble in organic solvents.

![Fig. 6.3. Comparison between size exclusion elution profiles of dehydrated-rehydrated (black line) and sonicated (gray line) liposomes. FRI is fluorescence relative units. Fluorescence signal comes from 0.1 mol% rhodamine-DPPE in liposomes.](image)

The impact of initial LPS-to-lipid ratio and the polysaccharides group length of LPS on the incorporation of LPS into the model membranes composed of *E. coli*-lipids was investigated. Four LPS chemotypes were selected: i) smooth LPS (*E. coli* O55:B5), ii) LPS-Ra (*E. coli* EH-100), iii) LPS-Rc (*E. coli* J5), and iv) LPS-Rd2 (*E. coli* F-583) with molecular weights of approx. 10 kDa, 5.5 kDa, 4.2 kDa, and 3.2 kDa, respectively. To minimize the repulsive effect of negative charges on both LPS, and *E. coli*-lipids, ionic strength was kept at physiological level (>150 mM NaCl equivalent). To promote incorporation, the concentration of LPS in all initial solutions was 400 μM and only the amount of *E. coli*-lipids was changed. In presence of 30 and more mol%, when the mixtures of phospholipids and LPS were used for
electroformation procedure without a purification step, the formation of GUVs was impaired. The most likely cause is the preference of LPS to non-lamellar phases, e.g., H_{II} or cubic, particularly above \( T_m \) [2, 3, 145]. The electroformation was performed at 55ºC, above all reported LPS (and lipid A) \( T_m \). Presence of lipid A in 50 mol\% concentration (labeled with LPS-Alexa488 as a fluorescent probe) revealed existence of probably non-lamellar phases (Fig. 6.4).

![Z-projection of LSM images (false color representation) of Pt wire surface, used for GUVs electroformation. The sample is a mixture of E. coli-lipids with 50 mol\% of lipid A doped with small fraction of LPS-Alexa488 as a fluorescent probe. Scale bar represents 20 \( \mu \)m.](image)

Therefore, only LPS concentrations below 30 mol\% were investigated. The dependence of the incorporation efficiency on the initial LPS to phospholipids ratio for four studied LPS chemotypes is shown in Fig. 6.5. The initial concentration of LPS is given by the molar ratio of LPS-to-phospholipids used for incorporation procedure. The concentration of LPS incorporated into purified vesicles was measured by determination of inorganic phosphate assay [115] and Kdo determination assay [116] (cf. section 4.1 and 4.2, respectively). The assay based on the chromogenic reaction of Kdo was chosen, because commonly used LAL (\textit{Limulus} amebocytes lirate) assay, although much more sensitive for low LPS concentrations, is based on the LPS toxicity, which varies for different LPS chemotypes, and is greatly reduced when LPS molecules are incorporated into the liposomes [4, 146].
The incorporation efficiency approaches 98% at 5 mol% initial concentration of LPS, but decreases with increasing initial LPS-to-phospholipids molar ratio. The efficiency is higher for LPS chemotypes with shorter polysaccharide groups: maximum incorporation of LPS was approx. 22 mol% for LPS Rc and Rd2. In case of LPS containing full core region or additional O-specific chain (LPS Ra and smooth, respectively), the incorporation efficiency was lower, and maximum incorporation reached approx. 12 mol%. The clear dependence of the incorporation efficiency on LPS polysaccharides group size may be linked to the LPS-phospholipids mixing during dehydration step. A larger polysaccharide group, which is hydrophilic, may hinder the mixing of LPS with membrane lipids. Purified vesicles containing LPS were used for GUVs electroformation according to aforementioned protocol, providing good yield of GUVs with average size of approx. 20 μm. In GUV samples containing deep rough LPS chemotypes (Rc and Rd2) above 10 mol% of LPS, and in all studied lipid A concentrations (5, 10, and 15 mol%) inhomogeneous distribution of rhodamine-DPPE was observed. Rhodamine-DPPE is known to be excluded from membrane domains in gel phase when phase separation is present. In GUVs containing LPS chemotypes
with full core region or an additional O-specific chain (LPS Ra and smooth, respectively), no visible phase separation can be seen in the GUVs at all explored concentrations, up to 12 mol %. The lateral organization of LPS-containing membranes, e.g., phase separation, is described and discussed in following section.

![Representative images of GUVs (Z-projection of LSM images, false color representation) composed of E. coli-lipids and selection of LPS chemotypes: A) LPS smooth (10 mol%); B) LPS-Ra (10 mol%); C) LPS-Rc (above 10 mol %); D) LPS-Rd (above 10 mol %); E) Lipid A (above 5 mol %). Scale bars are 5 μm.](image)

The protocol of fully developed method for the preparation GUVs containing high concentrations of LPS (up to 12 mol% for smooth LPS and LSP Ra, and up to 22 mol% for LPS Rc and Rd2) under physiological ionic strength is described in detail in section 4.7.

### 6.3. Preparation of GUVs from bacterial membrane vesicles

Bacterial membrane vesicles are small pieces of bacterial cell membranes in vesicular form, prepared by a ruption of bacterial cells in French press, and a separation in density gradient centrifugation: the vesicles formed mainly from outer or inner membranes can be separated [147]. Resulting inner membrane or outer membrane vesicles (IMVs or OMVs, respectively) consist all membrane components, including lipids and membrane-associated proteins, and are mainly in inside-out orientation. IMVs activity may be restored in certain conditions, therefore these structures were used to study protein systems of inner bacterial membranes in vitro, e.g., Tat (twin-arginine translocation) pathway [148, 149], or Sec membrane transport systems [150]. The IMVs were derived from E. coli cells, overexpressing the TatC-EcoGFP, the integral membrane protein (Sec-independent protein translocase, tatC) fused with green
fluorescent protein (EcoGFP mutant). Attempts to direct use IMVs for GUVs electroformation (Méléard et al. method [124]) failed. LSM examination of samples revealed existence of large aggregates of proteins and lipids (cf. Fig. 6.7).

Fig. 6.7. Aggregates formed from IMVs derived from E. colli cells overexpressing TatC-EcoGFP (Z-projection of LSM images, false color representation). Scale bars are 25 μm (left) 10 μm (right).

The most likely reason for the unsuccessful GUVs formation may be the large protein concentration in IM, reaching 70% of dry mass [45]. In order to overcome this obstacle, membrane proteins were “diluted” in membranes by a decrease of protein-to-lipid ratio. IMVs were mixed with liposomes composed of E. coli-lipids (unlabeled or labeled with 0.1 mol% rhodamine-DPPE). Three different mixing procedures were used: i) freeze-thaw of mixed vesicles; ii) co-sonication of mixed vesicles; iii) direct deposition of mixed vesicles onto the Pt-wire used for electroformation. The dilution of IMVs was in at least 1:1 (1:2, 1:5, 1:9) proteins to an added E. coli-lipids. Dilutions of higher factors resulted with higher yield of vesicles, but investigation of actual protein dilution in the GUV membranes was not performed. The IMV-liposomes mixtures were deposited on the Pt-wire and dried shortly (procedure was not repeated to minimize protein denaturation), and electroformation was performed in phosphate buffer at 37°C. The samples were examined with LSM; in the case of all mixing procedures, GUVs formed from mixed samples contained GFP (cf. Fig. 6.8). Mixing in the third case is probably due to mixing during dehydration and rehydration steps. The freeze-thawing of mixed vesicles was chosen for further testing, as this procedure assured the best mixing of IMVs and liposomes, and was accompanied by a smaller danger of
denaturing proteins than the sonication method.

Fig. 6.8. Representative images of GUVs (Z-projection of LSM images (A and B), and LSM image taken at GUV equatorial plane; false color representation) composed of IMVs (E. coli overexpressing TatC-EcoGFP) and E. coli-lipids, mixed (freeze-thawed) in (A) 1:1, (B) 1:2, and (C) 1:5 (IMVs proteins:E. coli-lipids wt ratio). GFP fluorescence was used to record these images. Scale bars are 10 μm.

Results from electroformation of GUVs containing inner bacterial membranes are preliminary, but seem promising. Although presence of proteins in GUVs membrane was confirmed, unfortunately no membrane enzyme activity assay was performed. The real membrane protein dilution factor and membrane proteins activity has to be tested before the value of this protocol can be fully assessed.

6.4. Discussion

The formation of GUVs under physiological conditions [124] has been successfully extended (with some modifications, cf. previous section) to different compositionally complex LPS/E. coli-lipids mixtures. This new protocol opens an interesting possibility for studies of these lipid mixtures. GUVs containing various amount of different LPS chemotypes can be successfully prepared, allowing the use of fluorescence microscopy techniques. The crucial part of the procedure leading to the GUVs formation is the incorporation of LPS molecules into the membrane and the proper evaluation of this process. It is particularly important, because unlike other lipid molecules, LPS contains large hydrophilic group composed of polysaccharides of different sizes. In case of smooth LPS chemotypes, the size of the polysaccharide group is manifold larger than hydrophobic lipid A. Due to a presence of this large hydrophilic group, most of the LPS chemotypes are insoluble in organic solvents, with
an exception for lipid A, which lacks entire polysaccharide group, and LPS Re, which contains only two Kdo monosaccharide units. In aqueous solutions, LPS is in a monomeric form and forms aggregates above CMC, which is in 1-10 μM concentrations range [127, 129, 130]. Model membranes containing LPS were used mostly for studies of the LPS toxicity on the host organisms or immune system cells. Incorporation of LPS into the liposomes greatly decreases its endotoxic properties relatively to the free LPS molecules [121, 122, 133, 142, 143]. The incorporation efficiency clearly depends on the phospholipid charge, particularly for the sonication method, when LPS and membranes cannot come in close contact, due to electrostatic repulsion, and LPS polysaccharide group size. This conclusion is completely in line with my experimental results, obtained for different phospholipids system, mimicking *E. coli* membrane. Moreover, I explored much larger LPS-to-phospholipids ratios (close to the ratios in natural bacterial membranes) and systematically evaluated the impact of polysaccharide group size on the incorporation efficiency. The dehydration of the lipid samples may bring phospholipids and LPS molecules closer, even though approx. 20 mol% of phospholipids are negatively charged, allowing for better mixing. Heating the samples above $T_m$ of LPS, which is known to promote a formation of $H_{II}$ and cubic LPS phase [2, 3] may have influence on increase in LPS and membranes mixing. At 50 mol% of LPS-Re in the DPPE/DPPG (8:2) liposomes, cubic phases dominates [145]. This is in agreement with my observation that the formation of GUVs from the non-purified lipid dispersions was completely impaired at high LPS-to-phospholipids ratios (above 30 mol%) and the non-lamellar phases were possibly formed (cf. Fig. 6.4). A preparation of GUVs containing DPPC/DOPC and smooth LPS conjugated with FITC in 1:1:1 molar ratio was reported by Henning et al. [144]. The method of Dijkstra et al. (dehydration-rehydration method) [4] was followed, but much above conditions from the original protocol (33 mol% and up to 1 mol% of LPS in Henning and Dijkstra protocols, respectively). Unfortunately, the final amount of LPS in the DPPC/DOPC/LPS membrane is not reported in this work. The authors assumed that all the LPS used in the initial mixture was incorporated into the GUV (cf. ref. [144]). This is unlikely, taking into account a general tendency of a smaller efficiency of the LPS incorporation at higher concentrations of LPS, particularly for smooth chemotypes (ref. [122] and my findings). The lack of qualitative data is hampering the final comparison with my method. My general finding is that the LPS incorporation efficiency decreases with an increase of LPS-to-phospholipids ratio and an increase of LPS polysaccharide group size.
(from 95-97% for 5 mol% of the initial LPS concentration, to 35% for LPS smooth or Ra, and 67% for LPS Re or Rd2 at the initial concentration of 33 mol%). The LPS chemotypes can be divided into two groups: i) smooth LPS and LPS Ra with lower incorporation efficiency, and ii) deep rough LPS chemotypes, e.g., LPS Re and Rd2, what can be extrapolated on LPS Re, and lipid A. The difference in the incorporation efficiency of different LPS chemotypes is accompanied by the difference in lateral organization of membranes. GUVs containing deep rough LPS chemotypes show the micrometer-size domain formation. Based on these results, I conclude that there is a critical size difference between the polysaccharide groups of LPS Ra and LPS Re. This difference determines the distinct behavior of LPS in membrane systems. This conclusion can be extrapolated for LPS containing larger (LPS smooth, various chemotypes) and smaller (LPS Rd, Re, and lipid A) polysaccharide group.

