Report on research work
Summary of research activity and discussion of the results
„Interaction of biopolymers with cell membrane using model systems with increasing complexity”

1. Design of cell membrane model systems

a) Lipid components to prepare monolayer model systems

Selection of lipid components was based on the occurrence frequency in the human cell membrane. The appropriate lipids could be applied in various experimental techniques. Lipid molecules with saturated alkyl chains (e.g. Dipalmitoylphosphatidylcholine, DPPC) are used to form molecularly ordered highly packed monomolecular films in the Langmuir-trough. DPPC was mixed with Dipalmitoylphosphatidylglycerol, DPPG at various ratios to study the electrostatic interactions. Other lipid components can also be introduced into the molecular layer. In-chain deuterated DPPC was also used to prepare lipid layer in Langmuir-trough in order to be able to investigate the structural order of molecules by surface sensitive spectroscopic technique (sum frequency generation spectroscopy, SFG). Unsaturated phospholipids as typical constituents of human cell membrane are also included in the model lipid layers.

b) Composition of supported lipid bilayers

Lipid bilayers were prepared on the surface of a quartz crystal microbalance, QCM sensor. There are different approaches to obtain supported lipid bilayers reported in the literature. One of those is the Langmuir-Schaefer method to be applied for lipids forming Langmuir films. Lipid bilayer was built by this method onto quartz surface of QCM sensor. Although its structure was proved to be satisfactory by AFM imaging, other *in situ* technique would have been more convenient for the QCM measurement. Therefore the bilayer formation using liposome spreading was chosen in further experiments. A method was developed to form lipid bilayer from monodisperse palmitoyl oleoylphosphatidylcholine, POPC liposomes on the hydrophilised QCM sensor surface in a reproducible manner. That bilayer was proved to be a proper model for interaction studies performed nanogravimetrically. In order to increase the range of lipids applicable for liposome spreading a temperature program was introduced allowing formation of bilayer from lipids with higher transition temperature (DPPC, DPPG).

c) Plasma membranes as model in studying membrane affinity

Plasma membrane components were obtained and separated. Spreading of membrane fragments on the surface of liquid subphase was performed in a Langmuir balance. Stability of the spread film was characterized and surface activity of the soluble components was measured.
Lipid extracts were prepared and surface pressure – area isotherm of that fraction was determined and compared to that of pure lipids, such as POPC, DPPC and their mixtures.

According to our experience the stability of the surface layer of this plasma membrane sample was not satisfactory to allow penetration experiments. The water soluble components were dissolved into the subphase and appeared on the rare side of the barrier in the Langmuir trough. That phenomenon made impossible to detect the real surface pressure characterizing the interaction of bioactive components with surface layer. One approach to solve this problem might be the extraction and use of lipid components from the cell membrane sample. We found that surface behaviour of this product is highly similar to that of designed lipid mixture which possesses a well defined composition. Therefore the designed lipid mixtures were applied in further experiments.
The other possibility to overcome this difficulty was the evaluation of the results obtained from model experiments comparing to the results of *in vitro* cell experiments.

2. Selection of bioactive compounds, drug-candidates

Preliminary experiments were carried out to select the compounds from large number of *in silico* identified drug candidates effective against *Mycobacterium tuberculosis*. Beside the determination of octanol/water partitioning ratio we measured the surface and interfacial activity of the selected molecules. These data and the solubility of the compounds helped us to design the chemical modification or conjugation of the base molecules.

The other type of bioactive material was a set of antibacterial cationic polyelectrolytes synthesized and characterized by our German cooperation partner (Group of Prof. M. Moeller at Leibniz Institute of Interactive Materialen, Aachen). This series of polyelectrolytes was conjugated with alkyl chains of varying length to provide a range in hydrophobicity.

The natural ancestors of the above antibacterial cationic polyelectrolytes are the cell penetrating peptides (cpp). Different natural peptides with antibacterial properties are identified with a common feature of multiply cationic charges in addition to their surface activity. Hence the main structural features of the cpp are the cationic character and amphiphilicity the widely used cationic surfactants represent this crucial character in the most simple molecular structure. A series of cationic surfactants as well as a group of cpp are also included in the membrane affinity investigations to allow revealing the influence of systematically changing properties on the interaction with lipid layers.