GUVs are excellent tool to study membrane model system using fluorescence microscopy related techniques. Various membrane proteins, including ABC-transporters were reported to be successfully reconstituted in small liposomes and GUVs, and used for the studies of the proteins lateral diffusion and activity [98, 151, 152]. ABC-transporters remained their activity and quantitative information is obtained about dissociation constants for ligand binding, number of binding-sites, transport affinities, rates of transport, and the activities of transporter molecules were measured [152]. The method used by Girard et al. is based on the overexpression of membrane proteins in bacterial system, e.g., E. coli, detergent extraction from the membranes, purification and renaturation of purified proteins back into the liposomes. Proteoliposomes can be used to form GUVs [151, 152]. The concentration of incorporated integral membrane protein, reported by Girard et al., at which the formation of GUVs was still possible was 40% (wt, approx. 1:1800 protein-to-lipid molar ratio for Ca\(^{2+}\)-ATPase) [151]. The reconstitution method seems to be excellent for studies of single protein species, particularly in diluted protein conditions. However, when system of interest consist of many components, the simultaneous renaturation of many different proteins in physiological ratios may be troublesome. An alternative for this problem is a formation of GUVs from native membranes, e.g., isolated tissues (stratum corneum, pulmonary surfactant, ref. [27, 28], respectively), cells, cell organelles, or membrane preparations (ref. [93] and this work). The method of preparing GUVs formed from erythrocyte ghosts was described by Montes et al. This method offers the possibility of GUVs formation at physiological ionic strength (150
mM NaCl) in contrast to aforementioned method, so the physiological-like conditions for proteins activity may be preserved, and provides almost unchanged protein orientation and leakage free vesicles fusion [93]. The method for GUVs preparation from IMVs is similar to the one of Montes et al., but requires an additional “proteins dilution” step, probably due to a high protein concentration (70%) impairing vesicle fusion. The approx. 40% protein concentration [151] may be a limit for the membranes fusion and GUVs formation. It is in line with out findings that the double dilution (to approx. 40 mol%) of IMVs proteins allows the GUVs formation, in contrast to the original IMVs samples. However, it was not proven that the protein-to-lipid ratio was preserved through the electroformation procedure. The dehydration of samples prior to electroformation is an important step in the GUVs electroformation, because full dehydration hinders proteins activity, when membrane-associated proteins are present. To ensure the maximum preservation of proteins activity, a partial dehydration or a dehydration in presence of sucrose (to provide hydrogen bonding for protein) is typically used [28, 151, 152]. In this case I used brief dehydration (5 min and no repeats of the deposition of vesicles on the Pt-wire, in contrast to LPS vesicles) and/or sucrose in the IMVs buffer. The results on GUVs formed from IMVs are preliminary and further characterization of the method, i.e., determination of a proteins concentration in GUVs membrane, the proteins orientation, and their activity is necessary to assess the value of this method.

6.5. Summary

In this chapter the method for efficient incorporation of LPS into the liposome membranes, and for an electroformation of GUVs composed of E. coli-lipids and LPS or bacterial IM components was characterized. The presented method explored in a systematic way the impact of the size of the LPS polysaccharide group size and the LPS concentration on the incorporation efficiency. This kind of systematic quantification of LPS presence is new, as the incorporation efficiency dependence on the membrane charge and concentration of LPS was explored at much lower concentration range (up to approx. 1 mM) [4, 122, 143]. I found that the incorporation efficiency is smaller for higher LPS-to-phospholipids ratios and larger polysaccharide groups. The LPS chemotypes can be divided into two groups: i) smooth LPS
and LPS Ra with lower incorporation efficiency, and ii) deep rough chemotypes, e.g., LPS Rc and Rd2. The characteristic of the second group can be generalized to LPS Re and lipid A. A difference in the incorporation efficiency between LPS smooth or Ra and Rc or Rd2 is accompanied by a distinct organization of GUV membranes. In case of GUVs containing deep rough LPS, the formation of micrometer-size domains can be observed. The phase behavior of *E. coli*-lipid membranes containing LPS is characterized in chapter 7.
Chapter 7.

Lateral organization of LPS-containing *E. coli*-lipid membranes

In chapter 6, the incorporation of LPS into *E. coli*-lipid membranes was characterized. The outcome of the incorporation experiments showed a qualitative difference between LPS smooth or Ra, and Rc or Rd2 in incorporation efficiency and membrane phase behavior (a formation of macroscopic domains). Both quantities will be described in this chapter. To quantify the physicochemical properties of membranes with various concentrations of LPS with different polysaccharide group, two fluorescence based techniques were used: i) LAURDAN GP to investigate the local packing of membrane lipids, ii) FCS to study the lateral diffusion of LPS molecules in the membrane. The data presented in this chapter are new, because the molecular packing and the phase behavior of LPS was studied either for pure LPS [2, 3, 153], or in the systems of small physiological relevance 145, 154]. The systematic study of the impact of the concentration and polysaccharide group size of LPS on the membrane organization is the first of its kind.
7.1. Investigation of phases in LPS-containing membranes with LAURDAN GP

LAURDAN fluorescence spectra are sensitive to the local packing of the lipids surrounding the fluorophore molecules because of the strong solvatochromic properties of the fluorescent probe. LAURDAN was used to investigate the membrane organization in liposomes, i.e., small liposomes and GUVs. Liposomes containing various amounts of different LPS chemotypes (cf. chapter 6) were labeled with 1-2 mol% of LAURDAN.

LAURDAN GP was measured as the bulk property of small liposomes in a fluorometer and spatially resolved by calculating the LAURDAN GP map from 2-photon LPM images. Collected data are plotted in Fig. 7.2. GUVs containing LPS smooth or LPS Ra show homogeneous distribution of LAURDAN (and rhodamine-DPPE) at all explored LPS concentrations (up to approx. 12 mol%). Results from both small liposomes and GUVs are in good agreement at all concentrations. The LAURDAN GP increases with rising LPS concentration, starting at approx. 0.24 for 5 mol% LPS, and reaching 0.29 and 0.34 for 12 mol % of LPS smooth and LPS Ra, respectively. This augmentation can be contributed either to a
continuous increase of the lipid order, induced by the presence of a component of higher \( T_m \), or to the clustering of LPS molecules. For comparison, the LAURDAN GP of pure *E. coli*-lipid membranes is approx. 0.2. A similar increase of the LAURDAN GP can be observed in samples containing less than 10 mol% of LPS Rc and Rd2. In addition to the previously described phenomenon, the samples containing more than 10 mol% of these LPS chemotypes show additional feature, a micrometer-size phase separation (a domain formation), similar to the phase separation observed in samples containing at least 5 mol% of lipid A. In contrast to LPS samples, LAURDAN GP of lipid A-containing samples does not vary significantly (0.23 for 14 mol% lipid A). The phase behavior of lipid A at concentrations below 5 mol% was not explored, and the immiscibility concentration in this system is unknown. The comparison of the measured LAURDAN GP values for the micrometer-size domains present in GUVs containing at least 10 mol% of LPS Rc or Rd (>0.55, cf. Fig. 7.2 C-D), and the LAURDAN GP of the surrounding lipids (approx. 0.2, Fig. 7.2) implies a gel/liquid-disordered-like phase coexistence, with the elongated domains being gel-like domains enriched in LPS or lipid A (cf. Fig. 7.1) because their \( T_m \) values are higher than the \( T_m \) of *E. coli* phospholipids (cf. section 5.4). This conclusion is supported by comparison of the LAURDAN GP differences between the coexisting domains in the LPS-containing samples with those previously reported for phospholipid samples displaying gel/liquid-disordered phase coexistence (LAURDAN GP values for gel phases are >0.5 and <0.2 for fluid phases) [87, 94].
The quantum yield of LAURDAN fluorescence rises as the polarity of the environment decreases. This results in higher fluorescence intensities of LAURDAN in more condensed membranes (membranes or membrane domains in gel or gel-like phases), provided that the partition of LAURDAN into liquid and condensed phases is similar, which is generally assumed as true (cf. ref. [76, 87] and Fig. 7.3 A). Surprisingly, I observed much smaller fluorescence intensities in gel-like regions, of GUVs showing phase separation (LPS Rc and Rd2 above 10 mol%, lipid A at all explored concentrations, cf. Fig. 7.3 B). This is most likely due to the partitioning of less LAURDAN into compacted LPS- and lipid A-rich phases. A photoselection effect of the polarized excitation light due to strict aligning of LAURDAN molecules in gel phases [76, 87, 91] can be excluded, as a circularly polarized laser beam is
used for excitation, and images are taken at the GUVs equatorial planes. Therefore, LAURDAN GP values in GUVs showing micrometer-size phase separation were calculated separately for each domain, using a ROI routine and different threshold values.

Fig. 7.3. Representative images of GUVs (false color representation) composed of DOPC/DPPC 1:1 (A) and E. coli polar lipid extract with 14 mol% lipid A (B). Images in the left and center column show fluorescence intensity images (taken at the equatorial plane of the GUVs using 2-photon LSM). $I_B$ and $I_G$ corresponds to the intensities measured using BP filters 438 ± 12 nm and 494 ± 10 nm, respectively, which were used to calculate the LAURDAN GP map (right column). Color coding of LAURDAN GP values is different for each row: -0.8 to 0.8 (A) and 0 to 0.8 for (B). Arrows indicate the gel-like domains (LAURDAN GP approx. 0.55), DPPC-rich domain (A) and lipid A-rich domain (B).

Fig. 7.4. Representative images of GUVs (false color representation) composed of DOPC/DPPC 1:1, labeled with a small fraction (approx. 0.001 mol%) of LPS-Alexa488 (A) and 0.005 mol% of rhodamine-DPPE (B). Rhodamine-DPPE indicates the position of the liquid, DOPC-rich domain. The gel-like, DPPC-riched domains are marked with arrows. Suprisingly, LPS (O55:B5) conjugated with Alexa 488 partitions to the liquid domain.

### 7.2. Investigation of LPS lateral diffusion by FCS

To investigate the behavior of mixtures of E. coli lipids and LPS that show no micrometer-size phase separation (LPS smooth and Ra), the lateral diffusion of LPS molecules was measured using the FCS technique. LPS O55:B5 (smooth chemotype) conjugated with Alexa Fluor 488 was used to follow the diffusion in GUVs with different concentrations of LPS smooth (O55:B5) and LPS Ra. LPS-Alexa488 was added in a small ratio (approx. 0.01 mol% of total lipids) to small liposomes already containing the desired concentration of non-labeled LPS (either LPS smooth or Ra) and the GUV electroformation procedure was performed (note
that no incorporation procedure was performed for LPS-Alexa488). Based on the G(0) values of the ACF measured in GUVs, the concentration of LPS-Alexa488 was estimated to be approx. 0.001 mol%. For FCS measurements, the laser beam was focused on the polar region of GUV and the fluorescence signal fluctuations were recorded and analyzed. Data were modelled using 1-species diffusion in a 2-dimensional 2-photon Gaussian excitation volume (cf. section 2.3.2). For each vesicle several measurements were taken and fitted globally. The diffusion values for each concentration are the average from 15 – 20 GUVs (Fig. 7.5).

Fig. 7.5. FCS experiments performed in GUVs containing LPS-smooth or LPS-Ra doped with fluorescently labeled LPS-Alexa488 (0.001 mol% with respect to total lipids). The figure shows the ACFs of the recorded data (dots) with the diffusion model fits (solid lines) and the corresponding LPS diffusion coefficient measured in GUVs (each point is an average of 15 to 20 separate vesicles; error bars are standard deviations). No microscopic phase separation was observed in these samples using both LAURDAN GP and rhodamine-DPPE.

The diffusion of single LPS-Alexa488 molecules was measured to be $5.58 \pm 0.62 \, \mu m^2 s^{-1}$, which is in agreement with diffusion coefficients measured for phospholipids in model membranes [98, 109]. The concentration of 0.001 mol% estimated for LPS-Alexa488 is well below the concentration of the LPS clustering reported by Trubetskoy et al. [143] which occurs above 0.125 mol% of LPS. At higher concentrations of LPS a significant reduction of the lateral diffusion of LPS molecules was observed, up to an almost 5-fold decrease of $D_{coef}$ in GUVs containing 12 mol% LPS ($1.36 \pm 0.23 \, \mu m^2 s^{-1}$ for LPS smooth and $0.98 \pm 0.21 \, \mu m^2 s^{-1}$ for LPS Ra). The lateral diffusion of LPS-Alexa488 in GUVs with 12 mol% LPS Ra is...
slightly slower than diffusion in GUVs containing smooth LPS. This difference may be due to an experimental error, but it matches well the higher values of LAURDAN GP in the corresponding lipid mixtures (0.29 for LPS smooth and 0.34 for LPS Ra).

There are two plausible explanations for the raised LARDAN GP observed in both GUVs and small vesicles, and the decrease in the lateral diffusion of LPS. One is a higher viscosity and larger membrane thickness caused by the introduction of lipids of much higher \(T_m\) (LPS) with respect to \(E. coli\) phospholipids. However, this scenario is unlikely to occur, because relatively small concentration of LPS molecules introduced to the membrane system is not expected to affect the membrane properties in such a dramatic way. The scenario that is more likely to occur is the clustering of LPS molecules into domains of sizes below the lateral resolution of the microscopy system (approx. 380 nm) used in this study. To discuss the effect of domain size, I used the Saffman-Delbrück model (cf. section 2.3.2):

\[
D = \frac{k_B T}{4 \pi \mu_m h} \left( \ln \left( \frac{\mu_m h}{\mu_w R} \right) - 0.577 \right)
\]

[7.1.]  
where \(h\) is the thickness of the membrane, \(\mu_m\) is the viscosity of the membrane, \(\mu_w\) is the viscosity of the surrounding solution, and \(R\) is the radius of the diffusing cylindrical object. The model assumes an object of \(R > h\) and diluted conditions [97]. The model was fitted with the following parameters describing membrane properties: \(h = 4\) nm (and 2.1 nm for single LPS molecule, which spans only half of the bilayer), \(\mu_M = 180\) mPa·s, \(\mu_W = 1.003\) mPa·s, \(k_B T = 4 \cdot 10^{-21}\) J. The values of these parameters are in agreement with typical membrane properties used in membrane diffusion studies (for examples cf. ref. [98, 99]). The membrane viscosity (\(\mu_M\)) was estimated using the diffusion coefficient of single LPS and their known radius (a radius of 0.7 nm and a molecular area of 1.53 nm\(^2\) assuming molecules with cylindrical shape [155]). The logarithmic dependence of the diffusion coefficient on the domain size (radius and estimated number of molecules) is shown in Fig. 7.6.
Fig. 7.6. Dependence of the diffusion coefficient on the size (radius and number of molecules, log scale) of a diffusing domain according to the Saffman-Delbrück model (solid line, the log scale on both X-axes is used to obtain the same slope for the radius and the number of molecules which is related to the square of the radius). The measured diffusion coefficients of LPS-Alexa488 in GUVs containing LPS smooth and Ra at different concentrations are shown.

Estimated domain size parameters (radius and number of clustered molecules) are grouped in Tab. 7.1. Numbers of clustered molecules ($N_{agg}$) are calculated according to Eq. 7.2, assuming a cylindrical shape of both LPS clusters and molecules:

$$N_{agg} = \frac{\pi R^2}{\pi r^2}$$

[7.2]

where $R$ is the hydrodynamic radius of the domain, and $r$ is the hydrodynamic radius of a single LSP molecule.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$D_{\text{eff}}$ ($\mu$m$^2$s$^{-1}$)</th>
<th>$R$ (nm)</th>
<th>$N_{agg}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS Alexa488</td>
<td>5.43 ± 0.62</td>
<td>0.71</td>
<td>1</td>
</tr>
<tr>
<td>LPS smooth 7 mol%</td>
<td>2.73 ± 0.28</td>
<td>1.7</td>
<td>7</td>
</tr>
<tr>
<td>LPS smooth 12 mol%</td>
<td>1.36 ± 0.23</td>
<td>32</td>
<td>2100</td>
</tr>
<tr>
<td>LPS Ra 9 mol%</td>
<td>1.99 ± 0.36</td>
<td>8</td>
<td>130</td>
</tr>
<tr>
<td>LPS Ra 12 mol%</td>
<td>0.98 ± 0.21</td>
<td>73.8</td>
<td>11200</td>
</tr>
</tbody>
</table>

Using the microscope system with a resolution of ~380 nm in the x-y plane I estimated that the clustering of $>10^6$ molecules per leaflet is needed to visualize LPS smooth or Ra-enriched domains. In all cases (cf. Table 7.1), the estimated domain size is below the resolution limit of the microscopy system. This observation is in agreement with my experimental data.
Taking into account the raised LAURDAN GP and the slowed lateral diffusion of LPS molecules accompanying an increase in LPS concentration, I speculate that there is a threshold of the LPS concentration, below which LPS molecules form small clusters of a size depending on the LPS concentration, and above which the micrometer-size domains appear. The value of this threshold concentration depends on the size of the LPS polysaccharide group. For deep rough LPS chemotypes (LPS Rc and Rd2), the critical concentration is close to 10 mol%, but lipid A forms micrometer-size domains even at 5 mol%. LPS chemotypes with large polysaccharide groups (smooth, Ra) cannot form micrometer-size domains in an achievable concentration range (cf. discussion in chapter 6).

7.3. Discussion

In this chapter, the lateral structure model of membranes containing high concentrations of LPS was quantified using LAURDAN GP imaging and FCS. The impact of the LPS concentrations and the size of the LPS polysaccharide group on the phase behavior, with special focus on the LPS clustering, was evaluated. I found that the physicochemical behavior of LPS (membrane packing, lateral diffusion, clustering) follows the general tendency observed in LPS incorporation studies. I found a major difference in behavior between LPS chemotypes with a large (LPS smooth or Ra) and small or no lipopolysaccharide group (Rc, Rd2, or lipid A). In general, rough LPS easily forms large (micrometer-size) domains, which are in a gel-like phase (LAURDAN GP of approx. 0.55, in line with LPS aggregates studied in the fluorometer, cf. section 5.4). Large domains are formed already at 10 mol% of rough LPS. Lipid A, which completely lacks the polysaccharide group, forms large domains even at 5 mol% (and possibly in even lower concentrations). In contrast to rough LPS chemotypes, LPS smooth and Ra do not form microscopically resolvable domains at all studied concentrations (up to 12 mol%). Higher concentrations were not accessible due to limitations of our incorporation method. Still, this systematic study of the impact of the polysaccharide group size on the membrane lateral structure at that high LPS concentrations (up to 12 mol% for smooth and 22 mol% for rough LPS chemotypes) is the first of its kind.

In model phospholipids mixtures, the formation of a large-scale phase separation requires a difference in $T_m$ of the membrane components, and the system temperature being inbetween the components $T_m$. The higher the difference, the easier large-scale phase separation occurs,
and this tendency can be observed in many binary phospholipid mixtures [87, 94]. The $T_m$ of different LPS chemotypes depends on the size of the polysaccharide group. Lipid A, which completely lacks the polysaccharide group, has a much higher $T_m$ of 45°C than LPS smooth. For fully hydrated LPS in the absence of divalent cations, LPS smooth has the highest $T_m$ of 37°C. The LPS chemotypes with a smaller headgroup have smaller $T_m$ than the LPS with larger polysaccharide groups, e.g., $T_m$ of LPS Re is close to 31°C [3]. This tendency of the ordering effect of the polysaccharide group on the LPS aggregates can be easily explained by the formation of electrostatic bonds between polar sugar units. This may be interpreted as the polysaccharide group has two contrary effects on the structure of LPS aggregates: in the first place, it disorganizes the structure of compacted fatty-acid chains of lipid A, what is reflected in the difference of the $T_m$ values (45°C for lipid A to 31°C for LPS Re). But the larger polysaccharide groups can form more electrostatic bonds between polar monosaccharide groups. This has an ordering effect on the LPS assembly. The ordering effect is even more pronounced in the presence of divalent cations ($\text{Ca}^{2+}$, $\text{Mg}^{2+}$), where the $T_m$ of LPS can be shifted even above 75°C [49].

The feature of LPS-containing membranes to show large-scale phase separation as observed in my system is against the $T_m$ tendency in investigated selection of LPS chemotypes. Except for lipid A, which forms large gel-like domains even at low concentrations (5 mol% and probably below), the micrometer-size domains are observed only in samples of smaller $T_m$ differences between their components (LPS Rc or Rd2 and $E. \text{coli}$ polar lipid extract), in contrast to samples containing fraction of LPS smooth or Ra (with higher $T_m$). Here, I propose a model explaining this peculiar behavior. Based on the reduction the lateral diffusion of LPS at higher concentrations of LPS (Fig. 7.5), accompanied by a raised LAURDAN GP in samples where no visible phase separation occurs (Fig. 7.2), I speculate that the formation of submicroscopic LPS-rich domains is responsible for these phenomena. Studies on the rotational mobility of fluorescently labeled LPS in small vesicles (egg PC/cholesterol 7:3) suggested the clustering of smooth LPS (from $Nissleria meningitidis$) starting at approx. 0.125 mol% (1:800 LPS-to-phospholipids molar ratio) [143]. Above this critical concentration LPS, starts to cluster into domains. The increase of the concentration of LPS in the membrane is followed by an enlargement of the domain size as more LPS molecules cluster together (cf. Tab. 7.1). Above a certain concentration of LPS, the next critical transition occurs, and
micrometer-size domains are formed. The critical concentration required for this transition depends on the size of the polysaccharide group: the smaller the polysaccharide group, the lower the concentration required for the large-scale clustering. LPS chemotypes with large polysaccharide groups (smooth or Ra) cannot form micrometer-size domains in the concentration range achievable in my model (12 mol%). Interestingly, the raise of LAURDAN GP and the reduction of the lateral diffusion of LPS molecules, as a function of increasing LPS concentration in the membrane is larger for LPS Ra than for smooth LPS, which supports further my hypothesis, because the polysaccharide group of LPS smooth is larger than LPS Ra. However, in the case of lipid A, the change of LAURDAN GP in the liquid phase is very small, which suggest that only a very small fraction of lipid A forms small domains and most of the lipid A forms large gel-like domains. This is in contrast to the other LPS chemotypes studied here, for which the LAURDAN GP in the liquid phase rises even after gel-like domains are formed (Fig. 7.2). The dependence of domain size on the amount and nature of LPS observed in these experiments is interpreted as an interplay between different interactions occurring at the level of the LPS polar head group and those occurring in the hydrophobic part of the LPS molecules. Since in my experiments, the hydrophobic part of the different LPS molecules is the same (i.e., all investigated LPS chemotypes come from *E. coli* strains), it is reasonable to assume that the interactions occurring at the level of the polar headgroup can regulate the dimension of these gel-like domains. I speculate that a change in the nature of the LPS polar headgroup can modulate supramolecular interactions through steric effects, electrostatic repulsions, or eventually changes in lipid miscibility. All these effects may compromise the size of LPS-enriched domains in the plane of the membrane (cf. Fig. 7.6 and 7.7).
Fig. 7.7. Sketch representing the different LPS-rich domain sizes observed in my experiments. LPS chemotypes (A: LPS smooth or Ra; B: Re or Rd2 - deep rough chemotypes of LPS) inserted in the lipid membrane (PE and PG, cardiolipin not shown). (A) the lateral organization of LPS-smooth (valid also for LPS Ra) is represented for a low concentration of this lipid (domains in the sketch correspond to a diffusion twice lower than the single LPS molecule, cf. Tab. 7.1 and Fig. 7.6). (B) Formation of large membrane platforms (micrometer-sized) by the rough LPS chemotypes above 10 mol% LPS concentration.

Although a rich set of data on phase diagrams of pure LPS was published (discussed in chapter 6), the behavior of LPS in the membranes is studied less extensively. Takeuchi and Nikaido showed by EPR experiments with spin-labeled phospholipids that LPS does not mix with phospholipids in model membrane systems [156]. Tong and McIntosh showed in a supported lipid bilayers system formed from LPS Ra, Rd, or Rd mixed with *E. coli* phospholipids (1:1 molar), that LPS Ra is less ordered than LPS Rd at the same temperature [157]. This effect, which is in contrast to the expectations form the $T_m$ of LPS, is in agreement with my results. Tong et al. explained their findings by the disordering effect of the larger polysaccharide group in LPS Ra. Interestingly, the authors did not observe a separation between LPS Rd and phospholipids, which may be due to the impaired lateral diffusion in supported membranes. Urban et al. show a peculiar feature in a system of DPPE/DPPG (8:2) and LPS Re. The DPPE/DPPG (8:2) lipid mixture has a $T_m$ at approx. 61°C, but the addition of LPS Re ($T_m$ ~31°C [3]) up to 10 mol% caused an appearance of the second melting transition. Both melting transitions, which showed a high degree of cooperativity at the low LPS concentrations, were almost completely diminished to a very broad transition below 40°C as the LPS concentration approached 50 mol% [145]. The authors interpreted these results as a phase separation on the LPS/DPPE-rich and LPS/DPPG-rich phase for lower LPS Re concentrations (up to 10 mol%). At an equimolar ratio of LPS and phospholipids, cubic phases dominated over lamellar
structures. This is in agreement with findings that deep rough LPS chemotypes (and lipid A) prefer the H\text{II} or cubic phase, particularly above their $T_m$ (~31-32°C) [2, 3, 158]. Although, the results of Urban et al. are very interesting, the biological relevance of this study is limited, as lipids composition used there is very different from the natural bacterial lipids, which, unlike DPPE/DPPG, have a very broad melting transition close to 20-25°C ($E. coli$ grown at 37°C) [46]. Another difference between the model of Urban et al. and bacterial membranes is the relation between $T_m$ of LPS and phospholipids, respectively ($T_m$ of LPS higher than $T_m$ of phospholipids in bacterial membranes). My results are not in conflict because I used LPS chemotypes of larger polysaccharide groups and at lower concentrations. Moreover, $E. coli$-lipids used in my experiments have much lower $T_m$ than the DPPE/DPPG mixture. Gel-liquid phase separation observed in GUVs is due to the demixing between LPS or lipid A and $E. coli$-phospholipids. The partition of $E. coli$-phospholipids to the gel-like domains is unknow if it occurs at all. Rhodamine-DPPE is entirely excluded from LPS or lipid A-rich domains, but this phospholipid is known to prefer less ordered phases. However, LAURDAN, which is usually assumed to partition equally to various lipid domains [76], shows smaller partitioning to LPS or lipid A-rich domains than to phospholipid phase (Fig. 7.3). This may be due to a very tight packing of the fatty-acid chains within lipid A molecules [159]. Henning et al. used GUV model membranes containing LPS to examine the partition of LPS between gel and liquid phases in DPPC/DOPC 1:1 mixtures. DPPC/DOPC is mimicking eukaryotic membranes, and according to the authors, LPS partitions into the DPPC-rich, gel-like domains, not changing the lipid packing greatly [144]. This is probably due to the low concentration of LPS incorporated into the membrane (cf. discussion in chapter 6). A full comparizon between the model of Henning et al. and the model presented in this thesis cannot be performed because of the poor characterization of the first one, but I found interesting that at very low concentrations of 0.001 mol%, LPS seems to rather prefers the DOPC-rich phase than the gel one (cf. Fig. 7.4).

Besides the membrane models, LPS was also studied in its native environment, bacterial cells. The crucial role of divalent cations, which bridge LPS molecules, for the barrier functions of the bacterial OM was shown in many studies [1, 49, 160, 161]. The effect of divalent cations on the phase behavior of LPS was discussed earlier (cf. ref. [1, 49, 141, 162]). A particularly interesting study, which may also give some insight into my model's behavior, was performed
by Amro et al. In that study, the examination of the *E. coli* cell surface by atomic force microscope revealed the existence of inhomogeneous LPS patches, containing 100-1000 LPS molecules each, and regions of high local curvature [161]. The depletion of divalent cations by the chelating agent EDTA destabilizes the LPS membrane, leading to the release of LPS and OM proteins from the membrane and the formation of the irregularly shaped pits. This result may be compared to my model systems containing LPS smooth and LPS Ra because Amro et al. used wild type *E. coli* producing LPS of the smooth chemotype. The destabilization of the LPS-containing membrane upon the depletion of divalent cations reported by Leive et al. and Amro et al. [160, 161] may be caused by an increase of the steric repulsion between the polysaccharide chains, which are normally bridged by the positively charged ions. For the same reason both the membrane incorporation and the large domain formation of smooth or Ra LPS may be impaired in my model system.