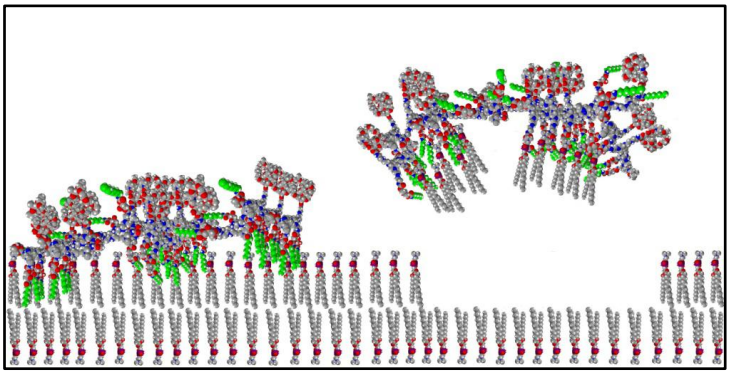
3. Membrane affinity studies

a) Penetration of molecular components

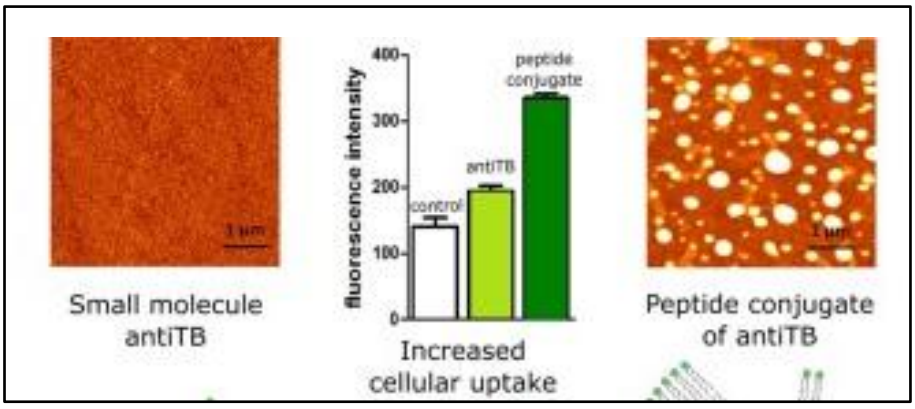
Detailed investigation of the degree of penetration, its kinetics and structural changes caused by the surfactant-lipid interaction are analysed. SFG technique was used to follow the adsorption to and insertion into the lipid monolayer of the dissolved component with molecular resolution. The influence of hydrophobicity was evaluated comparing the behaviour of cationic surface active agents with different alkyl chain length. The experimental findings were discussed together with the penetration ability of antitubercular drug candidates and their encapsulated forms.

Morphological features of the membrane layer transferred to a solid support by LB or LS technique was characterized by AFM.

Membrane affinity of antibacterial polyelectrolyte molecules was studied by penetration experiments using neutral and charged lipid monolayers. These investigations were continued by applying lipid double layer immobilized on an optical sensor surface. That arrangement allowed the *in situ* detection of the structural changes of surface lipid bilayer indicating the molecular interactions by the optical waveguide technique, OWLS.
We could give a possible scheme of mechanism of membrane disruption process induced by polymeric type antibacterial material. The results were published in a special issue Antibacterial Polymers of International Journal of Molecular Science.



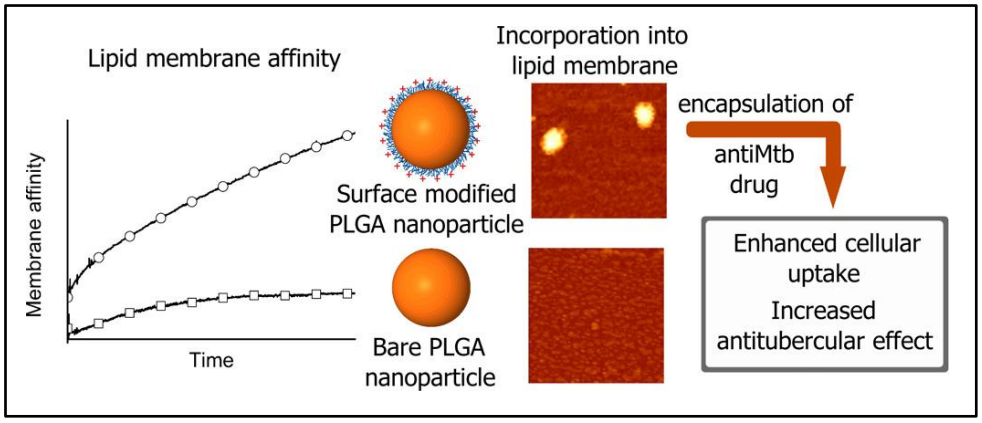
Interaction of small molecule antitubercular drug candidates and their peptide conjugates with the supported POPC bilayer was investigated and compared using the mass sensitive QCM technique. The results showed that the peptide conjugates presented higher non-specific membrane affinity applying at the same molar concentration. This behaviour is dependent on the peptide composition and is in correlation with the results found for lipid monolayer experiments. The conjugation with peptides significantly increased the degree of interaction with lipid layers, especially in the case of DPPC+DPPG system, which was in agreement with enhanced cellular uptake obtained by flow cytometry measurements. The structural change induced by the interaction was visualized by high resolution imaging technique AFM. Topographic characterization of the lipid layers was also done by optical techniques such as ellipsometry and reflectometry using liquid cells allowing the *in situ* monitoring of the structural changes. The construction being highly efficient against *MTb* (lower MIC value) showed a special membrane destabilization effect imaged by AFM.



b) Membrane interaction of particulate materials

In novel ways of drug administration the active material of a pharmaceutical product is incorporated into some nano- or microsize delivery system. The benefit gained by application of nanostructured drug delivery systems is highly influenced by their possible transport in the body and the therapeutic effect. The affinity to cell membrane is a crucial parameter to be optimized by tuning the surface properties of nanoparticles (NPs). Therefore it is important to characterize the interaction of these carrier particles with model lipid layers representing biointerfaces. Our investigations on such membrane – drug delivery particle interaction are among the first studies in this area.