The existence of gel-like phases of LPS in membranes as observed in my experiments is supported by studies using calorimetry proof the high $T_m$ of LPS in bacterial cells (>60°C in the presence of divalent cations [49, 163]). However, Vanounou et al. used LAURDAN (LAURDAN GP) and DPH (anisotropy) in regular bulk fluorescence spectroscopy experiments to evaluate the phase behavior of intact bacterial cell membranes. This last study interpreted the coexistence of two phases of different polarity and packing as the “lipid-rich” and “protein-rich” membranes [164]. This is consistent with the presence of the OM and the IM, but not related to gel-like LPS-enriched domains, which are known to exist in the outer leaflet of the OM [49, 163]. The conclusions made from bulk LAURDAN GP experiments, without further information from spatially resolved LAURDAN GP, but available in fluorescence microscopy experiments, can be misleading. As reported by Fidorra et al., bulk LAURDAN GP experiments with ceramide/POPC mixtures [165] suggest the absence of the gel/liquid-disordered phase coexistence, a situation that is in contrast to the data obtained on the same mixture using LAURDAN GP imaging, where the membrane domains can be spatially resolved. Because of the additive property of the GP function, the detection of the gel/liquid phase coexistence using bulk LAURDAN GP measurements requires a particular quantity of gel-phase areas in the membrane. The same arguments hold for the DPH anisotropy results, because the anisotropy shows the same additive property as the LAURDAN GP function [72, 166]. The intensity of the fluorescence signal coming from LPS
7.4. Summary

In this chapter, the impact of the LPS concentration and the polysaccharide chain size on the lateral physicochemical properties of E. coli -phospholipid membranes was investigated using GUVs and 2-photon microscopy techniques. Using LAURDAN GP imaging and FCS, the lateral packing of lipids and the lateral diffusion of LPS molecules were characterized. The lateral structure of the LPS-containing model membranes clearly depends on both studied parameters, i.e., the concentration and LPS chemotype. I found that rough LPS (Rc and Rd2) forms gel-like domains in E. coli-phospholipid membranes at concentrations above 10 mol%. This is similar to lipid A, which forms the gel-like domains already at 5 mol% and probably even below this concentration. In contrast to deep rough chemotypes, LPS smooth and Ra do not form micrometer-size domains up to 12 mol%, but my results (LAURDAN GP and FCS) suggest the existence of domains of sizes below the resolution of our microscopy system. I propose a scenario, in which LPS starts to form small clusters in the membrane already at low concentrations (possibly below 1 mol%) [143]. The raise of the LPS concentration is followed by an increase of LPS domain size up to a critical concentration where micrometer-size domains are formed. This critical concentration depends on the LPS chemotype and is higher for LPS with larger polysaccharide groups. This behavior is in contrast to the prediction based on the Tm of LPS and can be explained by the change of the structure and size of the LPS polar headgroup, which can modulate supramolecular interactions through steric effects, electrostatic repulsions, or eventually changes in lipid miscibility. All these effects may compromise the size of LPS-enriched domains in the plane of the membrane, as well as the overall membrane stability, particularly in the absence of stabilizing agents [1, 160, 161].

The ability of LPS to cluster and form gel-like phases is particularly important for the bacterial IM, which functions as a permeability barrier. The interface composed of
hydrophilic polysaccharide groups, which are stabilized by the bridging effect of divalent cations, and the tightly packed fatty-acid chains of lipid A is an excellent barrier against both hydrophilic and hydrophobic agents [1]. The presence of the membranes in the ordered phases is very often associated with a barrier function of the tissues they exist in, e.g, apical membranes of epithelial cells are mainly composed of sphingolipid/cholesterol [167] or skin stratum corneum membranes containing ceramides and cholesterol [27].
Chapter 8.

Interaction of SP-D with LPS

Pulmonary surfactant-associated protein D (SP-D) is, together with the related SP-A, a member of a protein group called collectins. Collectins are calcium-dependent lectins (sugar-binding proteins) containing collagenous and C-type lectin domains [59]. SP-D binds to many pathogens, including Gram-negative (via LPS) and Gram-positive (via lipoteichoic acid) bacteria, mycobacteria (via lipoarabinomannan), and some viruses (via high-mannose oligosaccharides associated with hemagglutynin of the influenza A virus) [60, 63]. The interaction with pathogens is mainly governed by their glycoconjugates. SP-D binds mannose and mannose-rich saccharides in a Ca\textsuperscript{2+}-dependent manner. The known mechanism of the SP-D action involves binding to bacteria (and other pathogens) via their LPS (or other surface saccharides) and cause the opsonization or agglutination of the pathogens. An alternative mechanism of the antimicrobial activity of SP-D (and other collectins), proposed by Kuzmenko et al. [69], is attributed to the permeabilization of the pathogens' membranes. The mechanism of this hypothetical phenomenon is unknown, and experimental data supporting this mechanism of SP-D action is very limited.

In this chapter, I focused on the interaction between SP-D and LPS, in particular the membrane-associated form of LPS. For this purpose the membrane models containing LPS, described in previous chapters (cf. chapter 6 and 7) are used. Various LPS chemotypes were chosen to evaluate the SP-D binding efficiency towards different antigens. Liposome models were used to measure the effect of SP-D on membranes containing different LPS by means of the FCS technique.
8.1. The relative efficiency of SP-D binding to various LPS chemotypes

SP-D is known to have different binding efficiencies towards different sugar units, i.e., the highest binding efficiency is towards mannose, smaller towards glucose, etc. (cf. Section 1.4.4 and discussion in this chapter). The efficiency of SP-D binding to LPS molecules depends on the presence of preferred monosaccharide units as well as on their availability for binding. The binding to smooth LPS is usually less efficient than to rough LPS. The selection of LPS chemotypes (smooth and rough) form *E. coli*, *Salmonella minnesota*, and *S. typhosa*, together with LPA from *Bacillus abortus* (Gram-negative bacteria) was used in an ELISA assays (cf. section 4.4) to quantify SP-D binding.

![Graph showing relative binding efficiency of mSP-D to various LPS chemotypes](image)

Fig. 8.1. Relative binding efficiency of mSP-D to various LPS chemotypes (various strains of *E. coli*, *S. minnesota* wild type, and *S. typhosa*) and LPA (from *B. subtilis*). The concentration of SP-D is in log scale. In the presence of EDTA, no binding was observed (not shown in the figure).

In agreement with the studies on the SP-D binding affinity towards different LPS chemotypes, SP-D shows almost no binding of the smooth LPS chemotypes, i.e., LPS from *E. coli* strains with different O-specific chain variants: O55:B5; O111, O127. LPS smooth from the *S. minnesota* (wild type) is bounded poorly, similarly to LPS Ra from *E. coli*. LPS Rd is bound with a similar efficiency as LPA from *B. subtilis*. SP-D reaches stronger binding against LPS Rc from a medium *E. coli* rough mutant and LPS from *Salmonella typhosa*. In the case of this
last LPS chemotype, the binding assay saturates already at approx. 0.2 ng/mL of SP-D. The binding saturation is either due to the limit in number of proteins in solution or LPS available for binding. The amount of LPS was not quantified, but equal mass of LPS was deposited in each well without further characterization. Differences in binding may be due to variations in the amount of LPS in each well. The difference in the relative binding efficiency of the same LPS chemotype (or LPA) between experiments (cf. Figs. 8.1 and 8.2) are most likely due to the variation in the LPS deposition efficiency in different experimental rounds. Within single procedure performances, the LPS deposition rates do not vary significantly. In the presence of EDTA (when all Ca$^{2+}$ was chelated) no binding of SP-D to LPS was observed, which proves that the binding was due to the specific collectin activity of SP-D.

Fig. 8.2. Relative binding efficiency of SP-D to various LPS chemotypes selected for highest binding efficiency. The concentration of SP-D is in log scale. No LPS was present in the control sample. In the presence of EDTA, no binding was observed, similarly to the sample with LPS smooth (E. coli O55:B5).

8.2. Aggregation of LPS-containing vesicles by SP-D binding

The model membranes containing LPS (described in chapter 6) were used to characterize the effect of SP-D binding to different LPS chemotypes presented on the vesicles' surface. Large unilamellar vesicles containing 1 mol% of LPS (S. typhosa LPS, E. coli LPS J5 Rc, and E. coli O55:B5 LPS smooth) were labeled with rhodamine-DPPE (0.05 mol%). The vesicles were extruded 30 times through a polycarbonate filter (50 or 100 nm pore diameter) at 55°C. The
reaction buffer contained either 1 mM Ca$^{2+}$ (required for SP-D binding) or 1 mM EDTA. Vesicle aggregation was quantified by FCS measurements determining the $D_{\text{coef}}$ of vesicles. Liposomes contain a substantial negative surface charge due to the presence of PG, CA, and LPS. Approx. 25-30 mol% of all lipids in the model system are negatively charged. LPS contains multiple negatively charged groups. Divalent cations may promote the fusion of negatively charged membranes, and although the concentration of Ca$^{2+}$ in the samples was relatively small (1 mM), a control experiment was performed. No change in the diffusion of a LPS-containing vesicles in the presence of 1 mM Ca$^{2+}$ and 150 mM NaCl was observed over the period a 65 hours (Fig. 8.3). This result indicates that no substantial change in the size of the vesicles can be attributed to the interactions of negatively charged lipids with divalent cations under the studied conditions.

Fig. 8.2. The experimental ACFs of the vesicles at different time points after extrusion. Vesicles were composed of the *E. coli* polar lipid extract with 0.05 mol% rhodamine-DPPE, and 1 mM Ca$^{2+}$ was present. No changes in the ACF position indicate that the $D_{\text{coef}}$ (and the vesicles size) remains the same during the experiment time (up to 65 h).

The vesicle cross-linking experiment was performed similarly to the SP-D binding assay (same concentration range of SP-D was used), vesicles were incubated in presence of SP-D and 1mM Ca$^{2+}$ (and EDTA in the control experiment) for approx. 2 h. The experimental ACF calculated from data obtained by FCS measurements were modeled with a single-species diffusion in a 3-dimensional Gaussian excitation volume. The diffusion coefficient was also used for calculating the hydrodynamic radius of diffusing objects (cf. section 2.3.2, Eq. 2.19).
Fig. 8.3. Diffusion coefficients (left plot) and hydrodynamic radius (right plot) of the LPS-containing liposomes (or aggregates of the liposomes): LPS from *S. typhosa* (black), *E. coli* J5 (Rc) (red), and *E. coli* O55:B5 (smooth) (blue) in the presence (open symbols) or lack (solid symbols) of Ca$^{2+}$ (1 mM). No aggregation was observed in absence of Ca$^{2+}$ or for LPS smooth (*E. coli* O55:B5) samples. The concentration of SP-D is given as the concentration of the SP-D dodecamers. Error bars are standard deviations of the experimental data.

Similarly to results obtained by ELISA, the most binding of SP-D was observed in case of LPS from *S. thyphosa*. Also, the largest aggregates were formed in the presence of this LPS chemotype (Fig. 8.3). Vesicles containing smooth LPS (*E. coli* O55:B5) showed no aggregation caused by SP-D, because there was no detectable binding of SP-D to this chemotype (and to all *E. coli* smooth LPS strains) (Fig. 8.2). Vesicles containing LPS Rc showed medium aggregation. In this experiment, the amount of LPS available for SP-D binding was the same in all samples, and the concentration of all components was kept constant (0.3 mM phospholipid concentration). This is contrast to ELISA experiment, where the amount of LPS in the system cannot be controlled. The difference in aggregation is therefore only due to the characteristic of LPS-SP-D interactions. The size of the aggregates in presence of approx. 2 nM SP-D (concentration of SP-D dodecamers, corresponding to ca. 10 μg/mL) was approx. 3 or 6 times larger than the single vesicles (for *E. coli* J5 and *S. typhosa* LPS, respectively). This 3- or 6-fold increase of the hydrodynamic radius corresponds to the aggregation of approx. 30 and 200 vesicles, respectively. Although the aggregation due to the cross-linking of the vesicles by SP-D is easily seen, it is unknown, if aggregation causes
a mixing of the membranes. This is, however unlikely because the proteins may act like spacers between membranes, with a size of approx. 100 nm. When the aforementioned samples were visualized using the microscope, micrometer-size aggregates where observed for the highest SP-D concentrations (Fig. 8.4).

Fig. 8.4. 2photon-LSM images (false color representation) of LUVs containing LPS from *S. typhosa* (A), *E. coli* J5 (Rc) (B), and *E. coli* O55:B5 (smooth) (C) in presence of different SP-D dodecamers concentrations (0, 0.5 nM, 1 nM, and 2 nM). The fluorescence intensity was not normalized and can be compared only within rows, but not in columns. The frame size is 10*10 μm. Each image is a sum of 100 frames.

In the case of LPS from *S. typhosa*, already at a 1 nM concentration of SP-D dodecamers, large aggregates of up to 1 μm can be observed. Vesicles containing LPS Rc are less aggregated, but also in this case aggregates can be observed using LSM. No change was observed in samples containing LPS smooth (*E. coli* O55:B5). Particularly in the sample containing LPS from *S. typhosa*, the decrease of the free vesicle concentration can be easily seen, visible as a decrease in the background signal intensity (Fig. 8.4. A).

### 8.3. Discussion

SP-D binds to many pathogens mainly via their glycoconjugates. SP-D binds mannose and mannose-rich saccharides in Ca^{2+}-dependent manner. Besides mannose, SP-D can recognize other saccharide units, such as glucose, fucose, N-acetyl-glucosamine, N-acetylmannosamine, and inositol [5, 168]. The binding efficiency of the pathogens carbohydrates depends on availability of preferred sugar residues. SP-D preferably binds to L-glycero-D-manno-heptose, which is the most common heptose found in the core regions of Gram-
negative bacteria, and to D,D-heptose. Other sugars L-D-heptose or D-maltose, are bound less efficiently. Interestingly, SP-D shows no binding of KDO, which is the only saccharide present in the LPS Re [168]. SP-D binds membrane-bound and free LPS at its core region, which contains heptose, but also at O-specific chains of some smooth phase variants of *Klebsiella pneumoniae* because of the presence of preferred saccharide units [65]. My results from SP-D ELISA are in agreement with already published data: I observed very weak or no binding of SP-D to smooth chemotypes of LPS. This is because the saccharide units preferentially bound by SP-D are in the core region of LPS, and in smooth LPS chemotypes, the preferred saccharide units are hidden under O-specific chains. The agglutination of bacterial cells upon the binding by SP-D was reported for certain strains of *E. coli* and other Gram-negative bacteria [65, 66]. The inhibition of a bacterial growth upon the SP-D binding was reported for rough strains, but not for smooth strains [169]. The aggregation potency of SP-D (cf. Fig. 8.4) follows the same trend, and is in line with the relative binding efficiency measured in the ELISA experiment. The aggregation potency can be associated with binding efficiency to a LPS chemotype synthesized by a given bacterial strain. The SP-D-dependent agglutination and/or internalization and killing of Gram-negative bacteria by macrophages are the most typical SP-D activity against pathogens [5]. In addition, the interactions of SP-D with some rough *E. coli* mutants, synthesising LPS Rc, was reported to mediate direct bactericidal effects due to the increase of bacterial membrane permeability. This effect was observed in bacterial cells and model membranes [69, 169], but little is known about possible mechanism of this phenomenon.

### 8.4. Summary

My results from SP-D ELISA are in agreement with already published data: I observed very weak or no binding of SP-D to smooth chemotypes of LPS. This is because the saccharide units preferentially bounded by SP-D are in the core region of LPS, and in smooth LPS chemotypes, the preferred saccharide units are hidden under the O-specific chains. The aggregation potency of SP-D towards LPS-containing membranes follows the same trend. The obtained results have a preliminary nature, and are a part of the still ongoing project. LPS -containing membranes (as small liposomes and GUVs) seems to be a valuable tool to quantify the interactions between the model bacterial membranes and SP-D, as the system
composition is very easy to control. Particularly, the changes in the membrane phase state upon binding of SP-D, and the hypothetical effect on the membrane permeabilization could be studied using methods and model system developed within this thesis.
Chapter 9.

Conclusion and future perspectives

This thesis contributes to the understanding of the behavior of lipopolysaccharide in model membranes, together with some technical aspects on the model system preparation. The major part of this thesis was development and characterization of model membranes containing lipopolysaccharides: small liposomes and giant unilamellar vesicles. Particularly the GUV models proved to be extremely useful to study model membranes with a variety of fluorescent techniques [76]. The novelty of this work is based on a systematic approach to study the effect of LPS size on (i) the incorporation of LPS into the membrane and (ii) the lateral structure of the model membranes. Most of the previously published work focused either on constructing phase diagrams of free LPS [2, 3, 162] or interactions with the immune system [58], and studies using model membranes often lack the full characterization of the system [144] or physiological relevance [145]. The work presented in this thesis provides thorough characterization of the system components and some new insight which may contribute to better understanding the characteristic of LPS in both model membranes and natural bacterial cells.

In this thesis work, the protocol to incorporate high LPS concentrations in model membranes was developed. The protocol was based on the already existing method of Dijsktra et al. [4] but the LPS concentration range was increased by an order of magnitude (up to 12 mol% for smooth and 25 mol% for rough LPS) and the selection of LP smooth S chemotypes was used to evaluate the impact of the LPS head group size. The purification and thorough
characterization provided full information on my system, therefore number of conclusions and hypotheses could be made. I found, that incorporation of LPS into the membrane is never trivial, particularly for the smooth LPS, and the incorporation efficiency is decreased at larger initial concentrations of LPS (from almost 100% at 1-5 mol%, till 36% in the extreme case of smooth LPS at high concentration), therefore the incorporation of LPS cannot be taken for granted, but should be characterized every time. I also found major distinction in the behavior between smooth or Ra LPS and deep rough (Rc and others) LPS in means of incorporation efficiency, but also the lateral physicochemical properties (phase coexistence, domain formation). I also found the limit of incorporation (app. 30 mol%), above which GUVs cannot be formed, probably because of the transition of LPS to non-lamellar phases. Partial insight of the aforementioned features of LPS-containing systems in the context of the incorporation of LPS into the membranes was spread over several reports [4, 122, 142, 143, 145]. The work in this thesis provides a more complete picture.

To characterize my model system, the set of techniques, mainly 2-photon fluorescence microscopy (LAURDAN GP imaging, FCS) was used. These techniques provide excellent insight into the membrane phase state and the (membrane) dynamics, with the spatial resolution of light microscopy. Both techniques were used to access the spatially resolved membrane phase state, the lipid demixing and dynamics. Similarly to the incorporation studies, smooth and rough LPS chemotypes showed very different behavior. Although the aggregation of LPS in the membrane seems to happen at relatively small concentrations (5 mol%), in the presence of more than 10 mol% of deep rough LPS chemotypes (Rc or Rd2) large scale phase separation is trigger and micrometer-size domains in a gel-like phase are formed. Lipid A forms gel-like domains already at 5 mol% and the presence of sub-microscopic lipid A clusters exist in a much smaller extent if they exist at all.

This study demonstrates the importance of the characterization of all system components, particularly the probes. If the fluorescent probes, which partition to the different lipid phases (LAURDAN, DPH) are in use, it is important to know if the assumption that those probes partition equally to both phases is true. Particularly in bulk fluorescence studies, the fractions of the probe in different phases have to be balanced in order to be observed. I found a very low partitioning of LAURDAN into the LPS-rich clusters in phospholipid membranes, which is likely the reason for the observation of no gel-like phases in bacterial cell membranes in the
bulk fluorescence studies [164]. However, it is well known that LPS prefers formation of gel-like structures under physiological range of temperatures (ref. [3] and my results). Similar case, describing the discrepancy between the result from bulk fluorescence and microscopy studies of the phase separation in the PC/ceramide mixtures was described in ref. [165].

The model of the LPS-containing membranes, developed during this thesis, was well characterize and will be used in the ongoing projects, e.g., on the effect on the membrane structure upon SP-D binding, or the effect of physicochemical conditions (as divalent cations concentration) on the later structure of LPS-containing membranes. The GUVs formed from bacterial membranes will be also used for studying the bacterial membrane-associated proteins at the single molecule level using fluorescence microscopy.

The development and characterization of the model described in this thesis required the testing of many different experimental techniques ranging from common molecular biology methods to sophisticated spectroscopic techniques. Especially, gaining experience of how to handle LPS and what is the best way of proceeding with LPS incorporation, purification, etc., was very laborious and time consuming, as that was a completely new project started in our group. The FCS technique was newly introduced to the group, and required a broad testing and a system characterization. The expertise in the membranes/liposomes preparation and handling, and in the fluorescence techniques gained, during the work on this thesis, was shared with other researchers, and contributed strongly to many ongoing research projects.


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Manuscript
Lipid lateral organization on giant unilamellar vesicles containing lipopolysaccharides

Jakubs Kubiak†‡, Jonathan Brewer†§, Søren Hansen¶ and Luis A. Bagatolli†§

†Membrane Biophysics and Biophotonics group/MEMPHYS - Center of Biomembrane Physics,
‡Department of Physics and Chemistry, §Department of Biochemistry and Molecular Biology,
¶Department of Cancer and Inflammation Research, University of Southern Denmark, Odense, Denmark.

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*Correspondence regarding this manuscript should be sent to Dr. Luis A. Bagatolli at the following addresses:

Membrane Biophysics and Biophotonics group / MEMPHYS – Center for Biomembrane Physics, Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark. e-mail: bagatolli@memphys.sdu.dk
Abstract
We developed a new a protocol to generate giant unilamellar vesicles (GUVs) composed of mixtures of a single lipopolysaccharide (LPS) specie and *Escherichia coli* (*E. coli*) polar lipid extracts. Four different LPSs differing in the size of the polar head group (i.e. LPS smooth > LPS-Ra > LPS-Rc > LPS-Rd) were selected to generate GUVs composed of different LPS/E.coli polar lipid mixtures. The new procedure contains two main steps: i) generation and purification of oligolamellar liposomes containing LPSs and ii) electroformation of GUVs using the LPS-containing oligolamellar vesicles at physiological salt and pH conditions. Analysis of LPS incorporation into the membrane models (both oligolamellar vesicles and GUVs) shows that the final concentration of LPS is lower than that expected from the initial *E.coli* lipids/LPS mixture’s proportion. Particularly, our protocol allows incorporation of no more than 15 mol % for LPS-smooth and LPS-Ra, and up to 25 mol % for LPS-Rc and LPS-Rd (respect to total lipids). These GUVs were used to evaluate the impact of different LPS species on the lateral structure of the host membrane (i.e. *E. coli* polar lipid extract). Rhodamine-DPPE labeled GUVs show the presence of elongated micrometer sized lipid domains for GUVs containing either LPS-Rc or LPS-Rd above 10 mol %. LAURDAN GP images confirm this finding and show that this particular lateral scenario corresponds to the coexistence of fluid disordered and gel (LPS enriched) -like micron sized domains, similar to that observed when LPS is replaced with lipid A. For those LPSs containing the more bulky polar head group (i.e. LPS-smooth and LPS-Ra), absence of micrometer sized domains is observed for all LPSs concentration explored in the GUVs (up to ~15 mol %). However, fluorescence correlation spectroscopy (using fluorescently labeled LPS) and LAURDAN GP experiments in these microscopically homogeneous membranes suggest the presence of LPS clusters with dimensions below our microscope’s resolution (~380 nm radial). Our results indicate that in these “bacterial” model membranes, LPSs can cluster into gel-like domains and the size of these domains depends on LPS’s chemical structure and concentration.
Introduction
Lipopolysaccharides (LPSs) are critical components of the outer membrane of Gram-negative bacteria (e.g. *Escherichia coli*, *Salmonella enterica*). LPS lipids are part of a specialized barrier against macromolecules (e.g. lysozyme and antimicrobial peptide), hydrophobic compounds (e.g. antibiotics, bile salts) and other chemical agents that caused stress to Gram negative bacterial cells. The negative charge contributed by LPSs and their association with divalent cations, for example, helps to maintain the structural integrity of the overall outer bacterial membrane (1-2). In addition to their importance in the structure of Gram negative bacterial cell’s membranes, LPSs are important toxic agents towards normal animal immune systems; i.e. LPSs act as pyrogenic endotoxins generating massive inflammatory responses by activating macrophages and other cell types (1).

The outer membrane of Gram-negative bacteria is highly asymmetric and the outer leaflet contains a high percentage of LPSs (covering ~75% of the gram negative surface) (1). LPSs represent a group of complex amphiphilic molecules composed of a lipophilic part called lipid A which anchors LPSs molecules to the membrane, and a poly- or oligosaccharide portion which may extend up to 10nm outside of the bacterial membrane surface. The Lipid A structure comprise a biphosphorylated β-(1→6)-linked glucosamine disaccharide substituted with fatty acids ester linkages at positions 3 and 3′ and amide linked at positions 2 and 2′. The total amount of acyl groups per lipid A varies from 4 to 6, and generally comprise C\textsubscript{10} to C\textsubscript{16} acyl chains (hydroxylated or unhydroxylated fatty acids) although longer chain exist depending on the bacteria class (2), (see Fig. 1).

Gram negative bacterial cells contain a diverse selection of LPSs particularly differing in the polysaccharides length. This polysaccharide core is divided in three parts, named inner core, outer core and an O-specific chain (knows as O-antigen polysaccharide groups), Fig. 1. These “complete” LPSs molecules (containing the inner and outer cores plus the O-specific chain) present in wild-type strains are generally called “smooth” LPSs. Typical LPS cores structures (inner and outer) for enteric bacterial LPSs consist of 8-12 often branched sugars (2), with the sugar at the reducing end always being α-3-deoxy-D-manno-oct-2-ulosonic acid (also known as keto-deoxyoctulosonate or Kdo) (2→6) linked to the glucosamine of lipid A. Other sugars present in the core are generally L-glycero-D-manno-heptose residues including glucose, galactose and their derivatives (2).

Rough-chemotype LPS are mutant LPS molecules and are named according to the size of the oligosaccharide domain. Ra, Rb, Rc, Rd and Re correspond to the first, second, third, fourth and fifth degrees of polysaccharide chain length in order of decreasing domain size (3), see Fig. 1. The variety of “rough” mutants producing LPS lacking the sections of polysaccharide group (LPS-Ra to Re) is obtained by blocking steps in LPS synthetic pathway. For example, minimal *E. coli* LPS, named LPS-Re, consists of lipid A linking two Kdo residues. In general rough mutants are more sensitive to hydrophobic agents like antibiotics, detergents or mutagens. They are also less virulent and more prone to detection by immune systems (4).

Most LPSs related studies have typically focused on two main aspects: structure/behavior of LPS containing membranes (see below) or interactions of LPS with relevant components of the mammal’s immune system. LPSs acts on the immune system when they are released from bacterial cells. Small amounts of LPS are released during cell division but massive LPS release can occur when bacteria are killed by antibiotics or the immune system of the host organism (1). The activation of the immune system is mainly due to the interaction with macrophages, therefore understanding the interaction of LPS with macrophage membranes is an important factor. To study this process (and also to test the use of LPSs containing liposomes as potential vaccine) LPSs containing model
membrane systems were developed by Dijsktra et al. (5) and successfully applied for various LPS strains (5-7). Particular protocols for LPS incorporation in membranes used PC/PS/Chol lipid mixtures (which mimic eukaryotic membrane) as host membranes. These LPSs containing liposomal formulations are generally less toxic than free LPSs (5, 7). Additional giant unilamellar vesicle models were further developed to study LPSs partition in lipid mixtures displaying coexistence of solid ordered/liquid disordered phases (i.e. DPPC/DOPC) (8).