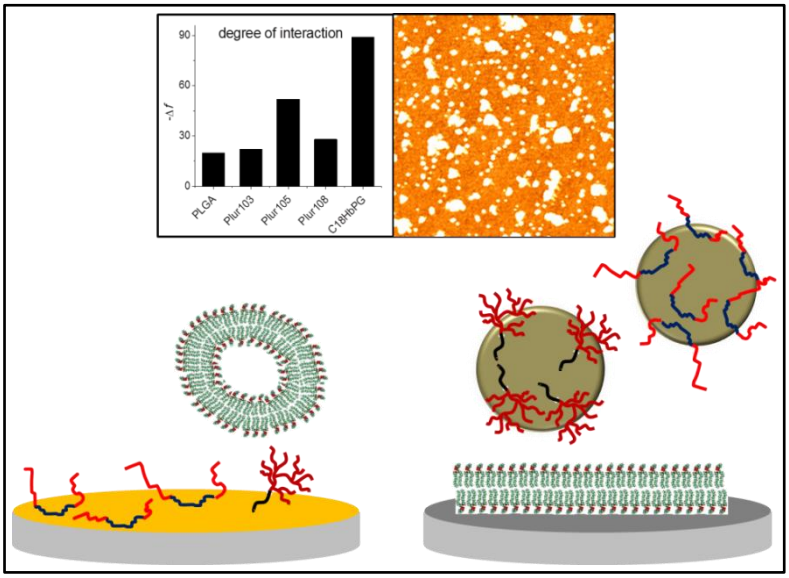
Colloidal drug delivery nanoparticles were prepared from biodegradable polymers. In order to increase the potential of targeted drug delivery the end group derivative of Pluronics was synthesized to obtain Pluronic-amines. Pluronic and Pluronic-amine stabilized PLGA nanoparticles were prepared by nanoprecipitation. The introduction of reactive primary amine groups into the surface layer of PLGA NPs while preserving the aggregation stability, resulted in improved membrane affinity of NPs while provides a possibility for coupling of various ligands allowing targeted delivery. Three papers were published reporting the results of *in vitro* and *in vivo* study of antitubercular drug candidates and their nanoparticulate forms in Bioconjugate Chemistry, Colloids and Surfaces A and Tuberculosis.



With the aim to improve further the membrane affinity and functionalization of NPs a new biocompatible polymer was applied as surface stabilizer in the nanoprecipitation method. Amphiphilic hyperbranched polyglycerols (HbPG) were synthesized by dodecyl and octadecyl alcohols as direct initiators for ring-opening multibranching polymerization of glycidol. These polymers possess molecular weight dependent surface activity, and are efficient surfactants and stabilizers for poly(lactic/glycolic acid) (PLGA) nanoparticles, opening new possibilities for functionalized drug carriers, targeting and imaging agents. Besides the determination of size and size distribution the surface charge and colloidal stability of nanoparticles (NPs) were also characterized at different electrolyte concentrations. The new amphipathic polymer which was applied as surface modifier provided satisfactory steric stabilization of the NPs, and as an additional advantage presented improved affinity to lipid layers.

PLGA NPs designed for drug delivery were stabilized with three different Pluronics (103, 105 and 108) as well as an amphiphilic monoalkyl hyperbranched polyglycerol (C18-HbPG). Their interaction with lipid bilayer was investigated in two types of arrangements using liposomes and

polymeric adsorbed layer, while in the other model polymer stabilized PLGA NPs and supported lipid bilayer (SLB) were applied. Degree of adhesion of liposomes and PLGA NPs were characterized by quartz crystal microbalance (QCM) measurements in combination with the visual analysis of the surfaces by AFM. The comparison of the various polymer coatings led to the conclusion that C18-HbPG resulted in the highest membrane affinity, followed by Pluronic105 with medium polarity within the Pluronic series.



Applying the new surface modification technique correlation between the membrane affinity, surface modification and cell uptake leading to biological activity was found and discussed in five posters or oral presentations exhibited at international conferences.

In another work targeting possibility of the PLGA NPs was exhausted when cell specific protein type recognition unit and targeting species were successfully immobilized on the surface of drug carrier nanoparticles. Two papers were published from this work.

Besides the papers summarizing the results of the work two young researchers participating in the project defended successfully their PhD thesis.

Budapest, 29 of Jan 2018

É. Kiss