Studies about structure and physical properties of LPS containing membranes such as phase behavior and aggregation properties were previously reported in the literature (3, 9-13). Different supramolecular assemblies such as spherical (normal or inverted micelles), lamellar, tubular (normal or inverted H_{I} and H_{II}) and cubic has been reported to exist in samples of pure LPS above the CMC. This largely depends on the molecular geometry of monomeric LPS and the environmental conditions such as hydration, temperature and ion content (3). Accordingly, the phase behavior of pure LPS containing membranes is also extremely dependent on the aforementioned factors. For example, it has been reported that the main phase transition temperature (T_{m} from order to disordered -like phases) for fully hydrated smooth E. coli LPS is 37ºC. It has been shown that this T_{m} decreases by shortening the polysaccharide group. However, in the case of lipid A, (which lacks the polysaccharide group), the T_{m} raise to 45ºC (9). Interestingly enough, since the LPSs T_{m} is substantially higher than the T_{m} of the phospholipids of E. coli or eukaryotic membranes, a connection of LPS with the “raft” hypothesis was proposed (14). In this context, existence of cardiolipin or LPS -rich membrane domains and its role on antimicrobial peptides action was reported (15).

Our work is focused on investigating the physical properties of LPSs/E. coli lipids containing membranes. Although these model systems lack lipid asymmetry, they has been previously used as model mimicking bacterial membranes (mixtures containing LPS plus PE/PG or LPS plus E. coli lipid extracts -PE, PG, cardiolipin ~ 81:17:2 molar) (16-18). In the first part of this report we present a modified protocol to generate GUVs containing LPSs under physiological salt and pH conditions. Part of this section includes studies devoted to ascertain the efficiency of LPSs incorporation into model membranes for high LPS-to-phospholipids rates since this information is generally lack in the existing literature. Finally, the GUVs were used to evaluate the impact of different LPS species in the lateral structure of these bacterial model membranes. By using multiphoton excitation fluorescence microscopy based techniques, spatially resolved information (lateral structure, dynamics) at the level of single vesicles was obtained as a function of the concentration of different LPS species.

Material and Methods

Materials

E. coli Polar Lipid Extract (referred as E. coli lipids in the rest of the manuscript) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). LPS from Escherichia coli O55:B5 (LPS-smooth), LPS Escherichia coli O55:B5 fluorescein isothiocyanate conjugate (LPS-FITC), E. coli EH-100 (LPS-Ra mutant), E. coli J5 (LPS-Rc mutant), E. coli F-583 (LPS-Rd mutant), Lipid A monophosphoryl from E. coli F583 (Rd mutant) were obtained from (Sigma-Aldrich, St. Louis, MO). 6-dodecanoyl-2-dimethylaminonaphthalene (LAURDAN), Lissamine-rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine-DHPE), and Alexa Fluor 488 hydrazide sodium salt were purchased from Molecular Probes (Eugene, OR).
Methods

Labeling LPS with Alexa fluor 488 hydrazide

LPS from *E. coli* O55:B5 (smooth) was labeled with Alexa Fluor 488 hydrazide according to a protocol described by Luk et al. (19) with modifications (see Section 1 in the Supporting Material).

Incorporation of LPS into oligolamellar vesicles

In order to incorporate LPS into the liposomal membrane a dehydration-rehydration method proposed by Dijkstra et al. (5) with some modifications was used. Dehydration-rehydration method is more efficient to incorporate LPS into liposomal membranes (particularly those containing negative charges) compared with prolonged sonication methods (Ref. 5 and our findings). The phospholipid vesicles were prepared from stocks of *E. coli* polar lipid extracts mixed with fluorescent probes (either rhodamine-DHPE or LAURDAN) in chloroform/methanol 2:1 (vol). Briefly, organic stocks of fluorescently labeled *E. coli* lipids were deposited in borosilicate-glass vials in various amounts (in order to obtain various LPS to *E. coli* lipids ratio), dried under a N$_2$ stream and exposure to low pressure in a desiccator for at least 2 hours. The lipid films were hydrated in 0.4-0.5 mM LPS (LPS-smooth, LPS-Ra, LPS-Rc or LPS-Rd) water solutions at 55°C for 20 minutes using continuous vortexing (Eppendorf Thermomixer Comfort R). These samples were further sonicated in bath sonicator (Branson 1510, Branson Ultrasonic Corporation, Danbury, CT) at 55°C for 20 minutes, freeze in liquid nitrogen and lyophilized for 5 hours. Dried samples were re-suspended in 150 mM NaCl to a final phospholipids concentrations of 2 mM, vortexed and further sonicated in bath sonicator (Branson 1510, Ultrasonic Corporation, Danbury, CT) at 55°C for 20 minutes. The LPS containing liposomes were separated from non-incorporated LPS (both aggregates and monomers) on size exclusion chromatography (Sephacryl S-400, from GE Healthcare). Separation of LPS containing liposomes from LPS monomers and aggregates on the column was evaluated using controls with fluorescently labeled liposomes and LPS, i.e. the liposomal fraction was detected in the column void volume and LPS eluted later (see Fig. S1 in the Supporting Material). After this procedure the liposomal fraction was taken for further analysis and finally used to prepare GUVs. The total phospholipid concentration and LPS concentration in these samples were determined by inorganic phosphate assay (20) and 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) assay (21), respectively. The preparation of oligolamellar vesicles containing Lipid A were done by mixing *E. coli* polar lipid extract with Lipid A in chloroform/methanol 2:1 (vol), followed by removal of the organic solvent, hydration with 10 mM phosphate buffer 150 mM NaCl pH 7.4 and sonication at 55°C as indicated above.

Preparation of giant unilamelar vesicles

GUVs were prepared from LPS-containing liposomes according to a recently reported protocol (22). Briefly, 0.1 mM solution of LPS-containing oligolamellar vesicles were deposited on the Pt electrodes (1 μl per electrode) of a custom built electroformation chamber (23) and dried under vacuum for 10 minutes. This procedure was repeated 3-4 times before electroformation. After final deposition of the oligolamellar vesicles on the Pt wires, 500 μL of 10 mM phosphate buffer 150 mM NaCl pH 7.4 was added to the electroformation chamber and alternate electric field was applied in three steps: 1) freq. 500 Hz, amp. 35 V/m for 5 min, 2) freq. 500 Hz, amp. 313 V/m for 20 min, 3) freq. 500 Hz, amp. 870 V/m for 90 min. Electroformation was performed at 55°C. Further visualization of GUVs was performed directly on the GUV electroformation chamber.
For the microscopy experiments ~25 vesicles per sample were analyzed.

**LAURDAN GP function and two photon excitation LAURDAN GP measurements**

The LAURDAN GP function is sensitive to membrane lateral packing (24-28). The spatially resolved GP information obtained from two-photon excitation microscopy allow to directly infer the local phase state (or phase coexistence) at the level of single vesicles at equilibrium conditions (27). Information regarding definition of the GP function and technical aspects of the two photon excitation microscope experimental setup (including data analysis) are described in the supporting material, Section 2.

**Fluorescence correlation spectroscopy of LPS-A488**

The FCS measurements were performed in a custom built two photon excitation fluorescence microscope previously described in (28). The measurements were taken using SimFCS (software package developed at the Laboratory for Fluorescence Dynamics at the University of California, Irvine). Fluorescence fluctuation data were globally fitted using Globals for Spectroscopy (Globals software package developed at the Laboratory for Fluorescence Dynamics at the University of California, Irvine (29)) for details regarding FCS experiment see Section 3 in the Supporting Material).

**Results**

Our preparation procedure for GUVs containing LPS renders a good yield of giant vesicles with an average diameter of 20 μm. The most laborious part of this protocol was the optimization of LPS incorporation into oligolamellar vesicles and further purification of these vesicles from the remaining LPS monomers and self-aggregates. Importantly, we observed that for values above 30 mol % of LPS respect to total lipids in the initial LPS/E. coli lipids mixtures, the formation of GUVs from the purified lipid dispersions was completely impaired. Therefore, we decided to characterize samples containing an initial LPS concentration below 30 mol % with respect to total lipid. Quantification of LPS concentration in oligolamellar vesicles containing LPS shows that not all the LPS is incorporated in the oligolamellar vesicles, in agreement with previously observations (5). In Fig. 2, the final concentration of LPS incorporated in the oligolamellar vesicles is compared with the initial concentration of LPS used to prepare the different LPS/E. coli lipids mixtures. These experiments show that no more than 15 mol % for LPS-smooth and LPS-Ra, and up to 25 mol % for LPS-Rc and LPS-Rd (respect to total E. coli lipids), are present in the oligolamellar vesicles.

In order to check if the incorporation of LPS in the GUVs is the same as in the oligolamellar vesicles, we performed LPS incorporation using fluorescently labeled LPS-FITC (corresponding to LPS-smooth) in membranes composed of E. coli lipids labeled with rhodamine-DHPE. We choose an LPS-smooth analogue since this lipid (together with LPS-Ra) shows the lower incorporation efficiency in the host membrane (see Fig. 2).

The rhodamine-DHPE/LPS-FITC fluorescence emission intensity ratio obtained in oligolamellar vesicle solution was comparable with that obtained in single GUVs experiments (0.67 ± 0.02 and 0.72 ± 0.09, respectively), showing that the lipid composition in both model membranes is very similar. This result is in agreement with that reported for GUVs composed of binary and ternary lipid mixtures, where the lipid composition before and after GUVs electroformation is invariable (30, 31). Another indication of LPS incorporation in the host membrane is shown in Fig. S2, where the fluorescence images were obtained using LPS-Alexa 488. In order to further validate our
protocol, we explored other lipid compositions (at physiological conditions in presence and absence of divalent cations, e.g. Ca\textsuperscript{2+} up to 2 mM) such as PE/PG containing mixtures or single PC species such as POPC. In all cases the formation of GUVs was successfully achieved at LPS concentrations similar to those observed for \textit{E.coli} lipids (data not shown).

After quantitative determination of LPS incorporation in the host \textit{E. coli} lipid bilayer membranes, we decided to explore the impact of the different LPS species in the lateral structure of these membranes using using GUVs/fluorescence microscopy related techniques. Our results can be divided in two groups, i.e. GUVs containing either LPS-smooth or LPS-Ra, and GUVs containing LPS-Rc or LPS-Rd (as well as lipid A).

\textit{GUVs composed of \textit{E. coli} lipid mixtures containing LPS-smooth and LPS-Ra}

GUVs containing LPS-smooth or LPS-Ra show homogeneous distribution of LAURDAN or rhodamine-DHPE probes at all the LPS concentration explored (up to \(~15\) mol %), Fig. 3, A and B. However, further analysis of LAURDAN emission signal using the LAURDAN GP function (both in GUVs and oligolamellar vesicles) show an increase in the GP function values as the concentration of LPS in the GUVs is increased (Fig. 4, A and B, similar to that observed for GUVs containing LPS-Rc and LPS-Rd below 10 mol % see below). In order to do a closer evaluation of this phenomenon we decided to perform FCS measurements using fluorescently labeled LPS. These experiments evaluate the diffusion coefficient (D) of fluorescently labeled LPS on the membranes as a function of LPS concentration. The diffusion coefficient shows a \(~5\) times -fold decrease when the LPS (LPS-smooth or LPS-Ra) concentration reaches the maximum attainable in our model systems (\(~15\) mol % respects to total lipids, see Fig. 5). The behavior observed in these mixtures for the FCS and LAURDAN GP data is attributable to formation of LPS lipids cluster with sizes below the resolution of our microscope images.

\textit{GUVs composed of \textit{E. coli} lipid mixtures containing LPS-Rc and LPS-Rd.}

Compared with the results obtained in LPS-smooth (or LPS-Ra), we observe a different LPS concentration-dependent effect on the lateral structure of the LPS-Rc and LPS-Rd containing GUVs. Using rhodamine-DHPE (or LAURDAN) as fluorescent markers, coexistence of distinct elongated micrometer sized lipid domains were observed only for LPSs concentration above \(10\) mol %, Fig. 3, C and D. Below a concentration of \(10\) mol\% LPS, a homogeneous distribution of the probes was detected in our fluorescence images (data not shown). The micrometer sized lipid domains observed for LPS-Rc and LPS-Rd above \(10\) mol %, resembled very much those shown in Fig. 3 \(E\) for Lipid A (lacking the inner and outer core sugars, see Fig. 1). However, the presence of domain coexistence in GUVs containing Lipid A is observed at lower Lipid A molar fractions (i.e. \(5\) mol %). Analysis of LAURDAN images using the LAURDAN GP function provided additional information about the local packing of these distinct membrane regions. By taking into account the measured GP value for these elongated domains (~0.55, see Fig. 4, C-E) and the GP of the surroundings (slightly below 0.2) we conclude that this scenario corresponds to a gel/liquid disordered -like phase coexistence, being the elongated domains gel -like domains enriched in LPS (or lipid A). This conclusion is supported by comparing the GP differences between the coexisting domains in our LPSs containing samples with those previously reported for phospholipid samples displaying gel/liquid disordered phase coexistence (GP values for gel and fluid phases, i.e. >0.5 and <0.2 respectively) (32, 33).

Although micrometer sized domains are not observed below 10 mol % of LPS-Rc and
LPS-Rd, the GP values measured in the GUVs increases to some extent relative to the GP measured in absence of LPS (Fig. 4, C and D). This behavior is also observed in the oligolamellar vesicles used to prepare the GUVs. These observations indicate an overall increase in the membrane packing in presence of these two LPSs before occurrence of micrometer sized lipid domains. Similar to the results obtained for LPS-smooth or LPS-Ra, this can be explained by assuming LPS clustering in the membranes below our microscope resolution. Notice that this effect is not observed for Lipid A containing GUVs, where the GP values for the fluid and gel-like phases is constant and independent on the Lipid A concentration in the mixture (Fig. 4 E).

Discussion

The formation of GUVs under physiological conditions (22) has been successfully extended (with some modifications, see methods section) to different compositionally complex LPS/E. coli polar lipids mixtures. This new protocol offers an interesting alternative for these lipid mixtures since appropriate free standing bilayer model system (i.e. GUVs) can be successfully prepared, allowing new studies using fluorescence microscopy related techniques. Additionally, our model system has been carefully characterized in order to provide quantitative information on the amount of LPS incorporated into the model bilayer membrane (E. coli lipid extract in this case). Determination of LPS molar fraction in the final model membrane is an important parameter, not only for the nature of our study but also because it has been reported that incorporation of LPS in bilayers models is generally not straightforward (5). A major consequence of this problem is the overestimation of LPS concentration in the GUV membrane, particularly if the initial concentration of LPS is assumed to be unchanged during the whole formation process. In fact, for all LPSs explored in our work, we found lower LPS concentration in the final membrane preparations respect to that anticipated from the initial E. coli lipid/LPS mixtures. This observation is in line with that reported by Dijkstra et al. (5), where a similar situation was found for the incorporation of Salmonella minesota wild-type LPS in PS/PC/cholesterol mixtures. By systematically exploring the incorporation of different LPSs at different concentrations we showed that no more than 15 mol % for LPS-smooth and LPS-Ra, and up to 25 mol % for LPS-Re and LPS-Rd (respect to total lipids), are possible to attain in our bilayer systems. These concentration ranges are wider compared to that reported for particular LPSs in previous works on small unilamellar or multilamellar vesicles, where the maximum incorporation reported was ~2 mol % (5, 6).

The efficiency of LPS incorporation is also relevant in the context of GUVs electroformation. We noticed that by using initial concentrations above 30 mol% of LPSs respect to total lipids, the electroformation of GUVs was impaired. This situation may be connected with a decrease in the tendency to form lamellar structures at higher LPS concentrations for each particular LPS/E. coli lipids mixture. In fact, formation of non-lamellar phases (cubic phases) has been previously reported for high concentration of a deep rough LPS mutant in mixtures of DPPE/DPPG (12). In our mixtures, the potential existence of non-lamellar structures can also be sustained with the decrease of LPS incorporation as a function of LPS polar head group size (particularly at high LPS concentrations, see Fig. 1 to compare the different LPS structures). This tendency can be rationalized by considering the influence of the critical packing parameter on the final membrane structure. For example, different supramolecular assemblies, such as spherical (normal or inverted micelles), tubular (normal or inverted H₁ and H₂⁾ and cubic has been reported to exist in samples of pure LPS above their critical micellar concentration.
(CMC). This largely depends on the molecular geometry of monomeric LPS and the environmental conditions such as hydration, temperature and ion content (3, 34). The formation of GUVs containing DOPC/DPPC/LPS (LPS-FITC from E. coli 0111:B4, Sigma Chemical Co) mixtures in 1:1:1 mol ratio using an electroformation based method has been recently reported in the literature (8). In this paper, the effect of Triton X-100 on DOPC/DPPC/LPS membrane and the impact of LPS on the lipid lateral organization are compared with the DOPC/DPPC/cholesterol 1:1:1 mol mixture (8). From the methodological point of view we noticed two main differences with our method: 1) the deposition of the lipid mixture on the Pt wires was directly done from CHCl₃ (in our case we used a suspension of oligolamellar vesicles containing known concentration of LPS) and 2) the electroformation of GUVs was done using non physiological conditions (1 mM Tris buffer, pH 8.0) by applying an alternate field with a frequency of 10 Hz (we used 500 Hz, see (23)). Unfortunately, the final amount of LPS in the DOPC/DPPC/LPS membrane is not reported in this work (the authors assume that all the LPS used in the initial mixture is incorporated in the GUVS, see Ref. 8) hampering a final comparison with our method. In any case we believe that our protocol offer a improved way to generate the GUVs containing LPS, particularly for three reasons: 1) the proper solubilization of LPS (particularly the more complex species, i.e smooth LPS) in CHCl₃ is not easily achievable (if not impossible), 2) quantitative information about the incorporation of LPS in the final membrane model is known, and 3) physiological conditions are attained.

**Impact of different LPS on the lateral structure of E. coli lipid bilayers**

The successful preparation of GUVs containing LPS allowed us to evaluate the spatial distribution of particular fluorescent parameters (i.e. LAURDAN GP for example) at the level of single vesicles. This experimental approach, that is very useful to study the lateral structure of membranes of diverse composition (27), was applied to evaluate the effect of LPS in GUVs composed of E. coli lipids/LPSs at different LPS compositions.

The LAURDAN GP values measured in E. coli lipid membranes in the absence of LPS (Figs. 4 and 5) suggest that in our experimental conditions (excess of water; high ionic strength, room temperature) the observed lamellar structures display a liquid disordered phase (Lα), in agreement with previous observations reported for phospholipid membranes (33). This conclusion is supported by measurements of the diffusion coefficient of fluorescent labeled LPS at very low concentrations in E. coli lipid membranes (0.001 mol % with respect to total lipid, D= 5.58 ± 0.62 \( \mu \text{m}^2 \text{s}^{-1} \)), resembling those measured from various fluorescent probes in phospholipid membranes displaying a single liquid-disordered phase (35). When LPS concentration is increased in the membrane, the impact of LPS on the lateral structure of E. coli lipid membranes is apparent (see Figs. 3, 4 and 5). From our observations, we conclude that LPSs show a tendency to cluster into gel-like domains in the explored model mixtures, and the size of these lipid domains depends on the chemical structure of the LPSs as well as their concentration. That is manifested by the presence of micrometer sized gel-like domains (for LPS-RC and LPS-RD containing membranes above 10 mol % of LPS), but also reflected by the increase of the LAURDAN GP values (below 10 mol % for LPS-Rc and LPS-Rd and up to 15 mol % for LPS-smooth and LPS-Ra) and the 5-fold decrease in the diffusion coefficient of fluorescently labeled LPS (up to 15 mol % for LPS-smooth and LPS-Ra containing membranes) at conditions were lipid domains are not visible in the GUVs. In order to obtain further information about these submicroscopic domains, our diffusion data was employed to compute the domain size (and number of molecules) using the Saffman-Delbrück model (36). From our calculations (see Supporting Material, Section 4) we found that a reduction of 5 times in the diffusion coefficient (in
interfaces containing LPS smooth or Ra) implies a clustering of ~10000 LPS molecules per leaflet (assuming that the domain span the bilayer). Taking into account the resolution of our microscope (that is ~380 nm in the radial direction) we estimate that a clustering of >1x10^6 LPS molecules per leaflet (assuming that the molecular area of LPS is app. 1.5 nm^2 (37)) is needed to directly observe the domains under the microscope. This last result is in line with our microscopy data. Using the same model we estimate that the diffusion of micrometer size domains is ~2 orders of magnitude lower compared to those of single LPS molecules (data not shown). The slow diffusion values for the LPS micrometer sized domains are in line with the diffusion coefficient of lipid domains measured in GUVs composed of ternary mixtures containing cholesterol (38).

The dependence of domain size with the amount and nature of LPS observed in our experiments (reflected in the low concentration of Lipid A respect to LPS-Rc and LPS-Rd observed to form micrometer sized domains and the lack of this phenomenon for LPS-Ra and LPS-smooth species; see Figs. 1, 3, 4 and 5) could be interpreted as an interplay between different interactions occurring at the level of the LPS polar head group and those occurring among the hydrophobic part of LPS molecules. Since in our experiments the hydrophobic part of the different LPS molecules is the same (i.e. all our LPS come from E. coli strains), it is reasonable to assume that the interactions occurring at the level of the polar head group can regulate the dimension of these gel -like domains. Although speculative, a change in the nature of the LPS polar head group can modulate supramolecular interactions through steric effects, electrostatic repulsions or eventually changes in lipid miscibility. All these effects may compromise the size of LPS enriched domains in the plane of the membrane (see Fig. 6). In agreement with our results, LPS clustering has been reported previously upon incorporation of LPS isolated from group B Neisseria meningitidis strain B125 in mixtures of egg PC/cholesterol at low LPS concentrations (39). Taking into account these aforementioned results it can be hypothesized that the clustering ability of LPS at low concentrations may similarly happens in compositional environments representative of both mammals and bacterial membranes. Since our protocol for LPS incorporation has been successfully extended to other lipid compositions (e.g. POPC) at similar LPS concentration ranges presented here, we believe that these model systems can be useful to test the aforementioned hypothesis.

Are LPS enriched gel -like domains relevant in bacterial membranes?

The phase behavior of intact bacterial cell membranes was studied using the fluorescent probes LAURDAN and DPH using regular “cuvette” GP and anisotropy fluorescence spectroscopy experiments (40). This last study interpreted the existence of 2 phases of different polarity and packing, related to 'lipid rich” and “protein rich” membranes, which is consistent with presence of outer and inner membranes, but not related to gel-like enriched LPS domains (40). These observations are in contrast to those from our results, where LPS enriched gel -like domains can be detected in model membranes. However, conclusions draw from LAURDAN GP experiments in cuvette without further exploration of spatially resolved GP information (available from fluorescence microscopy experiments), can generate potential data misinterpretation. In other words, presence of gel phase -like domains cannot be ruled out. As reported by Fidorra et al, LAURDAN GP experiments in cuvette (where solution of liposomes are used) using ceramide/POPC mixtures (41) suggest absence of gel/liquid disordered phase coexistence, a situation that is in contrast to the data obtained on the same mixture using LAURDAN GP imaging microscopy (since the membrane domains can be spatially resolved). As discussed in Fidorra et al, because the additive property of the GP function (41), the detection of solid ordered (gel)/liquid disordered phase coexistence using LAURDAN GP measurements in
cuvette requires a particular quantity of gel like -phase areas in the membrane. This situation is not achievable by ceramide containing mixtures since a maximum of ~25 mol% of this lipid is possible to be incorporated in the system, a situation that can be compared to the maximum amount of LPS incorporated in our model mixtures. Although the existence of membrane domains in bacterial membranes has been addressed in previous publications (15, 40), the potential existence of LPS enriched gel -like domains has not been considered. These organisms lack sterols and the presence of liquid ordered -like domains (as postulated in the raft hypothesis) can be ruled out (to our knowledge there is not molecular species reported to exist in bacterial membranes that can generate liquid ordered phase in model membranes). The lack of sterols may likely favors the potential existence of gel -like domains promoted by lipids with high phase transition temperature such as LPSs. This speculation may pose interesting questions about their connection with a potential biological role. Although very scarce, presence of gel -like phase organization has been proposed to exist for example in skin stratum corneum membranes (42) and connected to the tissue’s barrier function.

**Concluding remarks**

We introduce a new protocol to incorporate LPS in giant unilamellar vesicles composed of E. coli lipids. This method allows incorporation of no more than 15 mol % for LPS-smooth and LPS-Ra, and up to 25 mol % for LPS-Rc and LPS-Rd (respect to total lipids). By exploring the lateral structure of these membranes we demonstrate that LPS tends to form gel -like lipid domains with a size depending on the chemical structure and concentration of LPS. This phenomenon can be relevant for the organization and modulation of the outer membrane of Gram-negative bacteria and open the question if similar process can be connected with LPS partition in biological membranes upon bacterial infection. Additional we expect that the developed models (GUVs) will be also useful for microscopic characterization of the interaction of particular host molecules and antimicrobial drugs with LPSs (experiments in progress). There is a continuous call for new drug candidates targeting both new and well-known microbial molecules. This information is relevant particularly for multi resistant Gram-negative bacteria, e.g. methicillin-resistant *Staphylococcus aureus*, which are becoming an increasing problem in hospitals worldwide.

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References


Figure legends

**Fig. 1.** Molecular structure of LPS-Re of *E. coli* (left). Schematic representation of the different LPS used in this work (right). Gal is galactopyranose, Glc is glucopyranose, GlcN is 2-amino-2-deoxyglucopyranose, Hep is L-glycero-α-D-manno-heptopyranose, Kdo: 3-deoxy-α-D-manno-oct-2-ulopyranosonic acid, P is phosphate. For more details see ref. (2).

**Fig. 2.** Summary of LPS incorporation procedure for 4 studied LPS strains. X –axis represents the starting LPS concentration as a fraction of total amount of lipids in the sample, Y –axis shows the concentration of LPS in the oligolamellar vesicles as a fraction of total amount of lipids in the samples after the purification procedure. The solid black line indicates an ideal, 100% incorporation efficiency. These vesicles were used to form GUVs. The efficiency of LPS incorporation increases as the size of the polysaccharide length decreases. Error bars are standard deviations.

**Fig. 3.** Representative images of GUVs (false color representation) composed of *E. coli* polar lipid extract and LPSs: A) LPS smooth; B) LPS-Ra; C) LPS-Rc (above 10 mol %); D) LPS-Rd (above 10 mol %); E) Lipid A (above 5 mol %). The upper row shows fluorescence images (false color representation) of GUVs labeled with rhodamine-DHPE. The bottom row displays LAURDAN GP images of the GUVs. For LPS smooth (A) and LPS Ra (B), no visible phase separation can be seen in the GUVs at all explored concentrations (up to 15 mol %). For deep rough LPS strains, Rc (C) and Rd (D), coexistence of domains of high GP values (close to 0.6 indicating presence of a gel -like structure) is observed above 10 mol %. Scale bars are 10 μm.

**Fig. 4.** LAURDAN GP values of LPS containing membranes as a function of LPS concentration: A) LPS-smooth; B) LPS-Ra; C) LPS-Rc; D) LPS-Rd; E) Lipid A. (○ and ●) LAURDAN GP values calculated from GUVs. Double points at high LPS concentration for C, D and E represent visible phase separation can be seen in the GUVs at all explored concentrations (up to 15 mol %). These LAURDAN GP values were calculated separately for each domain, liquid-like (○) and gel-like (●). Each point represents an average of 15 to 25 separate vesicles, error bars are standard deviations. (Δ) LAURDAN GP values from the oligolamellar vesicles used to form GUVs (measured in a fluorometer in the concentration range where domains are not observed in the GUVs).

**Fig. 5.** FCS experiments performed in GUVs containing LPS-smooth and LPS-Ra respectively, doped with fluorescently labeled Alexa488 LPS-smooth (0.001 mol % respect to total lipids). The figure shows the corresponding LPS diffusion coefficient measured in GUVs (each point is an average of 15 to 20 separate vesicles, error bars are standard deviations). No microscopic phase separation was seen in these samples using both LAURDAN GP and rhodamine-DHPE.

**Fig. 6.** Sketch representing the different LPS-rich domain size observed in our experiments. LPS chemotypes (A: LPS smooth and Ra; B: Rc and Rd2 - deep rough chemotypes of LPS) inserted in the lipid membrane (PE and PG, cardiolipin not shown). (A) the lateral organization of LPS-smooth (valid also for LPS-Ra) is represented for a low concentration of this lipid (domains in the sketch correspond to a diffusion twice lower that the single LPS, see supporting material section 3). (B) Formation of large
membrane platforms (micrometer sized) for the rough LPS chemotypes above 10 mol % LPS concentration.
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Supporting Material

Section 1. Labeling LPS with Alexa fluor 488 hydrazide

LPS from *E. coli* O55:B5 (smooth) was labeled with Alexa Fluor 488 hydrazide according to a protocol described by Luk et al. (1). LPS was oxidized with 10 mM NaIO₄ in 100 mM carbonate buffer pH 5 for 20 minutes at 4°C. The reaction was stopped by addition of glycerol to final concentration of 15 mM. Oxidized LPS was purified by dialysis in 3.5 kDa cut-off Slide-A-Lyzer Dialysis Cassette (Pierce, Rockford, IL). Conjugation with Alexa Fluor 488 hydrazide was performed by overnight incubation in 10 mM phosphate buffer 150 mM NaCl pH 7.4 at 4°C. Labeled LPS was purified by size exclusion chromatography on Sephadex G-100 (Sigma-Aldrich). Purification was validated by performing fluorescence correlation spectroscopy (FCS) experiments (2), where the diffusion of single LPS molecules was measured below their critical micellar concentration (D_{coeff} = 26.0 ± 1.5 μm²s⁻¹, different to that measured for free Alexa Fluor 488 hydrazide, i.e. D_{coeff} = 430 μm²s⁻¹(3)). In order to evaluate the degree of LPS labeling, photon counting histogram (PHC) analysis was applied to our FCS data (data not shown) using the Globals software package developed at the Laboratory for Fluorescence Dynamics at the University of California at Irvine. The analysis of PCH allows to extract information about molecular brightness (number of photon counts per molecule) and the average number of molecules in microscope focal volume (4). FCS experiments were done in samples containing known concentration of free Alexa Fluor 488 hydrazide or Alexa Fluor 488 -labeled LPS. After data analysis the molecular brightness of Alexa488 hydrazide and Alexa488 hydrazide conjugated LPS were compared at the same conditions. These experiments had shown a LPS-to-Alexa Fluor 488 labeling ratio of 1:3 mol.

Section 2. LAURDAN GP function and two photon excitation LAURDAN GP measurements

2.1 LAURDAN GP function

The fluorescence emission properties of LAURDAN are sensitive to the water dipolar relaxation process that occurs in the probe’s environment, and the LAURDAN GP denotes the position of the probe’s emission spectrum (5). The energy of the excited singlet state progressively decreases when the extent of dipolar relaxation process is augmented. The extent of water dipolar relaxation observed in highly packed membrane regions (e.g. the solid-ordered phase in bilayers) is very low compared to what it is observed in less packed regions (e.g. the liquid-disordered phase in bilayers). For example when a solid-ordered/liquid-disordered phase transition occurs in the membrane, a prominent red shift in the fluorescence emission spectrum of the probe is observed (from blue to green; almost 50 nm shift) (5). The GP function was defined analogously to the fluorescence polarization function as:
\[ GP = \frac{I_B - I_R}{I_B + I_R} \]  

where \( I_B \) and \( I_R \) correspond to the intensities at the blue and red edges of the emission spectrum (440 and 490 nm) using a given excitation wavelength (5-7). At equilibrium conditions, this function is sensitive to the local phase state of the membrane (8).

2.2 Two photon excitation LAURDAN GP measurements

The LAURDAN GP measurements were performed in a custom built two photon excitation fluorescence microscope described previously (9). The objective used in the experiments was a 60X water immersion objective with an NA of 1.2 (Olympus). The excitation light source was a femtosecond Ti:Sa laser (Broadband Mai Tai XF-W2S with 10 W Millennia pump laser, tunable excitation range 710-980 nm, Spectra Physics, Mountain View, CA) and the excitation wavelength was 780 nm. The excitation light was circularly polarized to avoid photoselection effects in the image plane. In order to calculate the LAURDAN GP function, the fluorescence signal from the sample was split in two different channels using a dichroic mirror (splitting at 475 nm). Each channel contains one bandpass filters (438 ± 12 nm and 494 ± 10 nm, that correspond respectively to \( I_B \) and \( I_R \) in Eq. 1). The LAURDAN GP images were calculated using the program SimFCS (Laboratory for Fluorescence Dynamics, University of California at Irvine, USA). Corrections using the G factor were performed according to Brewer et al (9), using a 160 µM LAURDAN solution in DMSO as a reference (GP=0.006 at room temperature). The GP values obtained in the GUVs experiments (Fig. 5) are computed from the distinct membrane regions using a ROI routine. Approximately 20-25 GUVs per each concentration are analyzed and the average GP reported. The LAURDAN GP measurements for the reference solution and oligolamellar vesicles were done in a fluorometer (ISS Chronos, Champaign, IL, USA) using a 374 nm diode as excitation wavelength, and 440 nm and 490 nm (\( I_B \) and \( I_R \) respectively in Eq. 1) as emission wavelengths. The GP function in these experiments was calculated using the Vinci analysis software (ISS, Champaign, IL, USA) according to Eq. 1.

Section 3. Fluorescence correlation spectroscopy of LPS-A488

The FCS measurements were performed in a custom built two photon excitation fluorescence microscope previously described in (9). The objective used in the experiments was a 60X water immersion objective with an NA of 1.2 (Olympus, UPlanSApo 60x/1.20W). The excitation light source was a femtosecond Ti:Sa laser (Broadband Mai Tai XF-W2S with 10 W Millennia pump laser, tunable excitation range 710-980 nm, Spectra Physics, Mountain View, CA) and the excitation wavelength was 930 nm. The signal was collected through a bandpass filter of 525 ± 25 nm using a photomultiplier (Hamamatsu H7422P-40). The
measurements were taken using SimFCS (Globals software package developed at the Laboratory for Fluorescence Dynamics at the University of California, Irvine). The autocorrelation function (ACF) of temporal fluorescence intensity fluctuation was calculated using Eq. 2,

\[ G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} \quad (2) \]

where \( \delta F(t) = F(t) - \langle F(t) \rangle \). For the FCS experiments aimed to measure the diffusion of monomeric LPS in solution, concentrations of 1 to 10 nM of Alexa488-labeled LPS in 10 mM phosphate buffer 150 mM NaCl pH 7.4 were used. Calculated ACFs were globally fitted using Globals for Spectroscopy (Globals software package developed at the Laboratory for Fluorescence Dynamics at the University of Illinois at Urbana-Champaign) to the model of single species of molecules diffusing in 3-dimensional Gaussian Two-Photon excitation volume (Eq. 3),

\[ G(\tau) = \frac{\gamma}{N} \left( 1 + \frac{8D_\tau}{\omega_{3DG}^2} \right)^{-1} \left( 1 + \frac{8D_\tau}{z_{3DG}^2} \right)^{-\frac{1}{2}} \quad (3) \]

where \( D \) is diffusion coefficient, \( \gamma \) is instrumental factor, \( N \) is an average number of fluorescent particles in excitation volume, \( \omega_{3DG} \) and \( z_{3DG} \) are the radii of the excitation volume in the \( xy \)-plane and the \( z \)-direction, respectively (2).

Diffusion of LPS was also measured in GUVs. In these experiments GUVs were prepared using unlabelled LPS doped with Alexa488-labeled LPS (~100:1 molar ratio). The laser beam was focused on the polar region GUVs. Approximately 20 vesicles per sample were analyzed. Calculated ACF was fitted to the model of single species of molecules diffusing in 2-dimensional Gaussian Two-Photon excitation volume (Eq. 4),

\[ G(\tau) = \frac{\gamma}{N} \left( 1 + \frac{8D_\tau}{\omega_{2DG}^2} \right)^{-1} \quad (4) \]

where \( D \) is diffusion coefficient, \( \gamma \) is instrumental factor, \( N \) is an average number of fluorescent particles in excitation volume, \( \omega_{2DG} \) is the radius of the excitation volume in the \( xy \)-plane (2).

Section 4. Estimation of the sub-microscopic domain size

The equivalent of Einstein-Stokes equation for 3D diffusion in 2D systems, is described by the Saffman- Delbrück model (10):
where \( h \) is thickness of the membrane, \( \mu_m \) is viscosity of the membrane, \( \mu_w \) is viscosity of surrounding solution, and \( R \) is the radius of diffusing cylindrical object. The validity of Saffman-Delbrück model was confirmed for particles of relatively small radii (transmembrane proteins: \( R \) of 0.5-4 nm) \(^{11}\), as well as for much larger objects (microscopic-size domains: \( R \) of 0.5-10 \( \mu \)m) \(^{12}\).

Using this model, the diffusion coefficients of LPS-Alexa488 measured from FCS experiments were used to estimate the size of these nanoscopic domains. The set of parameters describing the membrane properties (see Eq. 5) used in our calculations is: \( h = 4 \) nm (and 2.1 nm for single LPS molecule, which spans only half of the bilayer), \( \mu_M = 180 \text{ mPa} \cdot \text{s} \), \( \mu_W = 1.003 \text{ mPa} \cdot \text{s} \), \( k_B T = 4 \cdot 10^{-21} \text{ J} \). Particularly, the membrane viscosity \( (\mu_M) \) was estimated using the diffusion coefficient of single LPS and their known radius (a radius of 0.7 nm and a molecular area of 1.53 \text{ nm}^2 \) assuming molecules with cylindrical shape \(^{13}\)). We found that this membrane viscosity value is on the range of already reported values for a fluid membrane \(^{14}\). The dependence of diffusion coefficient with the object size (radius) and number of molecules (assuming circular domain shape) is shown in Fig. S3. The computed values for each diffusion coefficient are showed in Table S1. Using our microscope resolution (which is ~380 nm in the x-y plane) we estimated that clustering of \( >1 \times 10^6 \) molecules per leaflet is needed in order to visualize the LPS smooth (or Ra) enriched domains. In all cases (see table S1) the estimated domain size is below our microscopy system resolution limit, observation that is in agreement with our experimental data.

**References:**


**Table S1.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>$D_{\text{coeff}}$ (μm$^2$s$^{-1}$)</th>
<th>R (nm)</th>
<th>Number of molecules</th>
</tr>
</thead>
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<tr>
<td>LPS Alexa488</td>
<td>5.43 ± 0.62</td>
<td>0.71</td>
<td>1</td>
</tr>
<tr>
<td>LPS smooth 7 mol%</td>
<td>2.73 ± 0.28</td>
<td>1.7</td>
<td>7</td>
</tr>
<tr>
<td>LPS smooth 12 mol%</td>
<td>1.36 ± 0.23</td>
<td>32</td>
<td>2100</td>
</tr>
<tr>
<td>LPS Ra 9 mol%</td>
<td>1.99 ± 0.36</td>
<td>8</td>
<td>130</td>
</tr>
<tr>
<td>LPS Ra 12 mol%</td>
<td>0.98 ± 0.21</td>
<td>73.8</td>
<td>11200</td>
</tr>
</tbody>
</table>

**Supplemental Figure legends**

**Fig. S1.** (A) Size exclusion chromatography elution profiles of oligolamellar vesicles prepared using the full incorporation protocol (described in the methods section). The vesicles were prepared by mixing LPS smooth/E. coli lipids with two fluorescence probes, i.e. 0.1 mol % rhodamine-DHPE (●) and 1 mol % of LPS-Alexa488 (○). (B) Control obtained by mixing rhodamine-DHPE labeled oligolamellar vesicles containing E. coli lipids with pure LPS aggregates containing LPS-Alexa488 (showing that pure LPS aggregates are separated from the oligolamellar vesicles). FRI account for fluorescence relative intensity.

**Fig. S2.** GUVs composed of *E. coli* polar lipid extract and LPS-Alexa488. The GUVs were prepared in 10 mM TrisHCl pH 7.4, 0.15M NaCl. Fluorescence images (false color representation of Alexa488 conjugated with LPS smooth) shows incorporation of LPS into GUVs. LPS concentration in the membrane is on the order of 0.1–0.01 mol %. Scale bars are 10 µm.

**Fig. S3.** Dependence of the diffusion coefficient on the size (radius and number of molecules, log scale) of a diffusing object according to Saffman-Delbrück model (solid line, see section 4). The measured diffusion coefficient for LPS (smooth and Ra) at different concentrations are included in the graph.
Figure. S1.
Figure S2
Figure S3.